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Cyclipostins and cyclophostin analogs inhibit the antigen 85C from *Mycobacterium tuberculosis* both in vitro and in vivo

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An increasing prevalence of cases of drug-resistant tuberculosis requires the development of more efficacious chemotherapies. We previously reported the discovery of a new class of cyclipostins and cyclophostin (CyC) analogs exhibiting potent activity against *Mycobacterium tuberculosis* both in vitro and in infected macrophages. Competitive labeling/enrichment assays combined with MS have identified several serine or cysteine enzymes in lipid and cell wall metabolism as putative targets of these CyC compounds. These targets included members of the antigen 85 (Ag85) complex (i.e. Ag85A, Ag85B, and Ag85C), responsible for biosynthesis of trehalose dimycolate and mycolylation of arabinogalactan. Herein, we used biochemical and structural approaches to validate the Ag85 complex as a pharmacological target of the CyC analogs. We found that CyC8p, CyC8sp, and CyC8p bind covalently to the catalytic Ser124 residue in Ag85C; inhibit mycolyltransferase activity (i.e. the transfer of a fatty acid molecule onto trehalose); and reduce triacylglycerol synthase activity, a property previously attributed to Ag85A. Supporting these results, an X-ray structure of Ag85C in complex with CyC8p disclosed that this inhibitor occupies Ag85C’s substrate-binding pocket. Importantly, metabolic labeling of *M. tuberculosis* cultures revealed that the CyC compounds impair both trehalose dimycolate synthesis and mycolylation of arabinogalactan. Overall, our study provides compelling evidence that CyC analogs can inhibit the activity of the Ag85 complex *in vitro* and in mycobacteria, opening the door to a new strategy for inhibiting Ag85. The high-resolution crystal structure obtained will further guide the rational optimization of new CyC scaffolds with greater specificity and potency against *M. tuberculosis*.

With 10.4 million new cases and 1.8 million deaths in 2016, tuberculosis (TB)5 continues to be a major global health problem. TB is caused by *Mycobacterium tuberculosis*, a resilient microorganism that persists through long courses of antibiotics and years of dormancy within the host. The emergence of multidrug-resistant and extensively drug-resistant TB has contributed to the difficulties in treating this bacterial infection (1). Chemotherapeutic treatments against TB remain very challenging and complicated, essentially because of the slow rate of growth of the bacilli and the presence of a thick, greasy, and relatively drug-impermeable cell wall (2). This mycobacterial cell wall consists of a complex skeleton comprising covalently linked macromolecules, such as peptidoglycan, arabinogalactan, and mycolic acids, in which non-covalently associated glycolipids are interspersed (3). The mycolic acid portion of the envelope is composed of very long fatty acids (C70–90) that are either covalently attached to the arabinan moiety of the arabinogalactan (AG) polymer or found esterified to trehalose as trehalose monomycolate (TMM) or trehalose dimycolate (TDM). Because several key antibacterial drugs, such as isoniazid, SQ109, delamanid, or ethambutol, target different aspects of the biosynthetic steps responsible for the cell wall attachment of mycolic acids (4–7), this pathway is of particular interest from a drug discovery perspective.

The three functionally and structurally related members of the antigen 85 complex, designated Ag85A, -B, and -C, are among the most abundantly secreted proteins in *M. tuberculosis* (8). These enzymes are responsible for the biosynthesis of TMM and TDM as well as the covalent attachment of mycolic acids to AG (9–11). Deletion of *fbpC2*, encoding Ag85C, resulted in a 40% decrease in the AG-bound mycolic acids but failed to affect the production of non-covalently linked myco-

5 The abbreviations used are: TB, tuberculosis; AG, arabinogalactan; CyC, cyclipostins and cyclophostin; DGAT, diacylglycerol acyltransferase; FAME, fatty acid methyl ester; MAME, mycolic acid methyl ester(s); MIC, minimal inhibitory concentration; TAG, triacylglycerol; TDM, trehalose dimycolate; TLM, thin layer chromatography; TMM, trehalose monomycolate; X8p, inhibitor molar excess leading to 50% inhibition; FP, fluorophosphonate; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); DEP, p-nitrophenyl phosphate; TAMRA, carboxytetramethylrhodamine; ILL, intracellular lipid inclusion; r.m.s., root mean square; PDB, Protein Data Bank.

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lates (10), whereas deletion of fbpA or fbpB, encoding Ag85A and Ag85B, respectively, led to reduced TDM levels (12–14), implying that although a level of functional redundancy exists in vivo between the three members, the contribution of each member is significant. The lack of double and triple knockout mutants might indicate that the loss of two or more Ag85 enzymes is detrimental to M. tuberculosis viability. An additional isoform, designated Ag85D or MPT51, has been characterized but found to be inactive due to the lack of catalytic elements required for mycolyltransferase activity (11, 15, 16). Ag85A/B/C share the same mycolic acid donor TMM, and their crystal structures present a highly conserved catalytic site, which further supports their similar enzymatic role (17–19).

