Potency boost of a *Mycobacterium tuberculosis* dihydrofolate reductase inhibitor by multienzyme $F_{420}\text{H}_2$-dependent reduction

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Triaza-coumarin (TA-C) is a *Mycobacterium tuberculosis* (Mtb) dihydrofolate reductase (DHFR) inhibitor with an IC$_{50}$ (half maximal inhibitory concentration) of $\sim$1 $\mu$M against the enzyme. Despite this moderate target inhibition, TA-C shows exquisite antimycobacterial activity (MIC$_{90}$ concentration inhibiting growth by $50\% = 10$ to 20 nM). Here, we investigated the mechanism underlying this potency disconnect. To confirm that TA-C targets DHFR and investigate its unusual potency pattern, we focused on resistance mechanisms. In Mtb, resistance to DHFR inhibitors is frequently associated with mutations in thymidylate synthase (*thyA*), which sensitizes Mtb to DHFR inhibition, rather than in DHFR itself. We observed *thyA* mutations, consistent with TA-C interfering with the folate pathway. A second resistance mechanism involved biosynthesis of the redox coenzyme $F_{420}$. Thus, we hypothesized that TA-C may be metabolized by Mtb $F_{420}$-dependent oxidoreductases (FDORs). By chemically blocking the putative site of FDOR-mediated reduction in TA-C, we reproduced the $F_{420}$-dependent resistance phenotype, suggesting that $F_{420}\text{H}_2$-dependent reduction is required for TA-C to exert its potent antibacterial activity. Indeed, chemically synthesized TA-C-Acid, the putative product of TA-C reduction, displayed a 100-fold lower IC$_{50}$ against DHFR. Screening seven recombinant Mtb FDORs revealed that at least two of these enzymes reduce TA-C. This redundancy in activation explains why no mutations in the activating enzymes were identified in the resistance screen. Analysis of the reaction products confirmed that FDORs reduce TA-C at the predicted site, yielding TA-C-Acid. This work demonstrates that intrabacterial metabolism converts TA-C, a moderately active “prodrug,” into a 100-fold more-potent DHFR inhibitor, thus explaining the disconnect between enzymatic and whole-cell activity.

*Mycobacterium tuberculosis* | DHFR | antibacterial | $F_{420}$

**Tuberculosis** (TB) is a major infectious disease killer globally. It affected 10 million and killed 1.4 million people in 2019 alone (1). The predicted impact of the COVID-19 pandemic is an additional 190,000 TB deaths in 2020, and it is expected in the next 5 y that there will be up to a 20% increase in the global TB disease burden (2), stressing the critical need for new safe and effective drugs against the causative agent, *Mycobacterium tuberculosis* (Mtb). In addition, controlling multidrug-resistant TB (MDR-TB) presents a huge public health challenge (1).

Dihydrofolate reductase (DHFR) is a ubiquitous enzyme in bacteria, parasites, and humans. The protein catalyzes the NADPH-dependent conversion of dihydrofolate into tetrahydrofolate, a methyl group shuttle required for the synthesis of many cellular building blocks including thymidylate, purines, and certain amino acids. Several DHFR inhibitors are in clinical use for the treatment of various infectious diseases and cancer (3, 4). However, approved DHFR inhibitors have only weak or no activity against Mtb, and there are no DHFR inhibitors used clinically for the treatment of TB (5).

Recently, DHFR was clinically validated as a vulnerable Mtb target. The old TB drug para-aminosalicylic acid (PAS) was long thought to inhibit dihydropteroate synthase (DHPs, FoP), the first enzyme in the folate pathway. However, it was uncovered that PAS, a hydroxy analog of DHPs’s substrate para-aminobenzoic acid, is a substrate of DHPs, which catalyzes the formation of hydroxy-dihydrofolate, which in turn inhibits DHFR (6–10). Thus, PAS is a prodrug that, upon metabolism by an enzyme of the folate pathway, is converted into a DHFR inhibitor.

With Mtb DHFR presenting an attractive target, the enzyme has been the subject of renewed drug discovery efforts (5, 11–13), reviewed recently by He et al. (14). A major hurdle encountered in many DHFR drug discovery programs consists in achieving selectivity toward human DHFR (14). Through ligand-based drug design starting from a Triazaspiro scaffold and a side chain extension approach, we previously identified selective inhibitors of Mtb DHFR (15). The most potent and selective analog resulted in a 100-fold enhancement of the potency pattern, suggesting that the intrabacterial metabolism of a Mtb dihydrofolate reductase (DHFR) inhibitor with moderate affinity for its target boosts its on-target activity by two orders of magnitude. This is a "prodrug-like" antimycobacterial that possesses baseline activity in the absence of intracellular bioactivation. By elucidating the metabolic enhancement mechanism, we have provided the basis for the rational optimization of a class of DHFR inhibitors and uncovered an antibacterial drug discovery concept.

**Significance**

Bacterial metabolism can cause intrinsic drug resistance but can also convert inactive parent drugs into bioactive derivatives, as is the case for several antimycobacterial prodrugs. Here, we show that the intrabacterial metabolism of a Mtb dihydrofolate reductase (DHFR) inhibitor with moderate affinity for its target boosts its on-target activity by two orders of magnitude. This is a "prodrug-like" antimycobacterial that possesses baseline activity in the absence of intracellular bioactivation. By elucidating the metabolic enhancement mechanism, we have provided the basis for the rational optimization of a class of DHFR inhibitors and uncovered an antibacterial drug discovery concept.

