Heterologous Expression of *ethA* and *katG* in *Mycobacterium marinum* Enables the Rapid Identification of New Prodrugs Active against *Mycobacterium tuberculosis*

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ABSTRACT Screening strategies for antituberculosis compounds using *Mycobacterium tuberculosis* are time consuming and require biosafety level 3 (BSL3) facilities, which makes the development of high-throughput assays difficult and expensive. *Mycobacterium marinum*, a close genetic relative of *M. tuberculosis*, possesses several advantages as a suitable model for tuberculosis drug screening. However, despite the high genetic similarity, there are some obvious differences in susceptibility to some tuberculosis drugs between these two species, especially for the prodrugs ethionamide and isoniazid. In this study, we aimed to improve *M. marinum* as a model for antituberculosis drug identification by heterologous expression of two common drug activators, EthA and KatG. These two activators were overexpressed in *M. marinum*, and the strains were tested against ethionamide, isoniazid, and a library of established antimycobacterial compounds from TB Alliance to compare drug susceptibility. Both *in vitro* and *in vivo* using zebrafish larvae, these genetically modified *M. marinum* strains showed significantly higher susceptibility against ethionamide and isoniazid, which require activation by EthA and KatG. More importantly, a strain overexpressing both *ethA* and *katG* was potentially more susceptible to approximately 20% of the antituberculosis hit compounds from the TB Alliance library. Most of these compounds were activated by EthA in *M. marinum*. Four of these compounds were selected for further analysis, and three of them showed obvious EthA-dependent activity against *M. tuberculosis*. Overall, our developed *M. marinum* strains are valuable tools for high-throughput discovery of potential novel antituberculosis prodrugs.

KEYWORDS *Mycobacterium*, *Mycobacterium tuberculosis*, animal models, antimicrobial activity, antimicrobial agents, enzyme activation

Tuberculosis, caused by *Mycobacterium tuberculosis*, is one of the deadliest infectious diseases and is responsible for more than 1 million deaths globally every year (1). With the emergence and spread of multidrug-resistant tuberculosis (MDR-TB) and extensive multidrug-resistant tuberculosis (XDR-TB), the disease has grown into one of the major threatening health problems worldwide (2). Current antimycobacterial compound screening strategies using pathogenic *M. tuberculosis* are limited and time consuming due to the bacteria's low growth rate and the requirement of biosafety level 3 facilities. As a result, there is an urgent need for an easy-handling model organism that mimics *M. tuberculosis*' drug sensitivity in an efficient high-throughput screening assay.

Mycobacterium marinum is a nontuberculous mycobacterium associated with opportunistic skin infections in humans (3). In zebrafish (*Danio rerio*), *M. marinum*

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mimics human tuberculosis-like infection in several features, including triggering necrotic and hypoxic granuloma formation (4, 5). As a biosafety level 2 pathogen and one of the closest genetic relatives of the *M. tuberculosis* complex, *M. marinum* has been utilized to investigate tuberculosis pathogenesis, ranging from identifying mycobacterial virulence determinants to host susceptibility factors (6). In addition, *M. marinum* has been proven to be a useful model for the identification of potential antimycobacterial compounds (7–10). However, *M. marinum* and *M. tuberculosis* possess distinct susceptibility levels to a number of antimycobacterial drugs, notably, ethionamide (ETH) and isoniazid (INH) (7, 11, 12). These observations indicate that there are significant differences between *M. tuberculosis* and *M. marinum*, which may hinder the efficacy of antimycobacterial drug discovery when using *M. marinum* as a screening model.

The first-line and second-line drugs INH and ETH are included in the backbone regimen for tuberculosis treatment (13). Both INH and ETH are prodrugs that require intracellular activation by catalase-peroxidase KatG and Baeyer-Villiger mono-oxygenase EthA, respectively (14, 15). The converted metabolites form adducts with NAD⁺ and subsequently bind to inhibit InhA, which is essential for mycolic acid synthesis (16). In several studies, the MICs of ETH and INH were observed to be among the most contrasting between *M. tuberculosis* and *M. marinum* (11, 12). For the activation of INH, this could be attributed to small differences between the KatG structures of *M. marinum* and *M. tuberculosis* (17).

In this study, by overexpressing *ethA* and *katG* of *M. tuberculosis* in *M. marinum*, we were able to mimic the susceptibility of *M. tuberculosis* in our model organism, *M. marinum*, toward ETH, INH, and other drugs derived from a library of antituberculosis hits. These established *M. marinum* strains will be a suitable and simple tool for the discovery of new antituberculosis drugs and the evaluation of their possible activation within bacterial cells.