Due to their importance in mycolic acid metabolism, the Ag85 enzymes have often been proposed as attractive targets for future chemotherapeutic developments against TB (9, 20–22). Because of their high structural conservation, it can be inferred that a single compound may inhibit all three enzymes of the complex at the same time and would make improbable the development of resistance to inhibitors, because resistant mutants would require the simultaneous acquisition of mutations in at least two fbp genes. In addition, because these proteins are secreted, targeting the Ag85 complex will minimize the effect of efflux mechanisms that may result in resistance phenotypes. Early inhibitors, such as trehalose analogs, were first designed as Ag85 inhibitors but were found to exhibit relatively poor activity on whole mycobacterial cells (9, 23). Another potentially selective fluorophosphonate α,α-d-trehalose inhibitor of the three antigen 85 enzymes has been reported to form a stable, covalent complex with the Ag85 enzyme following nucleophilic attack on the phosphorus atom of the catalytic Ser124 (24). In the same manner, the 2-amino-6-propyl-4,5,6,7-tetrahydro-1-benzothiophene-3-carbonitrile, designated I3-AG85, inhibits Ag85C, and exposure of M. tuberculosis to this compound was associated with reduced survival rates in broth medium and in infected primary macrophages. Moreover, I3-AG85 was active against a panel of multidrug-resistant/extensively drug-resistant strains, although it exhibited an MIC of 100 μM (25). By combining fragment-based drug discovery with early whole cell antibacterial screening, tetrahydro-1-benzothiophene analogs were discovered as potent Ag85C inhibitory molecules against drug-susceptible and drug-resistant M. tuberculosis strains (26). The selenazole compound ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) was found to inhibit the activity of Ag85C through an original mechanism by reacting with the conserved Cys209 residue located near the active site of the enzyme but not involved in the catalytic activity (27, 28). Ebselen was shown to directly impede the production of TDM and mycolylation of AG (27).

Recently, cyclipostins and cyclophostin (CyC), representing a new class of monocyclic enolphosph(on)ate compounds, have been discovered to act as powerful antitubercular agents affecting growth of M. tuberculosis both in vitro and in infected macrophages (29). Among the set of 27 CyC analogs previously evaluated against M. tuberculosis H37Rv, eight compounds exhibited potent anti-tubercular activities, particularly the cyclophostin analogs CyC7B and CyC8B as well as the cyclipostins-related molecule CyC17. Whereas CyC7B exhibited a strong activity against extracellular and intracellular mycobacteria (MIC50 of 16.6 and 3.1 μM, respectively), CyC8B was mostly found to be active against intracellular bacteria (MIC50 ~ 11.7 μM). In contrast, CyC17 was a potent inhibitor of in vitro growth (MIC50 ~ 0.5 μM) but failed to show activity against intracellular bacilli (29). To identify the putative target(s) of the CyC inhibitors, an activity-based protein profiling approach was used based on TAMRA-FP and desthiobiotin-FP probes and mass spectrometry analyses. This led to the capture of several active serine/cysteine enzymes in a complex proteome before mass spectrometry identification, among which Ag85A (Rv3804c) and Ag85C (Rv0129c) were identified.

The present study was undertaken to further explore and validate, through a combination of biochemical and structural approaches, the specificity of inhibition of the Ag85 activity by the CyC analogs, to determine their mode of action and to describe how they affect the mycolic acid profile in M. tuberculosis.

Results

CyC analogs inhibit TDM biosynthesis and transfer of mycolic acids to arabinogalactan in M. tuberculosis

CyCs are a new class of compounds demonstrating potent antitubercular activity, presumably involving inhibition of the Ag85 activity (29). The chemical structures of the cyclophostin analogs CyC7B and CyC8B and the cyclipostins CyC17, used in this study are provided in Fig. 1A. To test whether treatment with these CyCs alters the mycolic acid composition of M. tuberculosis mc²6230, cultures were exposed to increasing concentrations of CyC7B or CyC8B, the two inhibitors most active against extracellularly replicating M. tuberculosis (29), followed by metabolic labeling with sodium [2-14C]acetate and lipid analysis. Extraction and separation of the total mycolic acid methyl esters (MAME) by thin layer chromatography (TLC) revealed that neither CyC17 nor CyC8B altered the de novo biosynthesis of mycolic acid (Fig. 1B and C, left). In contrast, separation of the apolar lipid fraction by TLC showed a dose-dependent decrease in TDM levels associated with a concomitant increase in the production of TMM, which is the natural substrate of the Ag85 proteins (Fig. 1B and C, middle). To address whether CyC treatment also impacts the cell wall-bound mycolic acids, radiolabeled mycolic acids were extracted from delipidated bacteria (30). The autoradiography/TLC analysis confirmed a dose-dependent inhibition of [2-14C]acetate incorporation into all three forms of the AG-attached mycolic acids (α, methoxy, and keto), suggesting that treatment with CyC17 or CyC8B inhibits AG mycolylation at low concentrations (Fig. 1B and C, right). A quantitative analysis of these effects is provided in the corresponding graphs (Fig. 1B and C).

Overall, this suggests that in vivo inhibition of the mycolyltransferase activity by the CyC compounds results in decreased formation of the virulence-associated TDM and reduced transfer of mycolic acids onto the essential cell wall AG.

Covalent inhibition of the Ag85C mycolyltransferase activity

All three members of the Ag85 complex, sharing between 65 and 75% sequence identity, have been shown to possess a serine
hydrolase mycolyl esterase/transferase activity (17–19). To test the hypothesis that CyC analogs inhibit the activity of the three Ag85 members, we first cloned Ag85C (fbpcC2) into pET23b, and the recombinant protein was produced in Escherichia coli. The protein was then purified from lysates of E. coli by successive nickel-affinity, anion-exchange, and size-exclusion chromatography steps, leading to 3 mg of pure protein/liter of culture. Using a recently developed fluorescent assay based on resorufin butyrate as the acyl donor for Ag85C and trehalose as the acyl acceptor (27), we investigated whether CyC7/H9252, CyC8/H9252, and CyC17 inhibit the acyltransferase activity onto trehalose. In each case, a dose-dependent inhibition was observed with all three compounds, with CyC8 being the most efficient inhibitor (IC50 of 15 ± 5 μM), followed by CyC7 (IC50 of 43 ± 3 μM) and CyC17 (IC50 of 98 ± 6 μM) (Fig. 2A). Moreover, in terms of molar excess of inhibitor (xIC50 = IC50/[Ag85C]) (31), all three CyCs react almost in stoichiometry with pure Ag85C, as judged by their respective xIC50 values of around 0.3, 0.8, and 1.8, respectively.