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from attachment of a coumarin fragment to the triaza scaffold (Triazaspiro-Coumarin [TA-C]; Fig. 1). TA-C inhibited recombinant Mtb DHFR with an IC50 of ~1 μM and showed an acceptable selectivity index for the human enzyme (IC50 human DHFR/IC50 Mtb DHFR = 18). The selectivity indices for cytotoxicity (CC50 HepG2/MIC50) and hemolysis (LC50 red blood cells/MIC50) were ~4,000. Surprisingly, TA-C exhibited excellent whole-cell activity against Mtb and its closely related vaccine strain. Mtb encodes a second, ThyX-type thymidylate synthase. ThyX regenerates tetrahydrofolate instead of dihydrofolate following catalysis. In contrast to ThyX-mediated conversion of dUMP to dTMP, ThyA-mediated conversion results in an increased demand for DHFR activity to provide sufficient levels of tetrahydrofolate and therefore sensitizes the pathway to DHFR inhibition. Loss-of-function mutations in thyA reduce the demand for DHFR activity and are therefore associated with decreased susceptibility to DHFR inhibitors (4, 10, 16, 17, 20). It is interesting to note that missense resistance mutations in the dfrA gene encoding DHFR have not been reported in Mtb. Amino acid alterations that would prevent inhibitor binding to the active site of DHFR are likely deleterious to overall enzymatic function and are thus not tolerated by the bacterium (21).}

The whole-cell activity or MIC50 of TA-C is 50- to 100-fold higher than its IC50 against the isolated Mtb DHFR protein. Here, our aim was to confirm DHFR inhibition as the primary mechanism of action of TA-C and to shed light on the peculiar on-target/whole-cell potency disconnect. Resistance to the prodrug DHFR inhibitor PAS has been extensively studied and recently discussed in comprehensive reviews by Baughn and colleagues (4, 16). PAS resistance can emerge via multiple mechanisms that include preventing efficient bioactivation within the folate synthesis pathway, mitigating the impact of target inhibition, and limiting drug accumulation within the bacilli. A common mechanism, identified by transposon mutagenesis (17), isolation of spontaneous resistant mutants in vitro (18), and genetic works (19), is associated with mutations in thyA, one of the genes encoding thymidylate synthase. Thymidylate synthase is required for the methylene-tetrahydrofolate–dependent conversion of dUMP to dTMP. In most organisms, this reaction is performed by a ThyA-type thymidylate synthase that releases dihydrofolate following catalysis. Dihydrofolate must be reduced by DHFR to re-enter the folate metabolism. Mtb encodes a second, ThyX-type thymidylate synthase. ThyX regenerates tetrahydrofolate instead of dihydrofolate following catalysis.
show that loss of $F_{430}$ causes resistance to TA-C and that $F_{420}$ is required for TA-C's potent whole-cell activity but did not identify exactly how $F_{420}$ is involved in TA-C's activation.

**Low-Frequency TA-C Resistance Is Caused by Missense and Nonsense Mutations in thyA.** If TA-C indeed decreases folate synthesis by inhibiting DHFR, we expected TA-C-resistant mutants in folate metabolism genes at a frequency of $10^{-5}$/CFU (22). Thus, we speculated that out of 100 TA-C-resistant mutants emerging at a frequency of $10^{-5}$/CFU, 99 should carry a defective Fdor pathway, and one should harbor a mutation in the folate pathway. To test this hypothesis, we carried out a counter screen of a collection of 1,000 TA-C-resistant bacillus Calmette–Guérin strains for susceptibility to pretomanid. As expected, 994 strains were pretomanid resistant, presumably harboring lesions in $F_{430}$–biosynthesis genes. The six TA-C–resistant and pretomanid-sensitive strains showed a ∼100-fold increase in MIC$_{50}$ for TA-C (Table 2). Whole-genome sequencing, confirmed by targeted sequencing, revealed that all six strains carried missense or nonsense mutations in $thyA$ (Table 2). Genetic complementation of a representative strain confirmed that a $thyA$ polymorphism caused TA-C resistance (SI Appendix, Table S1). Furthermore, the $thyA$ mutations conferred cross-resistance to PAS but not to the control drugs isoniazid and rifampicin (Table 2). Together, these results show that $thyA$ mutations cause resistance to TA-C and emerge at a frequency of $10^{-5}$/CFU. These results suggest that the DHFR inhibitor TA-C exerts its antibacterial activity by interfering with folate metabolism.

**Chemically Blocking the Putative TA-C Site of Fdor Reduction Phenocopies $F_{430}$.** We first investigated whether $F_{430}$ could be involved in the reduction of TA-C by Fdors, as is the case with nitroimidazole prodrugs, generating a derivative with enhanced antibacterial activity. Revisiting our published structure–activity relationship around the triazaspiro scaffold, we noted that attachment of the coumarin group to the triazaspiro core resulted in a moderate increase of biochemical activity and a strong increase of whole-cell activity (15). We speculated that TA-C's coumarin group may be the site of intrabacterial reduction by Fdors. It has been shown that the α,β-unsaturated carbonyl motif of coumarins can be reduced by enzymes within the Fdor superfamily (30–32). Thus, we hypothesized that Mtb Fdors reduce the coumarin moiety of TA-C, producing an activated derivative with increased whole-cell activity (Fig. 1). If correct, a TA-C analog in which this “reducible” site is blocked by chemical modification should copy the $F_{430}$–dependent resistance phenotype. To test this hypothesis, we generated the TA-C analog TA-C-Met, in which reducibility of the double bond was blocked by methylation of the β-carbon (Fig. 1) (30–32). TA-C-Met retained an IC$_{50}$ of ∼1 μM against recombinant Mtb DHFR, similar to TA-C (Table 3). Thus, any altered whole-cell activity of TA-C-Met should be due to the loss of reduction since on-DHFR–target activity was preserved. As predicted, the MIC of TA-C-Met was ∼100-fold higher compared to TA-C (Tables 1 and 3), around 2 to 3 μM, reproducing the TA-C resistance level of $F_{430}$ mutants (Tables 1 and 3). Thus, the MIC of TA-C-Met was not affected by mutations causing loss of $F_{430}$ (Tables 1 and 3). Together, these results indicate that TA-C is converted to an active derivative via Fdor–mediated reduction of the α,β-unsaturated carbonyl motif of its coumarin moiety and that $F_{430}$–associated resistance is due to the loss of TA-C reduction.

