

Rv2466c Mediates the Activation of TP053 To Kill Replicating and Non-replicating *Mycobacterium tuberculosis*

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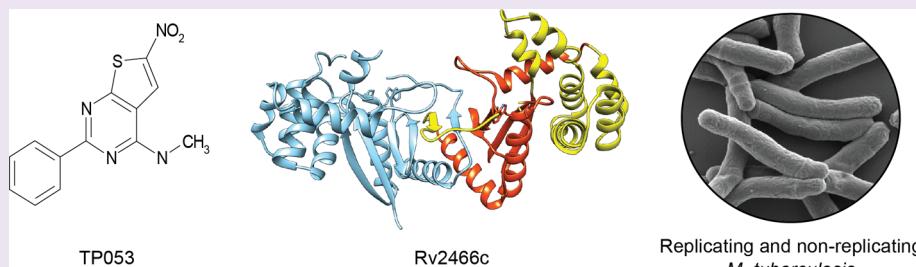
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Supporting Information



ABSTRACT: The emergence of multidrug- and extensively drug-resistant strains of *Mycobacterium tuberculosis* highlights the need to discover new antitubercular agents. Here we describe the synthesis and characterization of a new series of thienopyrimidine (TP) compounds that kill both replicating and non-replicating *M. tuberculosis*. The strategy to determine the mechanism of action of these TP derivatives was to generate resistant mutants to the most effective compound TP053 and to isolate the genetic mutation responsible for this phenotype. The only non-synonymous mutation found was a g83c transition in the *Rv2466c* gene, resulting in the replacement of tryptophan 28 by a serine. The *Rv2466c* overexpression increased the sensitivity of *M. tuberculosis* wild-type and resistant mutant strains to TP053, indicating that TP053 is a prodrug activated by *Rv2466c*. Biochemical studies performed with purified *Rv2466c* demonstrated that only the reduced form of *Rv2466c* can activate TP053. The 1.7 Å resolution crystal structure of the reduced form of *Rv2466c*, a protein whose expression is transcriptionally regulated during the oxidative stress response, revealed a unique homodimer in which a β-strand is swapped between the thioredoxin domains of each subunit. A pronounced groove harboring the unusual active-site motif CPWC might account for the uncommon reactivity profile of the protein. The mutation of Trp28Ser clearly predicts structural defects in the thioredoxin fold, including the destabilization of the dimerization core and the CPWC motif, likely impairing the activity of *Rv2466c* against TP053. Altogether our experimental data provide insights into the molecular mechanism underlying the antimycobacterial activity of TP-based compounds, paving the way for future drug development programmes.

Tuberculosis (TB) remains a worldwide health crisis, causing nearly 1.3 million deaths and 8.6 million new cases in 2012, with an estimated one-third of the human population carrying a latent infection.¹ Over 25% of TB deaths

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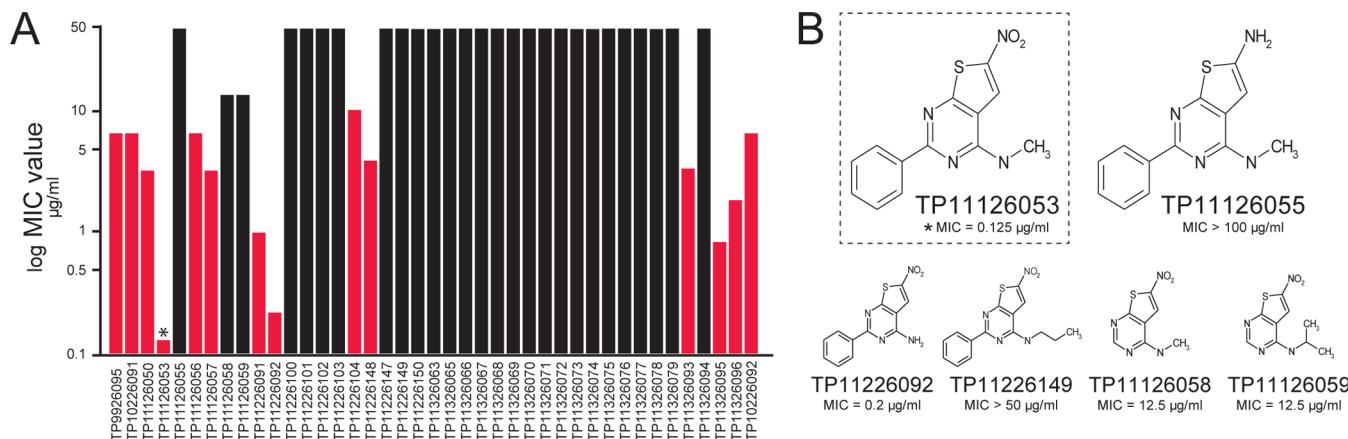


Figure 1. Identification of TP derivatives active against *M. tuberculosis* H37Rv growth. (A) MIC values for 41 TP scaffold derivatives against replicating *M. tuberculosis* H37Rv. The most active compound TP053, which displayed a MIC value of 0.125 µg/mL, is marked with an asterisk. (B) Chemical structures of TP053 compound and selective derivatives.

are associated with HIV co-infected patients, mostly occurring in low- and middle-income countries. Drug treatment is a laborious process, requiring daily dosage of a combination of four first-line drugs over 6 months: isoniazid (INH), rifampicin (RIF), ethambutol, and pyrazinamide. During recent years there has been an alarming rise of multidrug-resistant (MDR) and extensively drug-resistant (XDR) cases of TB. Specifically, 3.6% of new cases and 20% of previously treated cases have MDR-TB, showing resistance to at least the two most effective anti-TB drugs known to date, INH and RIF. Notably, 9.6% of MDR-TB cases are also resistant to any fluoroquinolone and any of the second-line anti-TB injectable drugs amikacin, kanamycin, or capreomycin. They were classified as XDR-TB and reported in at least 92 different countries.¹⁻³ Under such circumstances, the discovery of novel anti-TB drugs with bactericidal mechanisms different from those of currently available agents has become a worldwide priority.^{4,5}