To address the inhibitory effect on the mycolyltransferase assay, Ag85C (55 μM) was next incubated for 30 min in its native form with 500 μM (i.e. enzyme/inhibitor molar ratio of 1:9) of each CyC compound. As expected, the complete loss of activity was confirmed by comparing the pretreated versus non-treated

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**Figure 1. Ag85 complex mycolyltransferase activity is inhibited by CyC analogs in vivo.** Exponentially growing M. tuberculosis mc26230 was incubated with increasing concentrations of CyC17 or CyC7/H9252 in 7H9OADC/Tween 86 at 37 °C with agitation for 1 h. Subsequently, bacteria were labeled with sodium [2-14C]acetate for 6 h at 37 °C with agitation. The cultures were split, and from the first volume were extracted the total methyl esters of mycolates (MAME) and fatty acids (FAME). From the second volume, apolar and polar lipid fractions were obtained before derivatization of arabinogalactan MAME. A, chemical structures of the CyC analogs used in this study. B and C, effect of CyC7/H9252 (B) or CyC17/CyC7/H9252 (C) treatment on the mycolic acid profiles of M. tuberculosis mc26230. Equal counts (50,000 cpm) of MAME + FAME fraction were loaded on a TLC plate and resolved once using the solvent system hexane/ethyl acetate (95:5, v/v) run twice (far left). The apolar fraction was loaded (50,000 cpm), and TMM and TDM were visualized on a 1D TLC plate using the solvent system chloroform/methanol/water (40:8:1, v/v/v) (middle left). Equal volumes of arabinogalactan MAME fraction were loaded, and α, methoxy, and keto mycolic acids were visualized on a 1D TLC plate using the solvent system hexane/ethyl acetate (95:5, v/v) run twice (middle right). Densitometric analysis (far right) was performed on the TLCs shown in the left panels. Histograms and error bars, means and S.D. values calculated from at least two independent experiments.
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Ag85C. All three Ag85C-CyC adducts were treated with 10 μM TAMRA-FP fluorescent probe, known to bind to serine enzymes (32), for 1 h, and equal amounts of proteins were separated by SDS-PAGE and visualized by Coomassie staining (Fig. 2B, top) or in-gel fluorescence for TAMRA detection (Fig. 2B, middle). Pretreatment with either CyC7β or CyC8β resulted in a significant loss in fluorescence intensity (about 75%) as compared with the non-treated protein, whereas incubation with CyC17 abrogated TAMRA labeling. This suggests that reaction with the TAMRA probe is strongly impaired in the Ag85C-CyC adducts, resulting in a decrease/loss of fluorescence emission. To determine the implication of the conserved catalytic Ser124 in Ag85C in TAMRA labeling, this residue was replaced by an Ala residue, and the mutated protein was purified (Fig. 2B, bottom). Exposure of TAMRA to Ag85C<sub>124A</sub> failed to produce a fluorescence signal (Fig. 2B, middle), indicating that the catalytic Ser<sub>124</sub> is required for binding of the probe. As expected, no fluorescence emission was observed when pretreating the mutated protein with the CyC analogs (Fig. 2B, middle).

MALDI-TOF mass spectrometry was further used to study the (covalent) nature of the inhibition. Mass increments of +383.5 and +402.3 Da in the presence of CyC7β and CyC8β respectively, were observed within the global mass of treated Ag85C as compared with the global mass of untreated Ag85C (Fig. 2C). In contrast, no changes in the global mass were observed with the inactive Ag85C<sub>124A</sub> protein (Fig. 2D). These data thus support the formation of a covalent Ag85C-CyC complex, as the reaction between the catalytic Ser<sub>124</sub> and either CyC7β or CyC8β is expected to yield mass increases of +374.2 or +402.25 Da, respectively. Moreover, such results are consistent with the known and irreversible classical mechanism of action of phosphonate compounds, as demonstrated using pure mycobacterial lipolytic enzymes (31).

With respect to CyC17, the observed 322.1-Da mass shift increment was 124.18 Da lower than its expected theoretical molecular mass of 446.28 Da (Fig. 2C). This size difference may arise from the specific chemical properties of phosphate (i.e. CyC17) versus phosphonate (i.e. CyC7β and CyC8β) chemical groups. In all cases, the nucleophilic attack of catalytic Ser<sub>124</sub> at the phosphorus center induces ring opening. However, the reaction with CyC17 is very likely to form a new phosphate triester, which in turn becomes susceptible to hydrolysis. From these findings, it can be inferred that once the CyC17-Ser<sub>124</sub> adduct is formed, it becomes rapidly hydrolyzed in the presence of water, resulting in the cleavage and release of the methyl 2-acetyl-4-hydroxybutyrate (i.e. 124.1 Da), accounting exactly for the molecular mass discrepancy observed experimentally (Fig. 2C).

Taken together, these findings conclusively indicate that Ag85C is covalently modified by CyC analogs, leading to the inhibition of the mycolyltransferase activity and thus supporting the in vivo alteration of the mycolic acid pattern by these compounds.