RESULTS

ETH and INH susceptibility of *M. tuberculosis* H37Rv and *M. marinum* M^{USA}. To evaluate the suitability of *M. marinum* as a model for tuberculosis drug screening, we compared its susceptibility for a number of clinical antituberculosis drugs against *M. tuberculosis*. We utilized the commonly used *M. marinum* M^{USA} strain and *M. tuberculosis* H37Rv strain. The tested drugs were first-line medications, including rifampicin and INH, second-line medications kanamycin and ETH, and recently approved medications and compounds in clinical trials, including bedaquiline, nitazoxanide, macozinone, linezolid, and sutezolid. Most of the antituberculosis drugs exhibit comparable activity in both species, with usually less than 2-fold difference in MIC₅₀s, indicating that *M. marinum* is potentially a good model for tuberculosis drug testing. For the first-line medication rifampicin, the MIC₅₀ difference is somewhat higher, approximately 5-fold. However, the biggest differences were observed for the antibiotics ETH and INH, for which *M. tuberculosis* was 10-fold more susceptible than *M. marinum* (Fig. 1) (see also Table S5 in the supplemental material). We hypothesized that the lower expression levels of *ethA* and *katG* in *M. marinum* than in *M. tuberculosis* could play a role. Based on a previous transcriptomic analysis, *katG* is indeed upregulated in *M. tuberculosis* but not *M. marinum* when treated with INH at both 4-h and 24-h time points. Furthermore, *ethA* is already more abundantly expressed in untreated *M. tuberculosis* than in *M. marinum* (see Fig. S2). Although EthA proteins of *M. marinum* and *M. tuberculosis* are highly similar (85% identity), these small differences could affect activity (see Fig. S3A). Therefore, we also evaluated whether there is a difference between the overexpression of *M. marinum ethA* compared to that of *M. tuberculosis ethA*. As expected, *M. marinum* overexpressing *ethA* of *M. tuberculosis* or *M. marinum* from plasmid backbone pSMT3 showed higher susceptibility to ETH (see Fig. S3B). This effect was identical for both *ethA* variants, indicating that the differences in *ethA* expression levels are the main reason for differences in susceptibility. To substantiate this, we integrated the *ethA*

FIG 1 Comparison of susceptibilities of *M. marinum* and *M. tuberculosis* to different clinical and preclinical antituberculosis drugs. *M. marinum* M^{USA} and *M. tuberculosis* H37Rv were treated with antibiotics in serial dilutions. After a 4-day incubation for *M. marinum* and 7-day incubation for *M. tuberculosis*, bacterial viability was measured using the resazurin microtiter assay (REMA). MIC₅₀ of each compound was determined using GraphPad PRISM 8. Data are presented as fold difference in MIC₅₀ between *M. marinum* and *M. tuberculosis*, with the average and standard deviation between two repeats. KAN, kanamycin; RIF, rifampicin; ETH, ethionamide; INH, isoniazid; BDQ, bedaquiline; LNZ, linezolid; STZ, sutezolid; PBTZ169, macozinone; NTZ, nitazoxanide.

expression cassette into the genome, using the L5 bacteriophage integration site, which reduces the *ethA* gene copies compared to episomal expression by 12- to 25-fold (18). Overexpression of *M. tuberculosis ethA* from the integrated plasmid had only a mild effect on ETH activity, indicating that substantial overexpression is required (Fig. S3B). Taken together, these results show that expression levels of *ethA* and *katG* play a partial role in the susceptibility toward ETH and INH.

***M. marinum* strains overexpressing *ethA* and *katG* are more susceptible to specific antibiotics.** To mimic the drug susceptibility of *M. tuberculosis* in our model organism, *ethA* and *katG* of *M. tuberculosis* were together or separately cloned into the expression vector pSMT3 and overexpressed in *M. marinum*. In addition, to facilitate our strains for utilization in high-throughput testing, we coexpressed a gene coding for tdTomato as a growth indicator (19, 20). As expected, overproduction of EthA and KatG in *M. marinum* resulted in increased sensitivity to ETH and INH, respectively, by approximately 10-fold. The effects for the constructs containing *ethA* or *katG* alone or the combination construct were similar but strictly linked to the presence of the dedicated activator, i.e., *ethA* for ETH and *katG* for INH. Together, these results show that increased activity was strictly dependent on the overexpression of the required activation enzyme. Furthermore, the new resistance levels were comparable to those for *M. tuberculosis* (Fig. 1 and 2A). As a control, we also studied the effect of overexpressing these constructs on resistance to kanamycin and rifampicin and observed that resistance levels for these two antibiotics changed <2-fold compared to the that of the empty vector control, implying that the increased susceptibility was not caused by a general effect but was due to specific bioactivation (Fig. 2A).