An important prerequisite for the development of novel drugs with promise for TB treatment is drug activity against both replicating and non-replicating bacilli. Although considerable progress has been made in recent years in the discovery of such compounds, a limited number of candidates are currently under clinical trials.^{4,5} Among the group comprising new chemical entities, the nitroimidazoles (PA-824 and OPC67683) and bedaquiline (TMC207) were found active against persistence bacilli. TMC207 is a diarylquinoline that inhibits the *c* subunit of ATP synthase, thereby decreasing intracellular ATP levels, and has been recently approved by the Food and Drug Administration (FDA) for the treatment of MDR-TB in adults.⁶⁻⁸ The two newer nitroimidazoles, PA-824 and OPC67683 (delamanid), are prodrugs requiring intracellular activation mediated by a deazaflavin-dependent nitroreductase (Ddn).⁹ This enzyme reduces PA-824 and delamanid, generating lethal reactive nitrogen species, including nitric oxide, as the major effectors of their activity.¹⁰ An interesting case among the new drugs currently in preclinical development is BTZ043, which blocks arabinogalactan and lipoarabinomannan biosynthesis in *M. tuberculosis* and shows efficacy comparable with that of INH and RIF.¹¹ BTZ043 undergoes nitroreduction by the reduced form of the essential decaprenyl-phosphoryl- β -D-ribose DprE1 to form a nitroso-species that covalently binds to a cysteine residue in the active site, thereby quantitatively and irreversibly inactivating DprE1.^{12,13}

In that context, the search for alternative drug therapies to combat TB has led to our interest in the thienopyrimidine (TP) scaffold.^{14–16} A new series of TP derivatives was carefully synthesized and tested for anti-TB activity, with the most potent inhibitor TP053 displaying very low MIC values of 0.125 and 0.800 µg/mL in replicating and non-replicating models, respectively. Using a combination of chemical synthesis, isolation of *M. tuberculosis* resistant mutants, genetic validation, and biochemical and crystallographic studies we provide evidence indicating that TP053 is a prodrug activated by a novel thioredoxin-like protein (Rv2466c) in a redox-dependent manner.

RESULTS AND DISCUSSION

Identification of TP053 as a Potent Inhibitor of *M. tuberculosis* Growth. The elucidation of a new method for the chemical synthesis of TPs allowed the evaluation of the anti-TB activity of this scaffold.¹⁷ The initially synthesized compounds TP9926095 and TP10226091 already had moderate activity against replicating *M. tuberculosis* H37Rv with an MIC value of 6.3 µg/mL (Supplementary Table 1S). This encouraging data stimulated efforts to generate more potent TP analogues and to gain understanding of the structure activity relationship around chemical substituents at positions C6, C2, and C4. To this end, we synthesized 39 additional derivatives and determined the MIC value for each compound against *M. tuberculosis* H37Rv using the resazurin reduction microplate assay (Figure 1A; Supplementary Table 1S). TP11126053 (TP053 thereafter) and TP11226092 resulted the most active compounds, displaying MIC values of 0.125 and 0.2 µg/mL, respectively (Figure 1A,B; Supplementary Figure S1 and Table 1S). Both TP derivatives exhibited conserved substitutions at position 2 characterized by the presence of a phenyl group and a NO₂ group at position 6. In contrast, the position 4 displayed a N-methyl or a NH₂ group, likely accounting for the observed differences in the MIC values. Indeed, the NO₂ group at position C6 proved to be essential for anti-TB activity as any alternative substitution, including NH₂, carbonitrile, carboxylate, or carboxamide, resulted in an inactive TP compound (Figure 1B and Supplementary Table 1S). With regard to C2, the addition of a lipophilic phenyl ring improved the activity of the compound 100-fold (MIC from 12.5 to 0.125 µg/mL for TP058 and

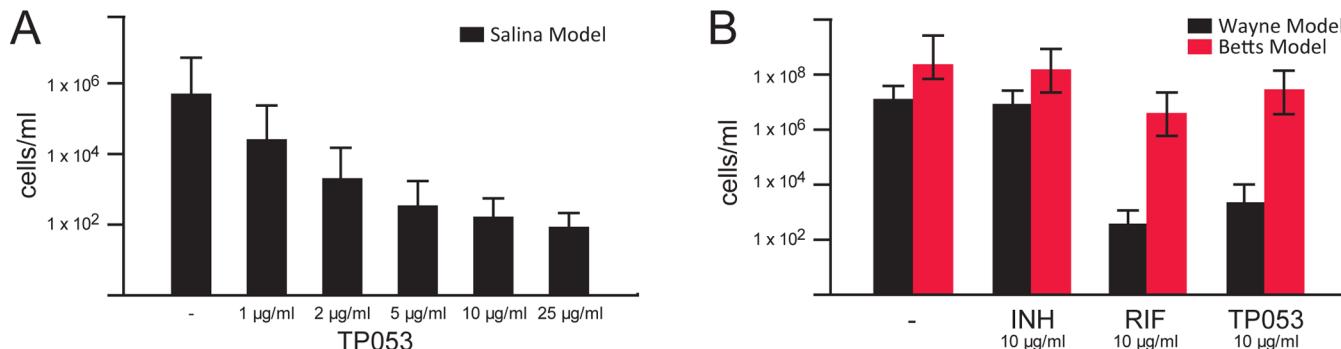


Figure 2. TP053 is active against dormant *M. tuberculosis* H37Rv cells. (A) The bactericidal effect of TP053 *M. tuberculosis* H37Rv cells determined by the Salina model. NC dormant cells were treated with different concentrations of TP053 for 7 days at 37 °C. Viability of both treated and untreated NC cells was tested by MPN assay. Bars represent 95% confidence limits. (B) The bactericidal effect of TP053, INH, and RIF on *M. tuberculosis* H37Rv cells obtained according to the Wayne hypoxia and Betts starvation *in vitro* models.