Figure 2. Inhibition of the Ag85C mycolyltransferase activity is mediated by the covalent binding of CyC analogs. A, the enzymatic activity of Ag85C was tested using a fluorescence-based assay in the presence of different concentrations of CyC<sub>7β</sub>, CyC<sub>8β</sub>, and CyC<sub>17</sub>. The inhibitory effect was determined at the maximum rate of the reaction. Error bars, S.D. calculated from three independent experiments. Curves for CyC<sub>7β</sub>, CyC<sub>8β</sub>, and CyC<sub>17</sub> were fitted using the EC<sub>50</sub> shift non-linear regression model in GraphPad Prism with R<sup>2</sup> values of 0.9675, 0.9508, and 0.9415, respectively. B, equal amounts of either Ag85C or Ag85C<sub>124A</sub> were pretreated with CyC<sub>7β</sub>, CyC<sub>8β</sub>, and CyC<sub>17</sub>, incubated with TAMRA-FP, separated by SDS-PAGE, and visualized by Coomassie Blue staining (top) or in-gel fluorescence visualization (middle). The merged image is shown at the bottom. TAMRA labeling of Ag85C is prevented by the covalent binding of the CyC analogs to the catalytic Ser<sub>124</sub>. No TAMRA-FP labeling is seen for the Ag85C<sub>124A</sub> variant, confirming Ser<sub>124</sub> as the TAMRA-binding site. C and D, global mass modification of Ag85C (C) and Ag85C<sub>124A</sub> (D) preincubated with CyC<sub>7β</sub>, CyC<sub>8β</sub>, and CyC<sub>17</sub> as determined using an Ultraflex III mass spectrometer (Bruker Daltonics) in linear mode with the LP, 66 kDa method. The mechanism of action of the phosphonates CyC<sub>7β</sub> and CyC<sub>8β</sub>, and of the phosphate analog CyC<sub>17</sub>, based on mass spectrometry analyses is illustrated in C, a.u., arbitrary units.
Ag85A, -85B, and -85C express DGAT activity

Although the mycolyltransferase activity of the Ag85 complex has been established for a long time (8, 9), more recent work suggested that Ag85A mediates the transesterification of diacylglycerol using long-chain acyl-CoA to produce triglycerides (TAG), which act as storage compounds for energy and carbon (33). Ag85A contains the same catalytic triad as Ag85C or Ag85B, formed by residues Ser126, His262, and Glu230, and possesses a deep substrate-binding groove near the active-site serine, suggesting that Ag85B and Ag85C, similarly to Ag85A, may also express diacylglycerol acyltransferase (DGAT) activity. To test this hypothesis, all of the genes were cloned into pET23b, and the recombinant proteins were produced in E. coli and purified from lysates by successive nickel-affinity, anion-exchange, and size-exclusion chromatography steps. Because Ag85B was poorly expressed in E. coli, a synthetic gene was produced by replacing low-usage codons with high-usage codons, as reported previously (34) and subsequently cloned into pET23a.

All three proteins were assayed for DGAT activity in the presence of acyl-CoA with various chain lengths (from C4 to C18) as acyl donors and 1,2-dipalmitoyl-sn-glycerol (1,2-dipalmitin) as the acyl acceptor, as illustrated in Fig. 3A. Transesterification in the presence of 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) leads to the formation of TNB, which can readily be measured at 412 nm (33, 35). In agreement with previous findings, Ag85A was found to express DGAT activity, but such
an activity was only detected with C12-C18 acyl-CoAs (Fig. 3B). Whereas Ag85B demonstrated lower activity than Ag85A, Ag85C showed the highest activity, which was optimal in the presence of C16-CoA. No activity was detected with the C4- or C8-containing acyl chains. We also expressed and purified the Ag85 complex-related MPT51 (FbpC1) protein, which possesses an overall structure similar to that of the Ag85 complex members but is defective in the catalytic elements required for mycolyltransferase activity (11, 16). As anticipated, MPT51 failed to express any DGAT activity, suggesting that residues important for mycolyltransferase activity are also key players in the DGAT activity, as proposed earlier for the Ser124 in Ag85A (33). Purified Ag85C5124A was next assayed with various acyl-CoA substrates, and, as shown in Fig. 3B (inset), the DGAT activity was abrogated in the mutant protein, implying that Ser124 plays a critical role in the enzymatic reaction.

Overall, these data extend insights from previous findings and indicate that all three members of the Ag85 complex express DGAT activity, with Ag85C exhibiting the most pronounced activity. This suggests that Ag85C may also make an important contribution in TAG synthesis in M. tuberculosis.

**CyC analogs inhibit the in vitro DGAT activity of Ag85C but not of Tgs1**

The above-mentioned results prompted us to investigate whether CyC analogs alter the DGAT activity of Ag85C. This was achieved by incubating the purified enzyme in the presence of increasing concentrations of CyC7 and purified Ag85C, and purified Ag85A, -B, and -C as positive controls (Fig. 4A, right). The 17/4 monoclonal antibody recognized a well-conserved epitope present in Ag85A and Ag85B but not in Ag85C (37). In contrast, the 32/15 antibody revealed all three antigens and the presence of more pronounced bands in the pMV261-Ag85C and pMV261-Ag85C5124A lysates, which, by comparison with the 17/4 blot, could clearly be attributed to Ag85C (Fig. 4B, right). This indicates that both Ag85C variants were overproduced at comparable transcriptional and translational levels and allowed us to investigate whether this may affect the intracellular TAG content of M. tuberculosis (38). TAGs are often stored in the form of ILIs, which can be visualized by staining with Nile Red (39, 40). As shown in Fig. 4B (left), although a punctiform labeling corresponding to ILIs is observed in the control strain carrying the empty pMV261, Nile Red staining was much more pronounced in the strain overproducing Ag85C, and the effect returned to control levels in the strain harboring pMV261-Ag85C5124A. Quantification of the fluorescence intensity over the entire length of the individual bacilli from each strain clearly indicates that large and numerous ILIs were present in the Ag85C-overexpressing strain, as compared with the control and Ag85C5124A strains (Fig. 4B, right).