**The Putative $F_{420}$-Dependent Reduction Product of TA-C, TA-C-Acid, is a 10-nM DHFR Inhibitor.** Analysis of the substrate range of FDors with coumarin substrates has shown that reduction of the α,β-unsaturated carbonyl motif generates an unstable product that undergoes spontaneous ring opening by hydrolysis, which in turn releases a carboxylic acid coumarin derivative (30–32). Thus, we speculated that Mtb FDors were the most likely enzyme class to carry out the analogous conversion of TA-C via a reduced intermediate (TA-C-Red) to generate TA-C-Acid (Fig. 1) and that TA-C-Acid may display increased activity against DHFR, thus explaining the enzyme/whole-cell potency disconnect. We synthesized TA-C-Acid and measured an IC$_{50}$ of ∼10 nM against recombinant Mtb DHFR, showing that the putative reduction product of TA-C was 100-fold more potent than its parent against the enzyme (Table 3). Consistent with these biochemical results, structural modeling, in which TA-C and TA-C-Acid were computationally docked into the active site of Mtb DHFR (SI Appendix, Table S2 and Fig. S2), suggested that the carboxylic acid moiety of TA-C-Acid increases molecular interactions with the DHFR binding site, thus allowing tighter binding (Fig. 2 A and B).

The MIC$_{50}$ of TA-C-Acid was 0.8 μM against bacillus Calmette–Guérin, lower than the MIC$_{50}$ of TA-C-Met but significantly higher than the MIC of TA-C (Table 3). However, this is likely due to decreased intrabacterial uptake caused by the (charged)

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**Table 1.** TA-C-resistant *M. bovis* bacillus Calmette–Guérin and *M. tuberculosis* strains emerging at a frequency of $10^{-5}$/CFU

| Exp* | Strain | TA-C | PAS | INH† | Rif† (nM) | PTM† | TA-C-Met | Gene with polymorphism | Mutation | Mutation type§ |
|------|--------|------|-----|------|-----------|------|----------|------------------------|----------|---------------|
|      | wt     | 0.02 | 0.8 | 0.4  | 1.6       | 0.2  | 3.2      | —                      | —        | —             |
| 1 Tac1.1 | 3.2 | 0.8 | 0.4 | 2 >100 | 3.2 | fbiC | Δ2269GC | fs |
| Tac1.2 | 3.2 | 0.8 | 0.4 | 3 >100 | 3.2 | fbiC | Δ472A | fs |
| Tac1.3 | 3.2 | 0.8 | 0.4 | 2 >100 | 3.2 | fbiC | Δ1142C | fs |
| 2 Tac2.1 | 1.6 | 0.4 | 0.3 | 1.6 >100 | 1.6 | fbiC | Δ1983G | fs |
| Tac2.2 | 3.2 | 0.4 | 0.4 | 1.6 >100 | 3.2 | fbiA | T326C, L109P | ms |
| Tac2.3 | 3.2 | 0.4 | 0.4 | 1.6 >100 | 3.2 | fbiA | G928t, G310Stop | ns |
| 3 Tac3.1 | 3.2 | 0.8 | 0.3 | 3 >100 | 3.2 | fbiA | C465A, Y155Stop | ns |
| Tac3.2 | 3.2 | 0.8 | 0.5 | 1.6 >100 | 3.2 | fbiC | Δ932A | fs |
| Tac3.3 | 3.2 | 0.4 | 0.4 | 1.6 >100 | 3.2 | fbiC | Δ1084C | fs |
| 4 Tac4.1 | 1.6 | 0.2 | 0.4 | 1.6 >100 | 1.6 | fbiC | Δ1976C | fs |
| Tac5.1 | 3.2 | 0.4 | 0.4 | 1.6 >100 | 3.2 | fbiC | Δ3086G, L103R | ms |
| 5 Tac6.1 | 1.6 | 0.2 | 0.4 | 1.6 >100 | 1.6 | fbiC | Δ2269GC | fs |

*Exp*, independent resistance selection experiments.
†MIC experiments were carried out three times independently and mean values are shown.
‡Inh, isoniazid; Rif, rifampicin; PTm, pretomanid.
§fs, frameshift; ms, missense; ns, nonsense mutation.