TP053, respectively). The presence of a bulkier substituent at position C4 strongly decreased potency of the TP derivative in the following order: primary amine > methoxy > N-methyl > N-ethyl group. On the basis of these results, we focused on TP053 for studying the mechanism underlying the anti-TB activity of TP derivatives.

TP053 Is Active against Non-replicating *M. tuberculosis* Cells. The anti-TB activity of several key TP compounds was tested against non-replicating *M. tuberculosis* using the streptomycin-starved 18b (ss18b) model.¹⁸ As depicted in Supplementary Table 1S, all tested TP derivatives were found to maintain their activity against non-replicating ss18b, including TP053. The effectiveness of TP053 was further studied in a model of “nonculturability” of *M. tuberculosis* cells (Figure 2A).¹⁹ As these “nonculturable” (NC) cells are unable to produce colonies on nonselective agar-solidified media, we applied the most probable number (MPN) assay on liquid medium to check the effect of TP053 on the viability of dormant NC cells. In contrast to colony forming units (CFU) counting, this approach is able to uncover a subpopulation of cells with decreased culturability as a consequence of antibiotic treatment. Incubation of NC cells with TP053 at different concentrations resulted in dose-dependent escalation of killing activity. Figure 2A shows that TP053 already kills these bacteria at 1 $\mu\text{g/mL}$ (a 1-log decrease in CFU), with maximum killing observed at 10 $\mu\text{g/mL}$ (a 4 log decrease in CFU), whereas these cells are highly tolerant to such first-line antimicrobials as RIF and INH.¹⁹ To confirm the effectiveness of TP053 on dormant *M. tuberculosis* cells, we utilized two other *in vitro* dormancy models: the Wayne hypoxia model and the Betts starvation model (Figure 2B).^{20,21} Dormant cells obtained in these two models were treated with 10 $\mu\text{g/mL}$ of TP053, RIF, or INH for 7 days. These dormant cells were rather susceptible to RIF, but also to TP053, showing a more than 3-log killing effect in the Wayne hypoxia model and a 1-log killing effect in the Betts starvation model. Thus, we conclude that TP053 is able to kill both replicating and non-replicating *M. tuberculosis* bacteria.

Rv2466c Gene Is Required to Activate TP053 in *M. tuberculosis* H37Rv. To elucidate the mechanism of action of this new class of TP compounds, mutants resistant against TP053 were generated, by plating *M. tuberculosis* H37Rv on solid medium containing 10X the minimal inhibitory concentration (MIC) value. The resistant mutants appeared at a frequency of 1.4×10^{-9} . One of these mutants, named 53.3, displayed a particularly high MIC value of 2.5 $\mu\text{g/mL}$ (20X

MIC). To identify the genetic mutation responsible for the TP053 resistance in 53.3, whole genome sequencing followed by bioinformatics analysis was used. This strategy had proven useful to study antibiotic resistance in *M. tuberculosis* and other human bacterial pathogens.^{22,23} Comparative analysis revealed a non-synonymous mutation (g83c) in the *Rv2466c* gene, resulting in the replacement of the tryptophan at position 28 by a serine (W28S). The *Rv2466c* gene has been reported as a nonessential gene by Himar1-based transposon mutagenesis in H37Rv strain.^{24,25}

To determine the role of *M. tuberculosis* *Rv2466c* in TP053 resistance, we evaluated the effect of overexpressing the wild type and mutated versions of the protein in *M. tuberculosis*. To this end, the *Rv2466c* and *Rv2466c-W28S* genes were cloned in the expression vector pSODIT-2, and *M. tuberculosis* H37Rv cells were transformed with the corresponding plasmids. In addition, the 53.3 strain was transformed with plasmid pSODIT/*Rv2466c* to verify that the overexpression of *Rv2466c* is able to restore the sensitivity of the resistant cells to TP053. As depicted in Table 1, overexpression of *Rv2466c*

Table 1. Determination of MIC values for TP053 in *M. tuberculosis* and *M. smegmatis* Strains

plasmids	TP053 MIC ($\mu\text{g/mL}$)		
	<i>M. tuberculosis</i> H37Rv	53.3 mutant	<i>M. smegmatis</i> mc ² 155
pSODIT-2	0.5	>5	64
pSODIT/ <i>Rv2466c</i>	0.125	0.25	32
pSODIT/ <i>Rv2466c</i> (W28S)	0.25–0.5		64
pSODIT/ <i>nfnB</i>			2

restored the TP053 sensitivity of the *M. tuberculosis* 53.3 mutant, thus confirming the complementation of the W28S mutation. Notably, the overexpression of *Rv2466c* in *M. tuberculosis* H37Rv increased the sensitivity to TP053. Indeed, the MIC value decreased 4-fold when compared to that of *M. tuberculosis* cells transformed with the empty vector or with the pSODIT-2/*Rv2466c*-W28S. Similarly, the overexpression of *Rv2466c* in *M. smegmatis* mc²155 also conferred sensitivity to TP053, but with a MIC 2-fold lower when compared to that of *M. smegmatis* cells transformed with the empty vector. Altogether, the experimental data strongly support the notion of *Rv2466c* being required to activate TP053.