Next, we confirmed the drug efficacy on zebrafish larvae (*Danio rerio*) infected with either *M. marinum* wild type (WT) or *ethA-katG*-overexpressing *M. marinum*. INH failed to inhibit the growth of *M. marinum* WT at any of the concentrations tested, while the same drug lowered the amounts of *M. marinum* bacteria overexpressing *ethA* and *katG* by approximately 100-fold (Fig. 2B). Similarly, ETH sensitivity was significantly increased for the *ethA-katG*-overexpressing strain at the lowest tested concentration (0.1 μ M). In contrast, only a slight decrease in WT bacterial loads was observed for treatment with ETH at 1 μ M. These results show that also *in vivo*, our overexpressing *M. marinum* strains had specifically improved sensitivity without an apparent change in virulence.

Screening of TB Alliance library using established *M. marinum* strain. To examine the added value of our *ethA-katG*-overexpressing *M. marinum* strain for tuberculosis drug screening, we used a large library of compounds from TB Alliance. This library comprises 772 compounds demonstrated to be active against *M. tuberculosis*. To

FIG 2 *M. marinum* strain overexpressing *ethA* and *katG* showed higher susceptibility to ETH and INH. (A) *M. marinum* strains carrying pMS2-*tdTomato* (WT+*tdTomato*), pSMT3-*ethA*-*tdTomato* (WT+*tdTomato*+*ethA*), pSMT3-*katG*-*tdTomato* (WT+*tdTomato*+*katG*), and pSMT3-*ethA*-*katG*-*tdTomato* (WT+*tdTomato*+*ethA*+*katG*) were treated with rifampicin (RIF), kanamycin (KAN), ETH, or INH for 4 days. Subsequently, bacterial viability was measured via *tdTomato* signal (ex, 554 nm; em, 581 nm). MIC₅₀ of each strain against each compound was determined using GraphPad PRISM 8. Data are presented as fold difference in MIC₅₀ between WT strain and indicated strain, with the average and standard deviation between two repeats. (B) Zebrafish were infected through the caudal vein with either *M. marinum* strain carrying pMS2-*tdTomato* (WT+*tdTomato*) or pSMT3-*ethA*-*katG*-*tdTomato* (WT+*tdTomato*+*ethA*+*katG*) and treated with either ETH or INH at the indicated concentrations. Infection efficiency was examined by fluorescence microscopy based on *tdTomato* signal. Each dot represents a single zebrafish larva, and each bar represents the average of fluorescent intensity and its standard error. Positive control with DMSO and negative control with noninjected zebrafish were included. Larvae with fluorescence intensity equal to 0 were set to 1 to allow log₁₀ transformation. ns, not significant; ***, $P < 0.001$; ****, $P < 0.0001$ compared to DMSO-treated controls calculated by one-way ANOVA with Bonferroni's *post hoc* test.

evaluate whether any of these compounds were more active when *ethA* or *katG* of *M. tuberculosis* is overexpressed, the ratio of *tdTomato* fluorescent signal between the WT (WT+*tdTomato*) and overexpression strain (WT+*ethA*+*katG*+*tdTomato*) with the same compound at 10 μ M was defined as the activation ratio. Dimethyl sulfoxide (DMSO) and rifampicin were included as negative controls. Furthermore, the cutoff value for the assay was chosen as the average activation ratio of bacteria treated with DMSO and rifampicin with six times the standard deviation. As expected, ETH- and INH-treated samples showed values above the cutoff, with 5- and 10-fold increases, respectively. The Z factors were determined to be 0.63 between DMSO and ETH samples and 0.57 between DMSO and INH samples (see Fig. S4). Both Z factors demonstrated that our screening assay is reliable for screening purposes. Strikingly, in our screen, *EthA* and *KatG* overproduction significantly increased the sensitivity of *M. marinum* to 156 of the 772 compounds, which is 20% of the total compound library (Fig. 3). Among the hits which are above the cutoff value, 83 compounds with the highest activation ratio were retested with *M. marinum* overexpressing *ethA*, *katG*, or both drug activators using a conventional resazurin microtiter assay (REMA) as a growth indicator at 2.5 μ M and 5 μ M. From those retested compounds, 70 compounds (84%) showed a similar activation pattern, whereas the others were considered false positives. As a result, these data confirmed that by elevating the expression of prodrug activators, the *M. marinum* drug susceptibility profile was remarkably improved, which potentially resembles the profile of *M. tuberculosis*. Furthermore, these data also show that a large percentage of compounds active against *M. tuberculosis* are probably prodrugs.