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activation can prevent evolution of resistance). If correct, this
suggests that if multiple enzymes can activate the drug, they would all
be relatively promiscuous and active with a range of synthetic
compounds (31, 32, 35). In the context of pretomanid activation, Ddn (24). This enzyme family is known to
include the nitroimidazole reductase responsible for
dependent enzymes (34), 15 of which belong to the FDOR su-
pport the F420 biosynthetic pathway and not in FDORs
that includes Ddn, as well as representatives of the A2, B1, B3, and
species, we also tested five recombinant enzymes from
that we followed the oxidation of F420H2 (SI Appendix, Table S3).
We identified two enzymes from the A1 subgroup that could ef-
ciently catalyze the reduction of TA-C in vitro, Ddn and Rv1558 (370 ± 14 and 152 ± 4 nmol · min⁻¹ · umol⁻¹ · enzymes⁻¹, respectively) (SI Appendix, Table S3 and Fig. S3A). We also used the change in
fluorescence of coumarin upon reduction to confirm that this ap-
parent activity was a result of the reduction of the coumarinyl
moiety (SI Appendix, Fig. S3B). We then measured the enzyme
kinetics of Ddn and Rv1558 with TA-C revealing k_cat/K_M values
of 1.71 × 10³ M⁻¹ · s⁻¹ and 3.09 × 10³ M⁻¹ · s⁻¹, respectively (Table 3). To examine whether this promiscuous activity of FDORs
with TA-C was likely to be common across other mycobacterial
species, we also tested five recombinant enzymes from Mycobacterium smegmatis, identifying two (MSMEG_5998 and MSMEG_2027) that are orthologs of Ddn and Rv1558, respectively, from the A1 subgroup that had activity (SI Appendix, Table S3). Interestingly,

### Multiple Mtb F420 Oxidoreductases Reduce TA-C to TA-C-Acid

Results so far indicated that TA-C is a weak inhibitor of DHFR and is
converted intracellularly by FDORs to the highly potent
dihydrofolate reductase inhibitor by

### Functional Redundancy Would Explain Our Failure to Isolate FDOR Mutants Resistant to TA-C

To test this hypothesis, we screened a panel of seven recombinant
FDORs from Mtb, including all members of the A1 subgroup
that includes Ddn, as well as representatives of the A2, B1, B3, and
B4 subgroups against TA-C using a spectrophotometric assay in
which we followed the oxidation of F420H2 (SI Appendix, Table S3).
We identified two enzymes from the A1 subgroup that could ef-
ciently catalyze the reduction of TA-C in vitro, Ddn and Rv1558 (370 ± 14 and 152 ± 4 nmol · min⁻¹ · umol⁻¹ · enzymes⁻¹, respectively) (SI Appendix, Table S3 and Fig. S3A). We also used the change in
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### Table 3. The Rate of Reduction of TA-C, TA-C-Met, and TA-C-Acid by FDORs, their MIC₉₀ against M. bovis bacillus Calmette–Guérin, and IC₉₀ against DHFR

| Compound | TA-C | TA-C-Met | TA-C-Acid |
|----------|------|----------|-----------|
| Mtb FDOR Kinetics | | | |
| Ddn | 5.95 × 10⁻⁴ ± 2.37 × 10⁻⁴ | N/D | N/D |
| k_cat (s⁻¹⁻¹) | 3.47 ± 0.51 | N/D | N/D |
| k_cat/K_M (M⁻¹ · s⁻¹⁻¹) | 3.09 × 10³ | N/A | N/A |
| Rv1558 | 3.09 × 10⁻³ ± 1.48 × 10⁻⁴ | N/D | N/D |
| k_cat (s⁻¹⁻¹) | 1.01 ± 1.6 | N/D | N/D |
| k_cat/K_M (M⁻¹ · s⁻¹⁻¹) | 3.09 × 10² | N/A | N/A |
| MIC₉₀ against bacillus Calmette–Guérin (µM) | | | |
| Wt | 0.02 | 3.2 | 0.8 |
| F₄₉₀ mutant (Tac’1.1) | 3.2 | 3.2 | 0.4 |
| thyA mutant (Tac’7.2) | 1.6 | 3.2 | >25 |
| DHFR over-expresser | 1.6 | 1.6 | >25 |
| DHFR IC₉₀ (µM) | | | |
| M. tuberculosis | 0.93 ± 0.12 | 0.63 ± 0.07 | 0.009 ± 0.001 |
| H. sapiens | 10.5 ± 1.9 | N/A | 1.73 ± 0.14 |

N/D = Below the level of detection.
N/A = Not analyzed.

*Experiments were carried out three times independently and mean values are shown.
†Refer to Tables 1 and 2.
‡Described in ref. 15.
the activity with TA-C appears to be limited to enzymes of the A1 subgroup, where Ddn and the 39% identical enzyme Rv1558 displayed significant activity. Despite reasonably high sequence identity (>34%), neither enzyme of the A2 subgroup (Rv1261c and MSMEG_5030) displayed activity. Because we could not obtain soluble, folded, and active protein for several of the FDORs, we cannot exclude the possibility that other enzymes in addition to Ddn and Rv1558 could activate TA-C. However, given the restricted activity to the A1 subgroup, the only plausible candidate is Rv3178, albeit unlikely given that it is less similar to Ddn than the inactive A2 enzymes (29% identity).