To check whether enhanced IIL formation coincided with increased de novo biosynthesis of TAG, metabolic labeling of M. tuberculosis cultures with sodium [2-14C]acetate was performed, followed by extraction and separation of the apolar lipid fraction by TLC. In the absence of CyC treatment, the stain carrying pMV261-Ag85C produced moderately higher amounts of TAGs than the control strain containing the empty pMV261 or the strain overexpressing Ag85C5124A (Fig. 4C, left), supporting the in vivo contribution of Ag85C in TAG production. Importantly, exposure to CyC17 inhibited TAG biosynthesis in a dose-dependent manner in the control strain, thus implying that the de novo biosynthesis of TAG is also targeted by CyC17 (Fig. 4C). In addition, a less pronounced decrease in TAG production occurred in the strain overexpressing Ag85C as compared with the control strain, presumably because of the inherent capacity of this strain to synthesize more TAG that partially overcomes CyC17 inhibition (Fig. 4C). Collectively, these results suggest that TAG production in M. tuberculosis is inhibited by CyC17 and that this is dependent upon Ag85C DGAT activity.

**Crystal structure of the CyC8p-bound Ag85C**

To gain insight into the mode of action of the CyC compounds, crystallization studies of Ag85C were undertaken in the presence of the three CyC inhibitors. However, diffracting crystals were only obtained with CyC8p for which the X-ray structure of Ag85C bound to CyC8p was solved at a resolution of 1.8 Å (Table 1). The asymmetric unit contains two molecules of Ag85C (Fig. 5A). Residues 6–282 and 8–282 for each subunit...
could be built, implying that the last 14 residues as well as the polyhistidine tag in the C terminus were not modeled. The structure of Ag85C has been extensively reported (17). In brief, the protein adopts a typical $\alpha/H\beta_2$-$\beta$ hydrolase fold made of a central $\beta$-sheet surrounded by $\alpha$-helices. The two monomers are nearly identical, as their superposition over 274 residues gives an r.m.s. deviation of 0.24 Å. However, whereas a clear electron density could be seen for the entire structure of CyC$_{85}$ in one monomer, this was only the case for the headgroup of the second molecule (Fig. 5B). It is noteworthy that the extra but non-interpretable electron density (Fig. 5B) in the vicinity of CyC$_{85}$ observed in all data sets collected from either co-crystallization or soaking experiments and in various crystallization conditions (data not shown), appears as a possible molecule interacting with Phe$_{150}$ and could be seen in both monomers. As Phe$_{150}$ was shown to be involved in stacking of the lipid chain of octylglucoside in the Ag85C-octylglucoside crystal structure (PDB entry 1VA5 (19)), we tried to place the acyl chain of CyC$_{85}$ in this extra electron density, but refinement of this alternate conformation of CyC$_{85}$ did not converge. Therefore, further modeling of this electron density blob was not pursued. Although CyC$_{85}$ has clearly reacted, as evidenced by the presence of an opened ring and the MALDI-TOF data, no covalent bond between the catalytic Ser$_{124}$ residue and the phosphonate group of CyC$_{85}$ was observed (Fig. 5, B and D). Therefore, CyC$_{85}$ was modeled in an opened conformation (Fig. 5, B and D).
Inhibition of Ag85C by cyclipostins and cyclophostin

Table 1

Data collection and refinement statistics

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Discussion

Toward the generation of new lead compounds with unexplored modes of action in *M. tuberculosis*, the CyC analogs were initially designed to inhibit mycobacterial lipases (31). In particular, by covalently binding to the catalytic serine, they fully inactivated the monoacylglycerol lipase Rv0183 and the triacylglycerol lipase LipY from *M. tuberculosis* but not the mammalian gastric and pancreatic lipases (31). Subsequent biochemical studies involving the selective labeling and enrichment of captured enzymes using appropriate fluorophosphonate probes in combination with CyC₁₇ resulted in the identification of 23 potential target lipolytic enzymes, all of which comprise catalytic serine or cysteine residues (29). Because they are multitarget-inhibitory compounds in mycobacteria, the use of CyC analogs could prevent the selection of drug resistance mechanisms. In addition, the lack of cytotoxicity in human cells (29) makes them attractive hits to be further evaluated.

Herein, we provide compelling evidence that at least some of the CyC analogs primarily act by inhibition of the Ag85 complex, resulting in decreased TDM formation and reduced mycolylation of AG, an essential polymer of the mycobacterial cell wall. Although one cannot rule out the possibility that the killing effect of the CyC on *M. tuberculosis* results from the simultaneous and net effect on multiple physiological targets, the inhibition of TMM and AG mycolylation is very likely to represent the major cause of growth inhibition of *M. tuberculosis*, at least in *in vitro* growing cultures. We demonstrate here that all three Ag85 members express DGAT activity *in vitro*, with Ag85C being the most active, thereby extending previous work reporting the DGAT activity of Ag85A (33). Importantly, the S124A site-directed mutation of the active site of Ag85C proved that this residue is involved in the DGAT activity of this enzyme and TAG synthesis. Although the synthesis of TAG relies on the presence of multiple TAG synthases, such as the well-characterized Tgs1 (Rv3130c) (36), our work extends the growing list of enzymes displaying DGAT activity in *M. tuberculosis*. The Ag85 proteins do not belong to the known DGAT families and do not possess the characteristic conserved heptapeptide acyltransferase motif of the mycobacterial Tgs enzymes involved in TAG biosynthesis (35, 41). Nevertheless, the DGAT activity of Ag85C, similarly to Ag85A (33), includes two consecutive reactions, the fatty acyl-CoA hydrolsisis (thioesterification) and the subsequent transfer of the acyl chain to the diacylglycerol (transesterification). Overexpressing Ag85C in *M. tuberculosis* was correlated with an increase in *de novo* TAG production and formation of lipid storage inclusions. These findings establish for the first time a connection between cell wall and TAG biosynthesis by Ag85C and expand our understanding of this important enzyme in the physiology of *M. tuberculosis*. However, a direct implication of the DGAT activity of Ag85C in pathogenesis and persistence of *M. tuberculosis* requires further studies. In addition, under conditions where Ag85C is overexpressed, *M. tuberculosis* was more refractory to TAG inhibition by CyC₁₇, further emphasizing the yet unexpected contribution of Ag85C as a player in TAG biosynthesis. Inhibition of the DGAT activity of Ag85C, and therefore TAG inhibition, by the CyC compounds is very unlikely to