EthA expression is required for the activity of identified hits. The activation ratio was calculated for the active compounds, and 16 compounds with the highest activation ratio (above average signal plus standard deviation) that efficiently blocked the growth of *M. marinum* overexpressing both *ethA* and *katG* and either *ethA* or *katG* were selected for further testing (Table 1). Nearly all of these compounds contain sulfur, which can be oxidized to sulfoxides or sulfones and could therefore represent a likely target for *EthA* activation (21, 22). The compounds show recurrent consistent structural features, and strikingly, all sulfur atoms are conjugated to an adjacent

FIG 3 Screening of compounds from TB Alliance library with WT and *M. marinum* overexpressing *ethA-katG*. *M. marinum* having pMS2-*tdTomato* (WT+*tdTomato*) and pSMT3-*ethA-katG-tdTomato* (WT+*ethA*+*katG*+*tdTomato*) were treated with DMSO, rifampicin (RIF), ETH, INH, or compounds from TB Alliance library at 10 μ M. After 4 days, bacterial viability was measured via *tdTomato* signal (ex, 554 nm; em, 581 nm). Activation ratio was calculated based on equating the fold difference of *tdTomato* fluorescence signal between two strains, WT+*tdTomato* and WT+*ethA*+*katG*+*tdTomato*, with the same compounds. Each dot represents the activation ratio of an individual control or sample. Cutoff value was identified as average activation ratios of wells treated with DMSO and RIF with six times the standard deviation.

nitrogen (e.g., imidoyl thiols or thioamides) (Tables 1 and 2). The structures were classified into three groups, namely, thiones, thioethers (sulfides), and thiophenes, or combinations thereof. Thiones consisted exclusively of thioureas (compounds 1 and 2). Thioethers were found as 2-(methylthio)pyrimidines (compounds 3 to 5) that could include a thiazole as well (compounds 6 to 8). The remaining thioethers were linked to five-membered heterocycles and included 1,3,4-oxadiazoles, a 1,2,4-thiazole, and a 1,3,4-thiazole (compounds 9 to 12, 13, and 14, respectively). One oxadiazole thioether had a thiophene substituent (compound 15). A (4-piperazinyl-3-nitro)benzamide (compound 16) was the only nonsulfur compound and was classified separately.

To further validate our results, six compounds, representing two structure groups activated by EthA, were chosen for further analysis. Subsequently, we tested these compounds for dose-dependent sensitivity using the single *ethA*- and *katG*-overexpressing strains. All compounds showed increased inhibition of *M. marinum* overexpressing both *ethA* and *katG* compared to that of the WT strain. Furthermore, five of them showed only more activity when the *ethA* gene was present in the construct, which makes them EthA-dependent compounds (Fig. 4A). For compound C3, the situation was less clear, as this compound also showed some increased activity when only *katG* was overexpressed, although the strongest effect was obtained by overexpression of *ethA*. Moreover, based on MIC₅₀ data, the sensitivity of *M. marinum* overexpressing *ethA* or *ethA* with *katG* toward our hit compounds was improved and closer to that of *M. tuberculosis* than to that of WT *M. marinum* (see Table S5B). Overall, EthA seems to be the most important drug activator of these two enzymes.

Next, we tested if we would observe a similar effect *in vivo*. After treatment with these compounds, zebrafish infected through the yolk with *ethA-katG*-overexpressing strains showed a significant reduction of infection burden for all compounds in a dose-dependent manner (Fig. 4B). Of these hits, C2 and C4 showed the most efficient inhibition of *M. marinum*, which was also observed in the treatment of zebrafish infected through the caudal vein (see Fig. S5). These compounds did not show toxicity to zebrafish larvae at 10 μ M, indicating that they are potent prodrug scaffolds.

To confirm our findings that EthA is important for the activation of these compounds and has a predictive value for *M. tuberculosis*, we tested four compounds on the *M. tuberculosis* WT strain, an *ethA* mutant, and the complemented strain. As

TABLE 1 List of confirmed compounds activated by EthA. Sulfurs that are proposed to be oxidized are color coded