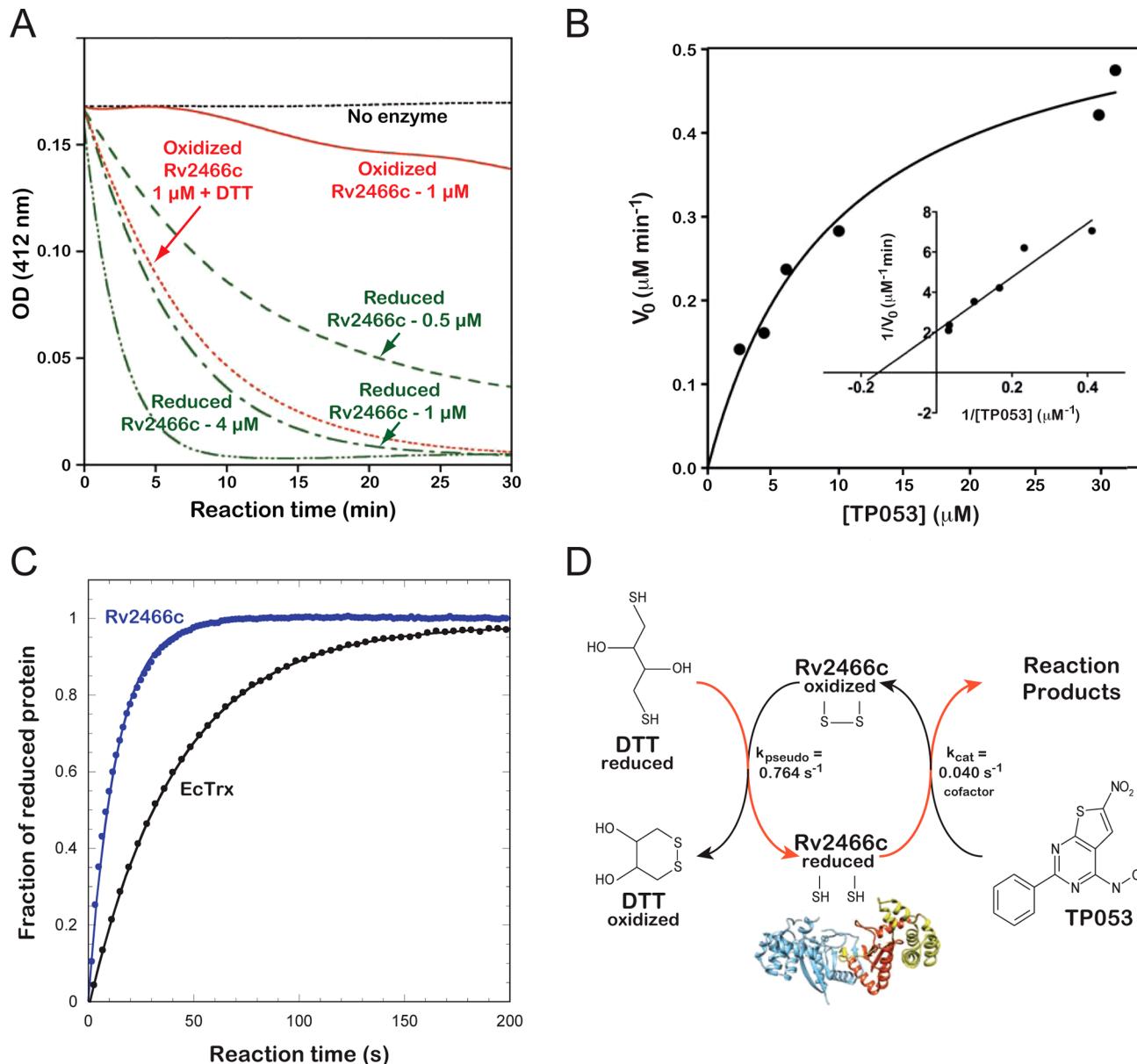


Figure 3. Rv2466c catalyzes the reduction of TP053 by DTT *in vitro*. (A) The reaction was monitored by the decrease in TP053 absorbance at 412 nm (initial TP053 concentration, 10 μM ; see Methods for details). The dotted black line corresponds to a control without Rv2466c. The solid and dotted red lines correspond to the incubation of TP053 with oxidized Rv2466c and Rv2466c after reactivation by DTT, respectively. Dashed green lines correspond to the reactions at Rv2466c concentrations of 0.5 μM , 1 μM , and 4 μM in the presence of 1 mM DTT. (B,C) Rv2466c is a catalyst of TP053 reduction by DTT. Rv2466c shows substrate saturation consistent with Michaelis–Menten kinetics. Initial velocities recorded at varying TP053 concentrations were plotted using the graphical representations of Michaelis–Menten and Lineweaver–Burk (inset). (C) Normalized stopped-flow fluorescence kinetics of the reduction of Rv2466c (blue) and *E. coli* thioredoxin (EcTrx, black) by DTT at pH 7.0 and 25 °C. Identical initial protein concentrations of 1 μM and an initial DTT concentration of 0.1 mM were used. Data were fitted according to pseudo-first-order kinetics (solid lines). (D) Model of the catalytic cycle of Rv2466c. Reduced Rv2466c reacts with TP053 to oxidized Rv2466c and reduced reaction products. Oxidized Rv2466c is then recycled to its reduced form by reduced DTT. The rate constants of both reactions at substrate saturation in the presence of 1 mM DTT are indicated. The proposed electron flow during redox exchange is indicated in red.