interacts through residues at the entrance of the Ag85C active site (Fig. 5, C and D). The polar head of CyC₈₅B is recognized through hydrogen bonds with the catalytic Ser¹²⁴ side chain as well as with the main chain of Leu⁴⁰ and the Asp⁴⁶ side chain via two water molecules. The Arg⁴¹ side chain completes the interaction with the headgroup of CyC₈₅B by van der Waals interaction (Fig. 5D). The long aliphatic chain of CyC₈₅B is stabilized by hydrophobic interactions involving the Ile²²², Pro²²³, Phe²²⁶, Val²²⁷, and Leu²²⁷ side chains (Fig. 5D). The distance between the phosphate of CyC₈₅B and Ser¹²⁴ of 3.6 Å clearly attests that in this crystal, the ligand is not covalently bound. Importantly, this loss of covalent binding was observed in multiple data sets collected, obtained either by soaking or co-crystallization experiments. However, the lack of covalent binding in the crystal structure does not rule out the well-known covalent inhibitory mechanism of the CyC analogs supported by MALDI-TOF mass spectrometry analyses (Fig. 2, C and D).

Furthermore, the polar headgroup of CyC₈₅B is located where trehalose, the natural substrate of the Ag85 proteins, binds, as seen in the crystal of the trehalose-bound structure of Ag85B (PDB entry 1F0P (18)) (Fig. 6A). Interestingly, the fatty acyl chain of CyC₈₅B is placed in a very hydrophobic cavity that was proposed to be part of the TDM/TMM fatty chain recognition site (17). In addition, structural comparison indicated that the important residues in Ag85C interacting with CyC₈₅B are fully conserved in Ag85B and Ag85A (Fig. 6B), strongly suggesting that CyC₈₅B, and presumably all of the other CyC analogs, may inhibit the three members of the Ag85 complex.
participate in growth inhibition of *M. tuberculosis* in vitro, but it may have important consequences for *in vivo* survival and/or for maintaining the bacilli in a non-replicating growth phase, such as in foamy macrophages in which *M. tuberculosis* is able to hydrolyze the host-derived TAGs from lipid bodies to fatty acids, which are then reprocessed as TAGs and stored within ILIs (42, 43). In these subcellular structures, TAGs represent the primary storage source of carbon and energy, allowing the bacteria to survive in a non-replicating state and to persist inside these foamy cells, which usually line the necrotic centers of tubercle granulomas and have been proposed to be the intracellular niche of *M. tuberculosis* during latent infection (42). Although this requires further exploration, inhibiting the DGAT activity of Ag85C may help in designing new classes of molecules that restrict entry of *M. tuberculosis* into dormancy, a strategy that would overcome mycobacterial persistence and prolonged chronic infections.

Biochemical studies involving the TAMRA-FP probe that binds to serine hydrolases along with mass spectrometry and structural analyses indicate that, in addition to covalently binding to the catalytic Ser124, the CyC analogs could also be competing with the binding of Ag85 substrate (*i.e.*, the trehalose and the acyl chain moieties of TMM). As the Ag85 complex members share similar substrate specificities, our results suggest that CyC analogs could target not only Ag85C but also Ag85B and Ag85A, an assertion reinforced by the fact that Ag85A was also identified as a potential target in the original proteomic screen approach (29). Comparison of the three structures...
strongly supports this hypothesis, as residues contacting CyC8 in Ag85C are strictly conserved in Ag85A and Ag85B. This is of interest, as the inhibitor I3-AG85 binding to the active site of Ag85C exhibits only strict specificity toward Ag85C and does not bind Ag85A and -B (25). Moreover, given their low \(x_{50}\) values, the three CyC compounds are able to act in near stoichiometry and alter both the mycolyltransferase and DGAT activities of Ag85C. It is noteworthy that, among the three CyCs investigated, the phosphate CyC17, which appears as the best inhibitor against extracellular \(M. \text{tuberculosis}\), was the least efficient when assayed on pure recombinant enzyme. However, when assayed on living bacteria, CyC17 clearly affected TDM synthesis and mycolylation of AG. The differences in activity with CyC7 and CyC8 may be related to the chemical properties of the phosphate versus phosphonate chemical groups. On the other hand, despite their high activity, phosphate inhibitors can be subjected to hydrolysis, rendering their covalent binding potentially reversible, as shown here in the case of the CyC17-Ser124 adduct (29). Interestingly, using a chemical proteomic approach, the EZ120 \(\beta\)-lactone compound exhibiting strong antitubercular activity and resembling an electrophilic mimic of mycolic acids was recently found to block several serine hydrolases essential for the mycomembrane biosynthesis (44). The polyketide synthase Pks13, whose \(\beta\)-keto mycolate is transferred onto trehalose and reduced to yield TMM, as well as Ag85A were identified as primary targets of EZ120. However, whether this \(\beta\)-lactone acts similarly to the CyC inhibitors in Ag85 awaits structural determination.

Comparison of the Ag85C-CyC8 structure with that of Ag85C-ebselen (PDB entry 4QDU (27)) shows that the mode of inhibition triggered by CyC8 is different. Ebselen indeed covalently modifies Cys209, which is 13 Å away from the catalytic domain.