Number	Name	Structure	Activating group	Remarks
1	4-bromo-N-[[[(4-fluorophenyl)amino]carbonothioyl]benzamide		Thiones	
2	5-(3-chlorophenyl)-N-[[[(4-methyl-1-piperazinyl)phenyl]amino]carbonothioyl]-2-furamide		Thiones	C1
3	2-Methylsulfanyl-pyrimidine-5-carboxylic acid (1-ethyl-2-methyl-1H-indol-5-yl)-amide		Thioethers	
4	2-Methylsulfanyl-pyrimidine-5-carboxylic acid [4-(benzo[1,3]dioxol-5-yloxy)-phenyl]-amide		Thioethers	
5	1-(4-tert-butylphenyl)-N-[[[2-(methylthio)-5-pyrimidinyl]methyl]-4,5,6,7-tetrahydro-1H-indazol-4-amine		Thioethers	
6	2-Methylsulfanyl-pyrimidine-5-carboxylic acid [4-(4-methoxy-2,5-dimethyl-phenyl)-thiazol-2-yl]-amide		Thioethers	
7	2-Methylsulfanyl-pyrimidine-5-carboxylic acid [4-(3-ethoxy-phenyl)-thiazol-2-yl]-amide		Thioethers	C5
8	2-Methylsulfanyl-pyrimidine-5-carboxylic acid [4-(3-fluoro-4-methoxy-phenyl)-5-methyl-thiazol-2-yl]-amide		Thioethers	
9	2-[5-(4-Methoxy-phenyl)-[1,3,4]oxadiazol-2-ylsulfanyl]-N-phenethyl-acetamide		Thioethers	
10	[5-(4-Methoxy-phenyl)-[1,3,4]oxadiazol-2-ylsulfanyl]-acetic acid propyl ester		Thioethers	C3
11	(5-m-Tolylloxymethyl)-[1,3,4]oxadiazol-2-ylsulfanyl)-acetic acid cyclohexyl ester		Thioethers	C4
12	(4-chlorophenyl)(5-[[[(5-phenyl-1,3,4-oxadiazol-2-yl)thio]methyl]-2-furyl]methanone		Thioethers	C2
13	4-[[[(2-[[[3-(methylthio)-1,2,4-thiadiazol-5-yl]thio]acetyl]amino]phenyl] acetate		Thioethers	
14	4,4'-[1,3,4-thiadiazole-2,5-diylbis(thio)]bis[1-(4-fluorophenyl)-1-butanone]		Thioethers	C6
15	(5-Thiophen-2-yl-[1,3,4]-oxadiazol-2-ylsulfanyl)-acetic acid cyclohexyl ester		Thioethers and Thiophenes	
16	N-(3-chloro-4-methylphenyl)-4-(4-methyl-1-piperazinyl)-3-nitrobenzamide		-	

TABLE 2 Structural features that are potentially activated by EthA

Activating Group	Name	Structure
1	Thiones
2	Thioethers/sulfides
3	Thiophenes
4	Unidentified	-

expected, all four compounds were active against *M. tuberculosis*. Importantly, the deletion of *ethA* resulted in resistance to ETH and also to compounds C1, C2, and C4. Susceptibility was restored when *ethA* was complemented (Fig. 4C). Interestingly, compound C3 was able to inhibit all strains, including the *ethA* deletion strain, at low doses. This might be due to the fact that C3 harbors antimycobacterial activity even in its nonactivated form. In addition, this result may also suggest an additional EthA-independent activation route of this compound in *M. tuberculosis*. In conclusion, EthA-dependent activation in *M. marinum* is representative of similar activity in *M. tuberculosis*. Combined with the rapid growth and robustness of the *M. marinum* model, this has the potential to deliver valuable data for tuberculosis drug discovery.

DISCUSSION

M. marinum has previously been used to identify antimycobacterial drugs, based on its genetic relationship with *M. tuberculosis* (9). However, several studies have reported the differences in sensitivity and resistance profiles to a number of clinical antituberculosis drugs (7, 12). This observation raised some concerns on the accuracy of *M. marinum*-based high-throughput screening, since potential active compounds can be discarded incorrectly.

In this study, we first corroborated these differences. Among the tested compounds, ETH and INH showed the highest fold difference in MIC₅₀s between *M. marinum* and *M. tuberculosis*. ETH and INH are well-known prodrugs that require conversion to their active form by the mycobacterial enzymes EthA and KatG, respectively. After being activated, ETH and INH inhibit mycobacterial mycolic acid synthesis by binding to one of the pathway core enzymes, InhA. Because InhA is highly conserved among mycobacterial species, the susceptibility differences between *M. marinum* and *M. tuberculosis* toward ETH and INH probably relate to the activities and/or expression levels of *ethA* and *katG* (23). For KatG, the story seems to be slightly different. Previously, small alterations of KatG were demonstrated to influence the activation of INH, which is also the most important basis for INH resistance (24). The KatG sequence from *M. tuberculosis* was also shown to diverge from its orthologue in *M. marinum*, leading to a higher binding capacity to INH (17). In addition, we observed that differential expressions of *katG* could also contribute to antibiotic sensitivity differences between *M. marinum* and *M. tuberculosis*. In wild-type *M. tuberculosis*, *katG* is constitutively expressed at a higher level, possibly due to the inactivation of stress regulator OxyR and the differential expression of stress regulator FurA, which explains its high susceptibility to INH (25, 26). On the other hand, we have shown that the low susceptibility of ETH in *M.*