To investigate the activation of TA-C by these enzymes in more detail, we solved the structure of Rv1558, in complex with F420, to a resolution of 2.47 Å (SI Appendix, Fig. S4 and Table S4). We were unable to capture the ternary complex between substrate, cofactor, and enzyme. As expected, Rv1558 adopts the split B-barrel fold typical of the FDOR superfamily (37) and is almost identical to the structure of Ddn at the level of the overall fold (rmsd C-α = 0.7 Å) (SI Appendix, Fig. S4). The N-terminal helix is fully resolved, unlike in the structure of Ddn, in which it is truncated. The active site is likewise very similar, with key catalytic residues such as Ser78, Tyr130, and Tyr136 (Ddn numbering) being conserved (38) and relatively conservative changes at other positions, such as Tyr65(Met) and Tyr130(Trp). TA-C was docked into the active sites of both proteins and adopts a catalytically productive pose (SI Appendix, Table S2 and Fig. S2), with the coumarin moiety positioned directly above the deazaflavin ring of F420 (Fig. 2 C and D). Triplicate 200-ns molecular dynamics simulations were then performed to confirm that these enzyme-substrate complexes were stable over a reasonable timeframe for catalysis to occur (SI Appendix, Fig. S5). These findings provided direct biochemical and supporting structural evidence that TA-C is recognized as a substrate and metabolized by FDORs. The finding that at least two different FDORs accept TA-C as substrate explains the absence of TA-C-resistant strains harboring mutations in an FDOR. We also tested whether our tool compound TA-C-Met, in which reducibility of the α,β-unsaturated carbonyl motif was blocked, is metabolized by Ddn or Rv1558. As expected, TA-C-Met was not reduced by either enzyme, confirming that methylation of TA-C’s coumarin motif blocks reduction (Table 3).

A key assumption of our model is that the Mtb FDORs Ddn and Rv1558 reduce the α,β-unsaturated carbonyl motif of TA-C’s coumarin ring to produce TA-C-Red and that TA-C-Red undergoes spontaneous hydrolysis to TA-C-Acid as the reaction end product. Using chemically synthesized standards of TA-C, TA-C-Red, and TA-C-Acid, we followed the enzymatic conversion of TA-C to TA-C-Acid by Ddn and Rv1558 using liquid chromatography/mass spectrometry (LC/MS). In the case of Ddn, analysis of the reaction products revealed that the majority of the substrate (TA-C) has been converted to product after 1-h incubation, which had an m/z of 591.3 Da, consistent with TA-C-Acid. Two smaller peaks were present, one of which corresponded to the mass of the reduced intermediate species TA-C-Red, which had a m/z of 573.29 Da, consistent with hydride transfer to the substrate peak (TA-C),
which had an n/z of TA-C (571.27 Da) (Fig. 2E and SI Appendix, Fig. S6). In the case of Rv1558, the conversion was slower, consistent with the enzyme kinetics (Table 3), and the intermediate peak was not observed; this would be expected if the spontaneous conversion of TA-C-Red to TA-C-Acid occurs more rapidly than the conversion of TA-C to TA-C-Red in this instance. Altogether, these results confirm that TA-C is indeed reduced by FDORs at the α,β-unsaturated carbonyl motif in TA-C’s coumarin moiety and that the reduced form of TA-C spontaneously undergoes a ring-opening reaction to form TA-C-Acid as the end product.

To confirm that the conversion of TA-C to TA-C-Acid also occurs in intact bacteria, we treated M. bovis bacillus Calmette–Guérin culture with TA-C and observed time-dependent generation of TA-C-Acid with concurrent reduction of TA-C levels (SI Appendix, Fig. S7).

Previously, we showed that TA-C had specificity toward the Mtb DHFR over the human DHFR (15). Although the intracellular activation of TA-C within Mtb will greatly increase the specificity of this compound (25), we tested whether TA-C-Acid retained selectivity toward Mtb by testing its inhibition of human DHFR (Table 3). We observed that although TA-C-Acid inhibited human DHFR with greater affinity than TA-C (1.7 ± 0.1 versus 10.5 ± 1.9 μM, respectively), the increase in affinity was less than observed for the multienzyme F420H2-dependent reduction (Table 3). Thus, the FDOR-dependent activation of TA-C increases the selectivity to 188-fold for Mtb DHFR versus human DHFR.

To lay the ground for further compound optimization, we determined the pharmacokinetic (PK) profile of TA-C and TA-C-Acid following administration via the intravenous and oral routes. We observed no detectable oral bioavailability (SI Appendix, Fig. S8), which appears to be due to poor permeability, based on the intravenous PK profile and previously published in vitro PK data (15).

Discussion

Intrabacterial metabolism plays a prominent role in whole-cell activity—or inactivity—of antimycobacterials (39, 40). On one hand, intrabacterial drug metabolism can cause intrinsic drug resistance, which involves drug molecules that are active against isolated Mtb targets becoming converted into derivatives lacking the ability to engage their target once inside the bacterium. As an example, sulfonamides show potent activity against isolated Mtb DHPS but weak whole-cell activity because they are metabolized by Mtb (6). On the other hand, a number of highly successful antibacterial prodrugs, including the first-line drugs isoniazid and pyrazinamide, the second line drug PAS, and the newly approved pretomanid (6, 10, 27, 41, 42) do not have detectable activity against specific bacterial targets in their parent form but are converted by Mtb into derivatives with activity against intrabacterial targets. Here, we show that the Mtb DHFR inhibitor TA-C represents a category of antimycobacterial prodrug. In contrast to established Mtb prodrugs, TA-C was designed as a selective Mtb DHFR inhibitor and as such does exert biochemical activity against its target (15). However, upon uptake, Mtb converts TA-C to a more-selective and 100-fold more-potent DHFR inhibitor, TA-C-Acid (Fig. 3).