Figure 6. Mode of inhibition of the Ag85 complex by CyC8. A, superposition of the Ag85B-trehalose (PDB code 1F0P, blue) and Ag85C-CyC8 (gray) crystal structures. The headgroup of CyC8 (yellow) occupies the same site as trehalose (green). B, Ag85C residues (gray) involved in the recognition of CyC8 are all strictly conserved in Ag85B (cyan) and Ag85A (magenta). C, superposition of the Ag85C-ebselen (PDB code 4QDU; blue) and Ag85C-CyC8 (gray) crystal structures. CyC8 binds far away from the ebselen-binding site and does not trigger structural rearrangement of helix 9. D, superposition of the Ag85C-DEP (PDB code 1DQY; cyan) and Ag85C-CyC8 (gray) crystal structures. CyC8 presents a similar mode of inhibition as DEP (cyan stick), a nonspecific \(\alpha/\beta\) hydrolase inhibitor.
Ser\textsuperscript{124} (Fig. 6C). Inhibition by ebselen and its derivatives (azido ebselen and adamantyl ebselen) is mediated by inducing structural rearrangements of helix α9 and the loop between helices 9 and 10 that ends in destabilizing the hydrogen bond network of the active site (27, 28, 45). Comparison of the crystal structures of Ag85C-CyC\textsubscript{ng} and the Ag85C native structure (PDB entry 3HRH) possessing the same space group and crystallized in similar conditions shows that the two structures are identical. The superposition of the two structures yields an overall r.m.s. deviation over 251 residues of about 0.19 Å. Furthermore, no local structural rearrangement was observed (data not shown).

As expected, the mode of inhibition of CyC\textsubscript{ng} consists of blocking the active site (31) and not of destabilizing the overall structure and stability of the protein as reported for ebselen and its analogs (27, 45). Furthermore, the mode of action of CyC\textsubscript{ng} is more related to that of the diethyl p-nitrophenyl phosphate (DEP), a nonspecific α/β hydrolase inhibitor that covalently modifies the Ser\textsubscript{124} catalytic residue (17). Superposition of the Ag85C-DEP (PDB entry 1DQY) and Ag85C-CyC\textsubscript{ng} structures highlights the similar positioning of the phosphonate groups of the two inhibitors (Fig. 6D).

In summary, the data reported here offer a first look at the potent inhibition of the M. tuberculosis Ag85C by cyclipostins and cyclophostin analogs, compounds that effectively inhibit growth of extracellularly and intracellularly replicating M. tuberculosis and their mechanism of action. Interestingly, a recent study indicated that these compounds were also effective against clinical isolates of the M. abscessus complex (46), mostly encountered in cystic fibrosis patients, and known to be intrinsically resistant to most antitubercular drugs. We anticipate that the high-resolution crystal structure of Ag85C-CyC\textsubscript{ng} will now open the way to the development, through structure-based drug design, of improved inhibitors that target the Ag85 complex in various pathogenic mycobacteria.

**Experimental procedures**

**Mycobacterial strains and growth conditions**

*M. tuberculosis* mc\textsuperscript{2}6230 (47) was grown on Middlebrook 7H10 agar plates containing OADC (oleic acid, albumin, dextrose, catalase) enrichment (Difco) and supplemented with 24 μg/ml pantothenic acid. Liquid cultures were obtained by growing mycobacteria in Middlebrook 7H9 (Difco) supplemented with 10% OADC enrichment, 0.2% (v/v) glycerol, 0.05% (v/v), Tween 80 (Sigma), 24 μg/ml pantothenic acid, and 25 μg/ml kanamycin when required.

**Plasmids and DNA manipulations**

The *fbpC2* gene, encoding Ag85C, was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA using the forward primer 5’-CTA CTT CAT ATG TCC CCT GGT CCC GTT CTA GCA G-3’ (Ndel site in boldface type) and the reverse primer 5’-GAG ATT CTC GAG AGC AGG AGC AGC AGG GAG G-3’ (Xhol site in boldface type). The PCR product was cloned into pET23b cut with Ndel and Xhol (New England Biolabs), enabling the incorporation of a polyhistidine tag in the C terminus of the Ag85C protein. The pET23b-*fbpA* and pET23b-*fbpC2* constructs carrying the genes encoding Ag85A and MPT51, respectively, were described previously (11). A codon-optimized version of the *fbpB* gene, encoding Ag85B, was synthesized (GenScript) and introduced within the pET23a plasmid thanks to the Ndel and Xhol restriction sites, enabling also the incorporation of a polyhistidine tag in the C terminus of the Ag85B protein. The Ag85C\textsuperscript{124AA} mutant was obtained by using the PCR-driven overlap extension method (48). Briefly, two separate PCRs were set up with the Phusion\textsuperscript{®} DNA polymerase (Thermo Fisher Scientific). The first one was set up with the forward primer used to amplify the wildtype *fbpC2* gene and the reverse internal primer 5’-AAG ACC CAC CGC GCG GTT-3’. The second one was set up with a forward internal primer, 5’-AAC GCG GCG GTG GTT CTT GCG ATG TTC GGG GGT TCC G-3’, overlapping the internal reverse primer and containing the nucleotide substitution (changed nucleotide in boldface type) with the reverse primer used to amplify the wildtype *fbpC2* gene. The purified PCR products were heterodimerized by heating to 95 °C for 1 min, followed by cooling to 60 °C for 10 min in the presence of Phusion\textsuperscript{®} DNA polymerase and dNTPs to generate a double-stranded hybrid. A last step of PCR was performed with the primers used to amplify the wildtype *fbpC2* gene with the hybrid product obtained in the previous step as template. The mutated *fbpC2* gene was finally cloned like the wildtype gene into pET23b and subjected to DNA sequencing to confirm the proper introduction of the mutation. The coding sequence of the gene *Rv3130c*, which encodes Tgs1 from *M. tuberculosis*, was PCR-amplified using the forward primer 5’-AGG GAG CCA CCA AGC CAT ACG CTA CCT AAC GAC ACT TGA CGC-3’ (Ncol site in boldface type, tobacco Etch virus protease cleavage site in lowercase type) and the reverse primer 5’-ACG AGG CTT CCA CCT AAC AAC GAC ACT TGA CGC-3’ (HindIII site in boldface type). The PCR amplicon was treated with NcoI and HindIII and ligated to Ncol-HindIII–linearized pET32a. This plasmid containing the polyhistidine and thioredoxin as fusion tags in the N-terminal position was used to produce soluble recombinant Tgs1.