FIG 4 Validation of compounds activated by EthA. (A) *M. marinum* transformed with pMS2 (empty) (WT), pSMT3-*ethA* (WT+*ethA*), pSMT3-*katG* (WT+*katG*), or pSMT3-*ethA-katG*-(WT+*ethA*+*katG*) was treated with six hit compounds and ETH with serial dilutions starting from (Continued on next page)

marinum was most likely due to its low expression of *ethA*, as *ethA* overexpressed by both *M. marinum* and *M. tuberculosis* showed the same efficiency in converting ETH to its active form. This discovery is further supported by the fact that many clinical ETH-resistant *M. tuberculosis* strains possess mutations in the *ethA* promoter region which eventually lower the expression level of *ethA* (27).

We overproduced EthA and KatG originating from *M. tuberculosis* in *M. marinum* to mimic the drug susceptibility of *M. tuberculosis*. As expected, EthA and KatG overproduction sensitized *M. marinum* to ETH and INH. This is in line with previous studies overexpressing either the *ethA* or *katG* gene in *Mycobacterium smegmatis* or *Mycobacterium bovis*, which indicated that the higher resistance to INH and ETH in these species is because of the lower expression levels of EthA and KatG, rather than the difference in the drug target (28, 29). Our new strain overexpressing these two prodrug-activating enzymes also showed higher susceptibility to ETH and INH *in vivo* in infected zebrafish larvae.

Subsequently, we established an assay for using *M. marinum* to examine the effect of overexpressing *ethA* and *katG*. The overexpression strain showed significantly increased sensitivity compared to that of the WT to approximately 20% of the 772 compounds. This result suggests that a large proportion of the compounds used in the library are probably prodrugs. In general, prodrugs can be activated either by host metabolic pathways or by bacterial enzymes. With bacterium-mediated activation, prodrugs are more advantageous, since they are stably delivered and selectively active in the target bacteria and often accumulate inside the target cell (22). As a result, the utilization of prodrugs can reduce undesired toxicity to host cells. The disadvantage of prodrugs is that the bacteria could develop resistance by mutating or downregulating the involved activators (30). This phenomenon is commonly observed for both ETH and INH (31). In addition, overexpression of EthR, a regulator that suppresses EthA expression, was also involved in ETH resistance (32). This screening assay can be used to identify possible prodrugs activated by EthA and KatG. In many cases, since *ethA* and *katG* mutations already exist in different forms in MDR-TB and XDR-TB, a specific drug that requires activation by EthA or KatG is most likely not the most suitable candidate for further advancement into clinical testing (33). However, in several studies, the usage of ETH in combination with an inhibitor of EthR has been proven to effectively tackle the ETH resistance mechanism mediated through EthA downregulation (34, 35).

Our screen showed a major bias toward EthA activation. The compounds activated by EthA in our screens are characterized by the frequent presence of sulfur. Recurrent motifs are thiones and thioethers, in which the sulfur is conjugated to a nitrogen atom. These observations are in agreement with reported EthA-activated compounds (22). In addition to the thioamide ETH, EthA was demonstrated to catalyze the oxidation of thioureas isoxyl or thioacetazones as well (thione class) (36, 37). Furthermore, thioethers and thiophene-based compounds were found to be oxidized by EthA to sulfoxides and sulfones, respectively (21, 38). Thiazolyl and thiadiazolyl groups were frequent side groups in the hit compounds (5 out of 16). Although these sulfur heterocycles structurally resemble thiophenes, their prominence might be due to a synthetic bias in the library, as no further EthA-mediated thia(dia)zole oxidations have been reported and no thia(dia)zoles without an additional activating group (i.e., a thioether) were found

FIG 4 Legend (Continued)