Rv2466c Encodes a Thioredoxin-Like Protein That Catalyzes the Reduction of TP053 *In Vitro*. To further investigate the ability of Rv2466c to metabolize TP053, we developed an *in vitro* assay in which the time-dependent decrease in TP053 absorbance at 412 nm was monitored in the presence of Rv2466c (Figure 3A,B). To this end, a recombinant version of Rv2466c, with a N-terminal TEV-cleavable oligohistidine tag was expressed and purified (see Supporting Information for details). Inspection of the primary sequence revealed that Rv2466c contains a CXXC active-site motif

(CPWC), characteristic for members of the thiol-disulfide oxidoreductase superfamily (Supplementary Figure S2).²⁶ TP053 (10 μM) was completely metabolized when incubated with catalytic amounts of Rv2466c in the presence of 1 mM dithiothreitol (DTT) and 2% methanol *M. smegmatis* cellular extracts ($k_{cat} = 0.0403 \text{ s}^{-1}$ and $K_M = 6.489 \mu\text{M}$; Figure 3A,B). In contrast, Rv2466c in its oxidized state (Rv2466c_{ox}) and in the absence of DTT was unable to metabolize TP053 within 5 min of incubation (Figure 3A). Only a small decrease in TP053 concentration after 5 min was observed under these conditions,

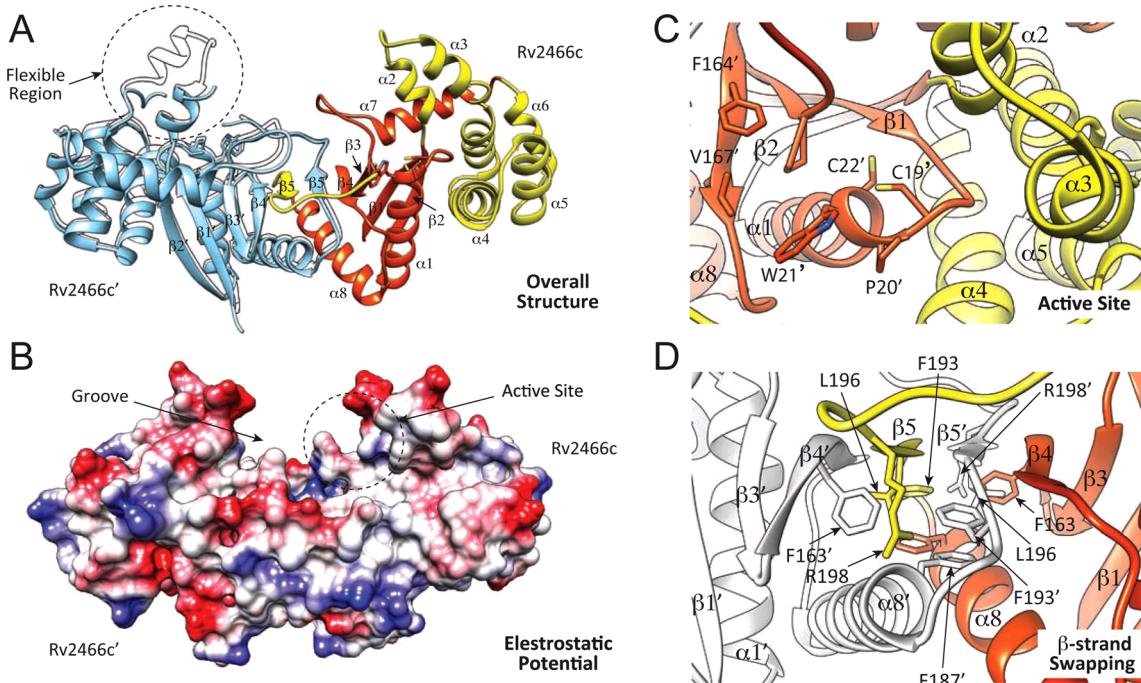


Figure 4. Overall structure of Rv2466c. (A) Cartoon representation showing the structure of Rv2466c. Monomer A (Rv2466c) is shown in yellow and orange, while monomer B (Rv2466c') is shown in light blue. To compare the conformational variability between monomers, monomer B has been superimposed onto monomer A, shown with white contour lines. The structural elements of the canonical thioredoxin fold are depicted in orange, including four β -strands (β_1 – β_4) and three α -helices (α_1 , α_7 , and α_8). Two insertions are depicted in yellow, the first one between β_2 and α_2 (residues 45–129) and a second one at the C-terminus (residues Tyr190–Asp207). The latest contains the β_5 , which is responsible for Rv2466c dimerization via a β -strand swapping architecture. (B) Surface representation of Rv2466c showing an electrostatic potential color scheme: negatively charged residues are shown in red, neutral residues in white, and positively charged residues in blue. For calculation of the Rv2466c surface, missing regions in monomer A were built based on the corresponding regions present in monomer B. (C) The catalytic motif (Cys19-Pro20-Trp21-Cys22) and residues in the vicinity are shown. (D) Rv2466c dimerizes following a β -strand swapping mechanism. Two structurally related enzymes show similar characteristics: the thiol:disulfide interchange protein DsbA from *Bordetella parapertussis* (PDB code 3HDS) shows an extended α -helix allowing the enzyme to trimerize. The dimerization of the protein disulfide isomerase DsbP is mediated through exchange of the strand β_1 .⁴²

most likely resulting from reductive reactivation of Rv2466c by a reducing agent in the *M. smegmatis* extract. A heat-stable cofactor of undetermined nature present in the boiled *M. smegmatis* extract was required for the reaction to occur. Initial velocities recorded at different TP053 concentrations in the presence of 1 mM DTT and catalytic amounts of Rv2466c (0.2 μ M) were consistent with Michaelis–Menten kinetics ($k_{\text{cat}} = 0.0403 \text{ s}^{-1}$; $K_M = 6.489 \mu\text{M}$) and confirmed that Rv2466c catalyzes the reduction of TP053 by DTT.