To characterize this prodrug paradigm, we isolated and analyzed spontaneous TA-C-resistant mutants in Mtb and bacillus Calmette–Guérin. Two types of resistance mechanisms were identified. As expected, TA-C resistance was caused by mutations in thyA at a frequency of ~10−8/CFU (Table 3 and Fig. 3). thyA mutations are known to cause resistance to other DHFR inhibitors by reducing the vulnerability of folate synthesis and susceptibility of DHFR to chemical inhibition (4, 16). The second resistance mechanism was the loss of enzymes required for the biosynthesis of the redox cofactor F420 (Table 3 and Fig. 3). TA-C-Met, a TA-C analog in which FDOR-mediated reduction of the coumarin moiety is blocked, copied the F420-resistance phenotype, suggesting that TA-C reduction by FDORs is required for full expression of antibacterial activity (Table 3 and Fig. 3). The putative product of FDOR reduction, TA-C-Acid, was 100-fold more potent against isolated Mtb DHFR (Table 3 and Fig. 3). Biochemical analyses using a collection of recombinant Mtb FDORs showed that at least two of these enzymes recognize TA-C as substrate, reduce the molecule at the predicted site, and generate TA-C-Acid (Table 3 and Fig. 3). This enhancement mechanism provides an explanation for the exquisite antibacterial activity of the DHFR inhibitor, which has an MIC90 of 10 to 20 nM despite moderate activity against its isolated target (Table 3). While these data are fully consistent with FDORs playing a role in activating TA-C, single- and double-knockout or overexpression strains of Ddn and Rv1558 were not generated and analyzed. Thus, one cannot unequivocally state that these two enzymes are indeed the FDORs responsible for the bioactivation of TA-C in intact bacteria.

TA-C in an F420-deficient background and TA-C-Met in wild-type background, which are phenotypically equivalent, both retained appreciable whole-cell activity (MIC90 2 to 3 μM), compatible with DHFR as their molecular target since both exhibit the same in vitro activity against DHFR (IC50 ~1 μM). However, TA-C-Met also retained the same activity in thyA mutants and DHFR-overexpressing strains (Table 3). This suggests that TA-C engages non-DHFR target(s) at this higher concentration. TA-C-Met–resistance selection experiments did not yield any resistant colonies up to 109 CFU of bacillus Calmette–Guérin. As TA-C-resistant mutations in thyA emerge at a frequency of ~10−7/CFU, this low resistance frequency (<10−9/CFU) 1) is consistent with a non-DHFR mechanism of action, which remains to be determined and 2) suggests that the compound engages more than one target and is not prone to (high-level) resistance development.
Rapid emergence of clinical resistance usually follows the clinical launch of drugs that target single enzymes, thus limiting their utility. A recent example is bedaquiline, approved by the US Food and Drug Administration in 2012 with clinical resistance reported shortly after its introduction (43, 44).

It may seem incongruous that the 2- to 3-μM antibacterial activity of TA-C-Met is not exerted via inhibition of DHFR despite an IC₅₀ of ~1 μM against the enzyme. However, most antibiotics inhibit their target at lower concentrations in biochemical assays than in bacterial cultures. Bedaquiline, which inhibits the F-ATP synthase with an IC₅₀ of 2.5 nM, has an MIC of 54 nM, a 20-fold potency reduction from enzyme to whole cell (45). This common shift is associated with intrabacterial PK (uptake, metabolism, nonspecific binding to macromolecules, and efflux) resulting in lower free drug concentrations at the cellular site of the molecular target compared to the culture medium. Considering these observations, it is less surprising that TA-C-Met’s inhibitory activity on the DHFR enzyme does not contribute to its 2- to 3-μM antibacterial activity.

In the same way that resistance can rapidly emerge against drugs that target a single protein, produgs are vulnerable to rapid evolution of resistance when a single activating enzyme is involved. For example, in the case of pretomanid, although we have shown that the fitness costs associated with mutations that cause loss of F₄₂₀ biosynthesis are too great for this to become a widespread resistance mechanism, mutations that result in loss of the promiscuous prodrug-activating activity of Ddn, without substantially affecting the native menaquinone reductase activity, can cause resistance (inhibit their target at lower concentrations) in biochemical assays (29). TA-C is interesting in the context of those results because it is activated by multiple enzymes: the likelihood of Mtb becoming resistant via simultaneous mutations in multiple activating enzymes is extremely low. Thus, compounds such as TA-C, with multiple targets and multiple activating enzymes, represent an intriguing class of antimycobacterial compounds that could be resilient to the development of drug resistance.

In summary, we have demonstrated that TA-C, an antibacterial designed as a selective Mtb DHFR inhibitor, exerts its superb whole-cell antibacterial activity by disabling the folate pathway, albeit only after intrabacterial “bioaugmentation” by FDORs, explaining the unusual 100-fold boost from enzymatic to whole-cell activity. TA-C also appears to possess a DHFR-independent mechanism of action at low-μM concentrations, as revealed by its residual whole-cell activity in F₄₂₀-deficient strains and in ΔthA mutants resistant to DHFR inhibition. This multitargeting property of TA-C and the functional redundancy of the activating enzymes could be exploited through medicinal chemistry to mitigate the development of high-level resistance in future drug discovery programs.