20 μ M. After a 4-day incubation, bacterial viability was measured by resazurin microtiter assay (REMA). Data points represent averages from duplicates with standard deviations. (B) Zebrafish (*Danio rerio*) embryos were infected in the yolk with *M. marinum* carrying either pMS2-*tdTomato* (WT+*tdTomato*) or pSMT3-*ethA-katG-tdTomato* (WT+*ethA*+*katG*+*tdTomato*) and treated with ETH, INH, and 6 hit compounds at the three indicated concentrations. Zebrafish infection efficiency was examined by fluorescence microscopy based on *tdTomato* signal. Each dot represents a single zebrafish larva, and each bar represents the mean of fluorescent intensity of the sample together with its standard error. Positive control with DMSO and negative control with noninjected zebrafish were included. Fluorescence intensities equal to 0 were set to 1 to allow $\log_{10}$ transformation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ compared to DMSO-treated controls calculated by one-way ANOVA with Bonferroni's *post hoc* test. (C) *M. tuberculosis* WT, *M. tuberculosis* with *ethA* deletion (Δ *ethA*), and *M. tuberculosis* with *ethA* deletion transformed with pMS2-*ethA* (Δ *ethA* + *ethA*) were dropped in serial dilutions on 7H10 plates supplemented with 10% ADS and DMSO, ETH (10 μ M), C1 (10 μ M), C2 (40 μ M), C3 (10 μ M), or C4 (40 μ M) diluted at 40 μ M. After 14 days, plates were imaged.

in the screen (39). The same was observed for our single thiophene hit (compound 15). Combined, this suggests either that EthA did not act on the sulfur-based heterocycles in the tested libraries or that their conversion did not enhance the antimycobacterial activity of the compounds. A non-sulfur-containing piperazinyl-nitrobenzamide (compound 16) appeared to be activated by EthA as well, which might relate to reported conversion of ketones by purified EthA (40).

Our selected hit compounds from the library showed increased activity upon overexpression of EthA in *M. marinum* both *in vitro* and *in vivo*, indicating that these scaffolds are interesting for further analysis. Furthermore, based on MIC₅₀ data and the drug susceptibility of a $\Delta ethA$ *M. tuberculosis* strain, these compounds also showed the same EthA dependency and high activity in *M. tuberculosis*. The only exception was compound C3. One explanation for the activity of C3 is that this compound may have an additional target in *M. tuberculosis* for which activation is not required. Another explanation is that this compound is activated by enzymes other than EthA. In several recent studies, activity loss of another Baeyer-Villiger oxygenase, MymA, or glycosyl-transferase MshA was shown to confer resistance levels equivalent to that with *ethA* deletion in *M. tuberculosis* (21, 41). In fact, we determined that the most homologous protein to MymA (Rv3083) based on amino acid conservation in *M. marinum* is in fact EthA, implying that MymA does not exist in *M. marinum* (data not shown). Collectively, it was proposed that under several certain conditions, enzymes other than EthA can play a more dominant role in converting ETH into active forms (42). Furthermore, in *M. marinum*, we already observed some effects of *katG* overexpression on C3 activity. Nevertheless, EthA is still the most dominant drug activator for ETH as shown by its high prevalence in MDR-TB and XDR-TB. Nevertheless, follow-up studies are necessary to confirm possible EthA-independent activation pathways for C3.

In summary, this study has successfully generated *M. marinum* strains as a screening model, showing improved sensitivity to not only clinical antituberculosis drugs but also potential new drugs, which are easily missed using normal *M. marinum* strains. In addition, with these strains, we can now also get a rapid evaluation of prodrugs within screening libraries. With their simplicity in handling and high-throughput property, these strains can be beneficial to readily uncover novel antimycobacterial prodrugs and, additionally, in early stages of TB drug screenings, to study which structural features are sensitive to metabolic conversion.

MATERIALS AND METHODS

Construction of plasmids and strains. All plasmids and primers used within this study can be found in Tables S1 and S2, respectively, in the supplemental material. Plasmids in this study were constructed using standard molecular cloning techniques summarized in Fig. S1A. The *ethA* deletion strain of *M. tuberculosis* was constructed by homologous recombination, as previously described, and confirmed by PCR using primer pairs B212/B136 and B211/B137 (Fig. S1B and C) (43).

Bacterial strains, media, growth condition and reagents. All bacterial strains used in this study are listed in Table S3. *Escherichia coli* DH5 α was used to propagate constructed plasmids. *E. coli* was grown in LB medium or on LB agar plates at 37°C with the addition of hygromycin (50 μ g/ml) when appropriate. *M. marinum* MUSA and *M. tuberculosis* H37Rv were routinely cultured at 30°C and 37°C, respectively, in 7H9 medium or on 7H10 (Difco) supplemented with 10% ADS (0.5% bovine serum albumin [BSA], 0.2% dextrose, 0.085% NaCl), 0.02% tyloxapol, and hygromycin (50 μ g/ml), where indicated. Isoniazid, ethionamide, rifampicin, kanamycin, ethambutol (all purchased from Sigma), bedaquiline, linezolid, sutezolid, nitazoxanide, and macozinone (all purchased from MedChemExpress) were dissolved in DMSO at the stock concentration of 10 mM. The TB Alliance compound library was a gift from TB Alliance (New York, NY, USA). Hit compounds were reordered for further validation from sources listed in Table S4.