Rv2466c showed an approximately 2-fold increase in tryptophan fluorescence upon reduction of its active-site disulfide (Supplementary Figure S3). Stopped-flow fluorescence kinetics of the reduction of Rv2466c with DTT revealed a 3-fold higher reactivity with DTT than that of *E. coli* thioredoxin (*EcTrx*) ($k_2 = 764 \text{ M}^{-1} \text{ s}^{-1}$ compared to $k_2 = 236 \text{ M}^{-1} \text{ s}^{-1}$, respectively; Figure 3C). This translates into a pseudo-first-order rate constant ($k_{\text{pseudo}} = k_2 \times [\text{DTT}]$) of 0.764 s^{-1} for the reduction of Rv2466c under the conditions used for recording the Michaelis–Menten kinetics in Figure 3B (1 mM DTT), which is more than 10-fold faster than TP053 transformation ($k_{\text{cat}} = 0.0403 \text{ s}^{-1}$). Consequently, the rate-limiting step in the catalytic cycle of Rv2466c, in the presence of 1 mM DTT, is the reduction of TP053 by reduced Rv2466c, and not the reduction of oxidized Rv2466c by DTT. The experimental data support a model in which the oxidized form of Rv2466c accepts electrons from a thiol reductant (DTT in the *in vitro* experiments), whereas the reduced form of Rv2466c transfers the electrons to TP053 (Figure 3D).

One of the major features making *M. tuberculosis* a successful human pathogen is its exquisite ability to survive under extreme anaerobic and aerobic conditions. *M. tuberculosis* effectively adapts to persistence under hypoxic conditions, such as those encountered in granulomas, by promoting an overall down-regulation of metabolism while up-regulating specific genes involved in respiration and central metabolism.^{27,28} In contrast, in order to survive and multiply within the oxidative background observed in macrophages, *M. tuberculosis* has acquired a variety of mechanisms to maintain its redox equilibrium, in particular reducing conditions in the cytosol.^{29,30} *M. tuberculosis* produces millimolar concentrations of multiple thiol buffers, including mycothiol and ergothioneine, which are essential to preserve standard metabolic activities and lipid/protein synthesis.³¹ Specifically, the intracellular levels of mycothiol are tightly regulated during exponential and stationary phases of growth and synchronized in the course of various stresses, including oxidative stress.^{32,33} Interestingly, other mycothiol producing bacteria such as *Streptomyces coelicolor* modulate the biosynthesis of mycothiol during oxidative stress via a thiol-specific antisigma factor (RsrA) and sigmaR (SigR) factors.^{34,35} *M. tuberculosis* contains a homologue of SigR, known as SigH, which under oxidative stress induces the expression of the thioredoxin reductase/thioredoxin (*trxB2/trxC*) system, essential for reducing unspecific disulfide bonds in the cell, and that of Rv2466c.³⁶

Several additional lines of experimental evidence strongly support the notion that Rv2466c is a thioredoxin-like reductase

that uses intracellular thiols as substrates. First, the TP053 reduction assay conclusively demonstrated that only the reduced form of Rv2466c interacts with TP053 ($k_{cat} = 0.0403 \text{ s}^{-1}$ and $K_M = 6.489 \mu\text{M}$), and that DTT reduced oxidized Rv2466c even faster than oxidized thioredoxin (Figure 3C). In contrast to thioredoxin, however, Rv2466c showed no activity as catalyst of insulin reduction by DTT (Supplementary Figure S4A) and was not a substrate of *E. coli* thioredoxin reductase (Supplementary Figure S4B). Second, we determined that the enzyme is likely located in the reducing environment of the cytosol (Supplementary Figure S5). We have not been able to obtain a redox equilibrium between Rv2466c and standard redox buffers such as DTT_{ox}/DTT_{red} (with a redox potential of -307 mV) and GSH/GSSG (with a redox potential of -240 mV).^{37,38} Furthermore, we could not observe thiol/disulfide exchange equilibrium between Rv2466c and EcTrx (Supplementary Figure S6). Why Rv2466c did not attain disulfide exchange equilibrium with standard thiol/disulfide redox buffers remains an open question, but the results suggest that reduced Rv2466c is only slowly reoxidized and has a reductive function in *M. tuberculosis*. Protection against fast Rv2466c oxidation may be particularly important in *M. tuberculosis* under oxidative stress conditions.

Finally, the activation of TP053 by Rv2466c to a toxic compound could be due to the reduction of the NO₂ group similar to other bactericidal antibiotics containing NO₂ groups that are reduced to nitro anion radicals and/or a variety of other highly reactive compounds including nitroso and hydroxylamine derivatives.³⁹ These products are extremely toxic, readily damage proteins, membrane lipids, and DNA and eventually lead to cell death as recently reported for anti-TB nitroimidazole compounds (PA-824 and delamanid). Interestingly, overexpressing in *M. smegmatis* of the nitro reductase NfnB, an enzyme absent in *M. tuberculosis*,⁴⁰ strongly increased the sensitivity of *M. smegmatis* to TP053 compound (MIC of 1 $\mu\text{g}/\text{mL}$ compared to 64 $\mu\text{g}/\text{mL}$ in the wild-type strain; Table 1). Together with our finding that the amine-derivative of TP053 was ineffective (see compound TP11126055; Supplementary Table 1S), these data also suggest that activation of TP053 by Rv2466c is linked to the reduction of its NO₂ group.