Materials and Methods

Bacterial Strains, Media, and Culture Conditions. M. bovis bacillus Calmette–Guérin Pasteur (ATCC 35734) and M. tuberculosis H37Rv (ATCC 27294) were obtained from the American Type Culture Collection. Strains were grown in Middlebrook 7H9 broth (BD Difco) supplemented with 0.05% (vol/vol) Tween 80 (Sigma), 0.2% (vol/vol) glycerol (Fisher Scientific), and 10% (vol/vol) Middlebrook albumin-dextrose-catalase (BD Difco) at 37 °C with orbital shaking at 80 rpm. For determination of CFU, bacterial cultures were spread onto Middlebrook 7H11 agar (BD Difco) supplemented with 10% (vol/vol) Middlebrook oleic acid-albumin-dextrose-catalase and 0.2% glycerol and grown at 37 °C. When appropriate, agar was supplemented with TA-C or TA-C-Met for isolation of resistant mutants or 25 μM kanamycin for the selection of transformed bacteria in the complementation experiments. Escherichia coli strains DH5α and BL21 (DE3) were used for propagation of plasmids and expression of recombinant proteins, respectively, and were cultured in Luria–Bertani (LB) broth or on LB agar (BD Difco).

Chemicals. The synthesis of TA-C has been described previously (15). Synthesis of TA-C-Red is described in SI Appendix, Supplementary Information. TA-C-Met and TA-C-Acid were purchased from BioDuro. PAS, pretomanid, isoniazid, rifampicin, and kanamycin sulfate were purchased from Sigma–Aldrich. All drugs and chemicals were dissolved in 100% dimethyl sulfoxide (DMSO) at 10 mM except kanamycin, which was dissolved to 50 μg/mL in deionized water and sterilized using 0.2-μm Minisart high-flow syringe filters (Sartorius).

Selection of TA-C-Resistant Mutants. Selection mutant was carried out as described previously (46). In brief, bacterial inocula (~10⁻⁶ to ~10⁸ CFU) from midlog cultures of M. bovis bacillus Calmette–Guérin or M. tuberculosis H37Rv were plated on 7H11 agar containing 3, 6, and 12 μM TA-C and grown for 4 wk at 37 °C. To verify resistance, colonies were picked and streaked alongside wild-type bacteria on agar containing the TA-C concentrations on which the putative mutants were selected. Single colonies were picked from the restreak plates, inoculated into 7H9 broth, expanded to midlog phase, and stored with 10% glycerol at −80 °C until used for subsequent studies.

Counter Screen of TA-C-Resistant Mutants with Pretomanid. To identify TA-C-resistant strains that harbor a wild-type F₄₂₀ pathway, first isolated 1,000 TA-C-resistant M. bovis bacillus Calmette–Guérin strains and then identified the subset that is susceptible to pretomanid via replica plating (47). Briefly, 10 independently grown bacillus Calmette–Guérin cultures were plated onto 7H11 agar containing 3 μM TA-C and grown for 4 wk. Resistant colonies were picked, inoculated into 7H9 broth in flat-bottom 96-well plates and incubated for 7 d. The culture in each well was adjusted to an optical density at 605 nm of 0.1 and plated onto 7H11 microplates. Cells were harvested, and sterile 96-well-pin replicator (STEM Corporation). Bacteria were spotted in duplicates on both drug-free and 20 μM pretomanid-containing agar. Wild-type bacillus Calmette–Guérin was included in each selection plate as control. TA-C-resistant colonies that did not grow on pretomanid agar were picked from the corresponding colony spots of drug-free replicates, expanded in 7H9 broth to midlog phase, and stored with 10% glycerol at −80 °C.

MIC Determination. MIC were determined as described previously by generating growth inhibition dose–response curves (15). Briefly, drugs were serially diluted in flat-bottom 96-well plates, and a midlog-phase culture was mixed with the drug-containing broth (final OD₆₀₀ = 0.005) and incubated for 10 d. Growth was monitored by measuring turbidity at 600 nm using a Tecan Infinite 200 Pro microplate reader (Tecan). MIC, the concentration that reduces growth by 50% compared to untreated control, was deduced from the generated dose–response curves.

Whole-Genome and Targeted Sequencing. Genomic DNA was extracted from bacillus Calmette–Guérin and Mtb strains using the phenol-chloroform method as described previously (46). Whole-genome sequencing was performed on the illumina MiSeq platform. Library preparation (NEBNext Ultra kit, New England Biolabs), sequencing, and bioinformatic analyses were performed by AIT Biotech. To verify mutations, target genes were PCR amplified using custom-designed primers (SI Appendix, Table S6), integrated DNA Technologies), and purified PCR products were Sanger sequenced (AIT Biotech).

Genetic Complementation Experiments. Representative TA-C-resistant M. bovis bacillus Calmette–Guérin mutants harboring polymorphisms in fbiC, fbiA, fgd1, or thA were complemented with the respective wild-type copies of the genes. The coding sequences were PCR amplified from the bacillus Calmette–Guérin genome using custom primers (SI Appendix, Table S6) and fused to the hsp60 promoter in pmV262 (48). The expression cassettes composed of the hsp60 promoter and the respective coding sequence were transferred into the integrative plasmid pmV306 (41) as either NotI/EcoRI or NotI/HindIII fragments (SI Appendix, Table S5). After confirming the integrity of the constructs by sequencing (AIT Biotech), the plasmids were electroporated into bacillus Calmette–Guérin, and transformants were selected on kanamycin agar as described previously (49).