High-throughput screening using Tb Alliance library. Compounds of the TB Alliance library were added to a final concentration of 10 μ M in 96-well round-bottom plates. *M. marinum* MUSA strains (transformed with pSMT3-*ethA-katG-tdTomato* or pMS2-*tdTomato*) were cultured as described above to mid-logarithmic phase. Cells were then collected, washed in phosphate-buffered saline (PBS)-0.02% tyloxapol and added to each well at the final optical density at 600 nm (OD₆₀₀) of 0.001. Plates were sealed and incubated at 30°C. Five days later, bacterial pellets were resuspended and shaken (30 min, 30°C). Finally, bacterial growth was determined using a Biotek plate reader at 30°C via *tdTomato* signal (excitation [ex], 554 nm; emission [em], 581 nm). Subsequently, cell viability was plotted on a dose-response curve and analyzed by GraphPad PRISM version 8.1.1 (GraphPad Software Inc., San Diego, CA, USA), and MIC₅₀ was determined using a nonlinear regression equation (log [inhibitor]) versus response,

with a variable slope. For the high-throughput screening of the TB Alliance library, the following formula was used to calculate the activation ratio of an individual compound:

$$\text{Activation ratio} = \frac{\text{tdTomato (WT + tdTomato)}}{\text{tdTomato (WT + tdTomato + ethA + katG)}}.$$

Dose-response assays. Selected compounds or antibiotics were 2-fold serially diluted in 96-well plates. *M. marinum* MUSA strains (transformed with pMS2, pSMT3-*ethA*, pSMT3-*katG*, or pSMT3-*ethA-katG*) or *M. tuberculosis* was grown to mid-logarithmic phase, harvested by centrifugation, washed in PBS-0.02% tyloxapol, and added to each well at the final OD₆₀₀ of 0.001. Plates were sealed and incubated for 4 days (*M. marinum*) or 7 days (*M. tuberculosis*). Subsequently, development solution (200 μM resazurin, 10% Tween 80) was added to each well. Plates were then incubated at 30°C for 3 h (*M. marinum*) or overnight (*M. tuberculosis*) at 37°C. After incubation, fluorescence intensity which reflects bacterial viability was measured using a Biotek plate reader (ex, 560 nm; em, 590 nm). Percent bacterial viability was calculated by setting DMSO-treated wells to 100%.

To compare susceptibilities of different *M. tuberculosis* strains, the bacteria were grown to mid-logarithmic phase, normalized by optical density, and plated in 10-fold serial dilution on 7H10 plates supplemented with ADS. Plates were incubated at 37°C and imaged after 14 days.

In vivo drug testing on zebrafish infected with *M. marinum* by caudal vein injection or yolk injection. Injection stocks of *M. marinum* M^{USA} strains (transformed with pMS2-*tdTomato*, or pSMT3-*ethA-katG-tdTomato*) were prepared in PBS with 20% glycerol, aliquoted, and stored at −80°C. For caudal vein injection, zebrafish (*Danio rerio*) embryos were injected with approximately 280 CFU/nl as previously described (44). For yolk injection, infection of zebrafish embryos was performed by using an automated microinjection system (Life Science Methods BV) as described previously (45). Zebrafish embryos were injected at the 2- to 8-cell stage with 1 nl of bacterial suspension (95 to 120 CFU) mixed with the fluorescent dye fluorescein (2.5 μg/ml) to visualize the injection process. Successfully injected embryos were selected by detection of the green fluorescence signal. Infected embryos were washed and incubated in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.4 mM MgCl₂) with 3 × 10^{−6}% methylene blue. At 1-day postinfection, zebrafish larvae were treated with test compounds, which were diluted in chorion water (60 μg/ml instant ocean sea salts) at the designated concentration and incubated at 28°C. At 3 days and 4 days postinfection, the infection efficiency was examined for yolk-injected and caudal vein-injected samples, respectively, and documented using an inverted Olympus IX83 fluorescence microscope. Granuloma formation was quantified using CellProfiler 3.15 (Broad Institute, Cambridge, MA, USA) with a custom-made pipeline to count pixels as previously described (46).

Statistical analysis. During zebrafish infection experiments, the data points were log-transformed before a one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test was used to determine differences between DMSO-treated zebrafish and zebrafish treated with tested compounds. Statistical analysis within the study was performed using GraphPad Prism version 8.1.1 (GraphPad Software Inc., San Diego, CA, USA).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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We declare no conflicts of interest.

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