Crystal Structure of Rv2466c: A Thioredoxin Fold with an Unusual CXXC Motif. The crystal structure of the reduced form of Rv2466c was solved using an N-terminal TEV-cleavable histidine tag construct of the protein (see Supporting Information for details). The crystallographic data collection and model statistics are summarized in Supplementary Table 2S. We could not obtain crystals of the completely oxidized form possibly due to a conformational change in the protein. Rv2466c adopts a canonical thioredoxin fold, which consists of four strands that form a β -sheet surrounded by three α -helices (Figure 4A).⁴¹ The Cys19-Pro20-Trp21-Cys22 active-site motif is located at the N-terminus of the first α -helix (α_1 ; Figure 4B,C and Supplementary Figure S2 and S7). Similar to other members of the thiol–disulfide oxidoreductase superfamily, Rv2466c displays an insertion (residues Met45 to Tyr132) comprising a helical bundle of five α -helices between the second β -strand and the second canonical α -helix (α_7 ; shown in yellow in Figure 4A).

Rv2466c crystallized as a dimer with an extensive contact area of ca. 2163 \AA^2 , representing ca. 39% of the total accessible surface of the isolated monomers. The dimer interface comprises two main regions of the protein: an extension of 17 residues (Tyr190 to Asp207) located at the C-terminus of

the canonical thioredoxin fold (shown in orange, Figure 4A) and the helix α_8 (Glu175 to Ser189). Interestingly, the C-terminal extension contains a single β -strand (β_5) that inserts between two β -strands of the second subunit of the dimer (β_4' and β_5' , primed/unprimed numbers indicate residues from each monomer in the homodimer), allowing the protein to dimerize by β -strand swapping (Figure 4B,D and Figure 5).

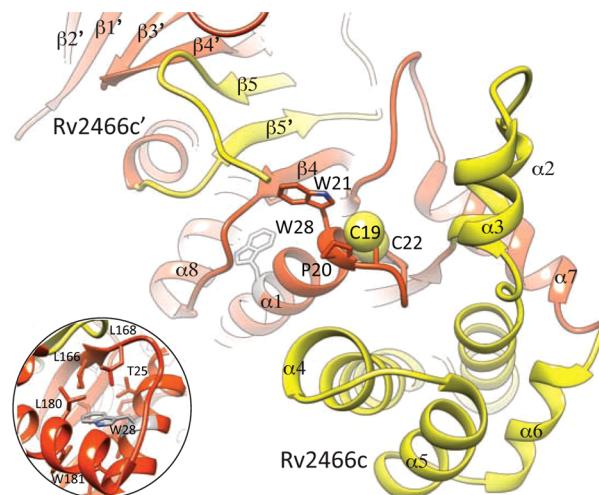


Figure 5. Trp28 is located at the core of the thioredoxin fold domain of Rv2466c. Cartoon representation highlighting the position of Trp28 (gray) in the structure of Rv2466c. The thioredoxin core and the accessory insertions are shown in orange and yellow, respectively. Trp28 localizes in the middle of the helix α_1 , which also contains the catalytic motif Cys19-Pro20-Trp21-Cys22 at its N-terminus. As shown in the inset, Trp28 plays a key role in the stabilization of the thioredoxin core of Rv2466c.

Thus, the individual twisted β -sheet of each monomer forms an extended β -sheet through the observed dimer (Figure 4A,C and Figure 5). The helix α_8 is essential for assembly and stabilization of the dimer, establishing an important network of hydrophobic interactions with other secondary structure elements of the thioredoxin core and both β_5 and β_5' . Interestingly, the exposed face of the extended β -sheet forms a large, mostly hydrophobic groove flanked by the array of five α -helices on each side, where the active site is located (Figure 4A,B). Structural superposition of the two monomers of the protein dimer indicates that the helical subdomain (residues Met45–Tyr132) displays a high degree of structural flexibility (rmsd of 1.7 \AA for $C\alpha$ atoms of 82 equivalent residues; Figure 4A). The crystal structure of the reduced Rv2466c homodimer is consistent with analytical gel filtration and dynamic light scattering data showing that both redox forms of Rv2466c are homodimers in solution (apparent molecular weights of 56 and 45 kDa, respectively; Supplementary Figure S8A,B).

Trp28 plays a central role in stabilizing not only the three-dimensional structure of the thioredoxin fold but also the dimeric architecture of the oxidoreductase (Figure 5). Trp28 is completely buried in the core of the protein, localizing in the middle of the canonical α_1 helix, which also contains the catalytic motif Cys19-Pro20-Trp21-Cys22 at its N-terminus (Figure 5). It makes important interactions with three main regions of the protein: the strands β_3 , β_4 , and α_8 . Specifically, the indole nitrogen makes an electrostatic interaction with the main chain of Ala177 in helix α_8 . The lateral chain of Trp28 also makes hydrophobic interactions with the lateral chain of

Leu180 and Trp181, in the same α -helix. Moreover, the side chain of Tyr28 is oriented toward the β_3 and β_4 strands, with the lateral chains of Ile156 and Pro166/Leu168, respectively. Consequently, the mutation of Trp28Ser clearly predicts structural defects in the thioredoxin fold, likely including the destabilization of the dimerization core and the CPWC motif, likely impairing the activity of Rv2466c against TP053.