F₄₂₀ Measurement. F₄₂₀ contents of bacillus Calmette–Guérin cultures were determined by measuring crude cell extracts for F₄₂₀-specific fluorescence as described with minor modifications (50). In brief, late log-phase culture was harvested at OD₆₀₀ = 1.0, washed in 0.9% (wt/vol) NaCl, and resuspended in disodium phosphate and potassium dihydrogen phosphate solution buffer (50 mM each at pH 5.7). The suspension was incubated in boiling water for 15 min, placed on ice for 10 min, and centrifuged at 13,000 × g for 40 min at 4 °C. To the supernatant, an equal volume of ice-cold 2-propanol was added, the mixture was spun (13,000 × g for 40 min at 4 °C), and the supernatant was dispensed in triplicate into a black flat-bottom 96-well plate for measurement
were flash frozen and stored at 50% power. The soluble extract was obtained by centrifugation at 8,500 × g at 80 °C in 20 mM Tris pH 7.5, 0.5 M NaPO4, 0.5 M lactate, 0.5 g/l ampicillin and stored at −80 °C.

**Enzyme Activity Assays.** FadP was reduced overnight with 10 μM FGD and 10 mM glucose-6-phosphate in 20 mM Tris, pH = 7.5 under anaerobic conditions. FGD was removed by spin filtration in a 0.5-ML 10K molecular-weights cutoff (MWCO) spin filter (Millipore), and FadP was used in 1/100 of FGD being removed. Enzyme assays with FDR and TA-C were performed according to previously published methods (36, 37) in 50 mM Tris–HCl, pH = 7.5, 25 mM FADH2, 25 μM TA-C, and 1 μM enzyme at room temperature. Activity was monitored using fluorescence following the oxidation of FADH2 (excitation/emission: 400/470 nm). Specific enzyme activity of FadP for oxidation was calculated using a standard curve of FadP.

**LC/MS Assay.** Enzymatic reactions for LC/MS analysis were done with 50 mM Tris pH = 7.5, 1 μM FGD, 5 μM FADH2, 5 mM glucose-6-phosphate, 5 μM enzyme, and 25 μM TA-C for 1 h. All protein was removed by spin filtration in a 0.5-ML 10K MWCO spin filter (Millipore). Initial rates were corrected for nonenzymatic reduction, and enzymatic activity was determined using the NADPH concentration (620 μM cm−1) as a reference. Initial rates were corrected for nonenzymatic reduction, and enzymatic activity was determined using the NADPH concentration (620 μM cm−1) as a reference.

**FADH Inhibition Assay.** Inhibition assays were done as previously described (15) with modification. All inhibition assays were performed in 150 mM potassium phosphate, pH = 7 containing 50 μM DHF, 60 μM NADPH, 10 mM DHFR, and 0 to 100 μM TA-C in 1/2 or 1/3 serial dilution. Enzyme velocity was determined by measuring the reduction of NADPH by absorbance at 340 nm for 10 min at room temperature using an Epoch microplate spectrophotometer (BioTek). Initial rates were corrected for nonenzymatic reduction, and enzymatic activity was determined using the NADPH concentration (620 μM cm−1) as a reference. Initial rates were corrected for nonenzymatic reduction, and enzymatic activity was determined using the NADPH concentration (620 μM cm−1) as a reference.

**Molecular Docking.** Molecular docking was performed in the Schrödinger suite (Schrödinger release 2020-2, LLC). DHFR (PDB ID: 6NND) (63), Ddh with FAD (PDB ID: 3SR5) (58) and with a reconstructed N terminus as described previously (38), and 1R158 with FadP (this study) were prepared in Protein Preparation Wizard (64). Briefly, any ligand present in the structure was removed, and each protein was preprocessed with bond order assigned, hydrogens added, waters removed, termini capped, and the ionization and tautomeric states of any het groups present were generated at pH = 7 using Epik. Hot groups such as FadP were reviewed, and the lowest energy state with the appropriate bond order was chosen to use in the docking. Hydrogen bonds were assigned and optimized at pH = 7 using PROPKA (65), and the structure was optimized with restrained minimization using the OPLS3e force field (66). The structures of TA-C, TA-C-Met, and TA-C-Acid were created using CnDraw (PerkinElmer Informatics) and prepared for docking by Ligprep (Schrödinger Release 2020-2: LigPrep, Schrödinger, LLC). Briefly, the ligands were minimized using the OPLS3e force field. Ionization of the ligands were generated at pH = 7 using Epik, and each ligand retain their

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specified chirality specified in the input files created from ChemDraw. All ligands were docked into each protein using induced Fit Docking (67–69). A set of 10 different complexes were generated, and docking was performed using the OPLS3e force field. A 12-Å grid box was generated on the central site of each protein. Docking was performed with no constraints, ligands sampled ring conformation within an energy window of 2.5 kcal/mol, amide bonds were penalized for nonplanar conformations, and van der Waals radii scaling was set to 0.5. Residues that were within 5 Å of the docked ligand were refined using Prime with sidechains being optimized. Ligands within 30 kcal/mol of the best protein:ligand complex or within the top 20 were then redocked. The top 10 of each ligand:protein complex were analyzed, and the highest scoring result with an appropriate binding mode for either catalysis (Rv1558/Ddn) or inhibition (DHFR) was used for further analysis.

Molecular Dynamics. Molecular dynamics simulations were performed using Desmond (70), as implemented in the Schrödinger software suite (Schrödinger Molecular Dynamics). Analysis (Rv1558/Ddn) or inhibition (DHFR) was used for further analysis. the highest scoring result with an appropriate binding mode for either catalysis (Rv1558/Ddn) or inhibition (DHFR) was used for further analysis.

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