Conclusions. *M. tuberculosis* is the second most deadly infectious agent in the world after HIV. The recommended treatment of drug-susceptible TB was introduced in the 1970s and requires the administration of four first-line drug combinations during a period of 6 months. Lengthy treatment regimens, unpleasant side effects, and patient noncompliance provided conditions for the generation of drug-resistant *M. tuberculosis* strains. The development of novel anti-TB drugs with bactericidal mechanisms different from those of currently available agents has become an urgent need. Here we present a new class of anti-TB TP derivatives that kill both replicating and non-replicating *M. tuberculosis*. This is a major finding since to date only a few drugs are capable of effectively killing non-replicating bacilli, responsible for TB reactivation. The selection of resistant mutants, whole-genome sequencing, and genetic validation revealed that TP053 is a prodrug activated by Rv2466c, a thioredoxin-like protein with the unusual active-site motif CPWC. Biochemical studies demonstrated that only the reduced form of Rv2466c activates TP053. The crystal structure of reduced Rv2466c revealed a homodimer of two thioredoxin domains stabilized by β -strand swapping. The mutation Trp28Ser seems to play a central role in the stabilization of the dimerization core and the CPWC motif, likely impairing the activity of Rv2466c against TP053. The experimental data strongly suggest a mechanism of activation involving the reduction of the nitro moiety of TP053. Our findings provide insights into the molecular mechanism underlying the antimycobacterial activity of TP053 and a promising basis for future drug development programmes.

METHODS

Synthesis of TP Derivative Compounds. The key scaffold 4,6-dichloro-2-phenylpyrimidine-5-carbaldehyde was synthesized in two consecutive chemical steps from benzamide hydrochloride and diethyl malonate with the formation of 2-phenylpyrimidine-4,6-diol. After treatment with the Vilmeier complex, the dichloropyrimidine was isolated. Successive replacement of a first chloro atom on the corresponding amine group and a second chloro atom on the thiocyanate group led to the production of 5-formyl-6R-2-phenylpyrimidin-4-yl thiocyanate. Its unusual reaction with active methylene compounds in the presence of triethylamine gives the 4-R-6-R'-2-phenylthieno[2,3-*d*]pyrimidines. The complete synthesis of TP053 compound is described in Supplemental Experimental Procedures.

MIC Determinations. MIC determinations were performed both in liquid and solid medium as described in detail in Supplemental Experimental Procedures.

Isolation and Characterization of *M. tuberculosis* 53.3 Resistant Mutant to TP053 Compound. *M. tuberculosis* H37Rv resistant mutants were isolated by plating $\sim 10^{10}$ cells from a late exponential growth phase wild-type culture ($OD_{600} = 1$) onto complete Middlebrook 7H11 agar plates containing different concentrations of TP053, ranging from 5- to 20-fold the MIC of the wild-type strain. The agar plates were incubated at 37 °C for 3–4 weeks. The phenotype of the resistant colonies was confirmed/validated by repeating the MIC evaluation three times. Genomic DNA of *M. tuberculosis* 53.3 resistant mutant and that of the H37Rv wild-type strain were isolated and sequenced by using Illumina HiSeq2000 technology at IGA Technology Services S.R.L. (Udine, Italy), resulting in a 77-fold genome coverage. Repetitive PE and PPE gene families

were discarded as well as all synonymous mutations. Furthermore, the Rv2466c gene (genolist.pasteur.fr/TubercuList/), was amplified by standard PCR using oligonucleotide primers Rv2466cseqFOR (5'-GGAACAGGTGCGGGCGG-3') and Rv2466cseqREV (5'-GCTCGGGCAGGTCGTCAC-3'), *Pfu* DNA Polymerase (Promega), and genomic DNA from *M. tuberculosis* H37Rv and 53.3 strains as templates. The PCR products were purified by using the Wizard SV Gel and PCR Clean-Up system (Promega), and their sequences were confirmed by the Sanger method (Eurofins MWG Operon). Genetic validation procedures are described in detail in Supplemental Experimental Procedures.

Rv2466c Cloning, Expression, and Purification. The Rv2466c gene from *M. tuberculosis* H37Rv was cloned in the T7 promoter-based expression vector pET29a. The recombinant Rv2466c was expressed in *E. coli* BL21(DE3)pLysS cells and purified following a four-step procedure carefully described in Supplemental Experimental Procedures.

Rv2466c Enzyme Kinetics. TP053 reduction was followed by change in absorbance of the compound in solution, in the presence of Rv2466c_{red}. Absorption spectra were recorded in 100 mM NaH₂PO₄–NaOH pH 7.0, 1 mM DTT, 10% DMSO, 2% *M. smegmatis* methanol extract, 10 μ M TP053 at different concentrations of Rv2466c_{red} (ranging from 4 to 0.5 μ M). Spectra were collected at 412 nm on a CARY-300 Bio UV-vis spectrophotometer at 25 °C. TP053 (molar extinction coefficient is 17962 M⁻¹ cm⁻¹ at 412 nm in DMSO) reduction capacity of oxidized Rv2466c (Rv2466c_{ox}) was tested at 1 μ M concentration in the absence of DTT. Activity recovery was tested after incubating Rv2466c_{ox} with 1 mM DTT during five min. For determination of initial velocities (V_0), 0.2 μ M of Rv2466c was used with varying concentration of TP053 ranging from 1 to 30 μ M in 100 mM NaH₂PO₄–NaOH pH 7.0, 1 mM DTT, 10% DMSO, and 2% methanol *M. smegmatis* extract. Rv2466c kinetic parameters V_{max} and K_M with respect of TP053 were determined by Lineweaver–Burke linear regression approximation.

Rv2466c Crystallization and Data Collection, Structure Determination, and Refinement. Details on crystallization, X-ray data collection, structure solution, and refinement parameters for Rv2466c can be found in Supplemental Experimental Procedures.

ASSOCIATED CONTENT

Supporting Information

Supplementary tables, figures, and experimental procedures, and references. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

TB, tuberculosis; INH, isoniazid; RIF, rifampicin; MDR, multidrug resistant; XDR, extensively drug resistant; TP, thienopyrimidine; ss18b, *M. tuberculosis* streptomycin-starved 18b; NC, nonculturable; MPN, most probable number; MIC, minimal inhibitory concentration; DTT, dithiothreitol

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