**Expression and purification of the individual Ag85 antigens and MPT51**

All four plasmids harboring the *fbpA*, *fbpB*, *fbpC2*, and *fbpC1* genes were used to transform the *E. coli* C41 (DE3) expression strain. Transformed bacteria were grown inuria-Bertani medium containing ampicillin (200 μg/ml) until the A\textsubscript{600} reached 0.6. Bacterial cultures were then placed on icy water for 30 min before induction with 1 mM isopropyl β-D-thiogalactopyranoside and further incubated at 16 °C for 20 h. Bacterial pellets were collected by centrifugation (6,000 × g, 4 °C, 1 h) and resuspended in lysis buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 1 mM benzamidine). Lysates were sonicated and clarified by centrifugation (27,000 × g, 4 °C, 45 min) before purification by nickel-affinity chromatography with nickel-nitrilotriacetic acid–Sepharose beads and elution with lysis buffer containing 250 mM imidazole without benzamidine (GE Healthcare). Proteins were next dialyzed against 50 mM Tris–HCl, pH 8.0, and 5 mM β-mercaptoethanol buffer and loaded on an anion-exchange HiTrap\textsuperscript{®} Q Fast Flow column (GE Healthcare). The protein was eluted with a linear NaCl gradient. The final step of purification was by
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size-exclusion chromatography using a Superdex™ 75 10/300 GL column (GE Healthcare). Proteins were eluted in potassium phosphate buffer (50 mM KH₂PO₄/K₂HPO₄ pH 7.6) for DGAT activity assessments. Ag85C was eluted in a sodium phosphate buffer (50 mM NaH₂PO₄/Na₂HPO₄ pH 6.0) for mycolyltransferase activity assessments and in 50 mM Tris-HCl pH 8.0, 200 mM NaCl for crystallization experiments and stored at 4 °C.

Expression and purification of Tgs1

The *M. tuberculosis* Tgs1 was overproduced in *E. coli* and purified. Briefly, *E. coli* BL21 Rosetta™ 2 was freshly transformed with pET32a-Tgs1. Exponentially growing bacteria cultured in 2 liters of NYZ Broth (BD Biosciences) were cooled on icy water for 30 min, and 1 ml isopropyl β-D-1-thiogalactopyranoside was added before incubation at 16 °C for 16 h with agitation (200 rpm). Bacteria were then collected by centrifugation, the medium was discarded, and the pellet was resuspended in lysis buffer containing 10% glycerol, which was maintained for all subsequent buffers used. Lysates were produced and subjected to purification via nickel affinity chromatography. His-tagged tobacco etch virus protease was added to the eluted protein solution at a 1:50 (w/w) ratio, and the mixture was dialyzed overnight before again being subjected to nickel-affinity chromatography. The fraction that flowed through the nickel-nitrilotriacetic acid column, containing tagless Tgs1, was concentrated and subjected to size-exclusion chromatography using a Bio-rad ENrich SEC 650 (Bio-rad) and as buffer 100 mM K₂HPO₄/KH₂PO₄ pH 7.5, supplemented with 400 mM NaCl and 10% glycerol. The fractions containing active Tgs1 were pooled and concentrated to 0.1 mg/ml.

RNA extraction, cDNA production, and quantitative real-time PCR

Mycobacterial RNA was purified using the Nucleospin RNA kit (Macherey Nagel) and assessed for purity on a NanoDrop spectrometer and for integrity using a BioAnalyzer (Agilent). Subsequently, RNA was treated by DNase I (Life Technologies) spectrometer and for integrity using a BioAnalyzer (Agilent). Kit (Macherey Nagel) and assessed for purity on a NanoDrop spectrometer and for integrity using a BioAnalyzer (Agilent). PCR product was digested with BamHI (Promega) and cloned into pET32a (BamHI site in boldface type). After amplification, the open reading frame under control of the hsp60 promoter to mc26230 wildtype or overexpressing Ag85C were prepared and subjected to Western blot analysis as described previously (49).

DGAT and mycolyltransferase assays

The DGAT activity assay was performed for 1 h at 37 °C using a protocol reported earlier (33). Briefly, the reaction mixture was composed of 400 μM 1,2-dipalmitoyl-sn-glycerol and a 500 μM concentration of the different acyl donor molecules tested (butanoyl-CoA, octanoyl-CoA, lauroyl-CoA, palmitoyl-CoA, and oleoyl-CoA (Sigma-Aldrich)) in 50 mM potassium phosphate buffer, pH 7.6, containing 2% DMSO. The enzyme concentration in the reaction was 3 μM (0.5 μM in the case of Tgs1). At the end of the assay, an equal volume of DTNB (360 μg/ml) was added to the reaction, and the absorbance was measured at 412 nm with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific), enabling the calculation of the specific activity of the enzymes (nmol of TNB produced × min⁻¹ × mg of protein⁻¹).

The mycolyltransferase activity assay was performed for 15 min at 35 °C based on a procedure described previously (27). Measurements were taken every 15 s using a Multimode Microplate Reader POLARStar® Omega (BMG Labtech), and the activity of Ag85C was calculated at the maximum rate of the reaction. The reaction mixture was composed of 50 mM sodium phosphate (pH 6.0) containing 2% DMSO, 4 mM trehalose, and 12.5 μM resorufin butyrate (Sigma-Aldrich). The resorufin butyrate was dissolved in DMSO and diluted 100-fold in the reactions. The enzyme concentration in each reaction was 5.5 μM. Data presented were obtained from three independent experiments and analyzed by non-linear regression using GraphPad Prism version 5 software.

Inhibition of the DGAT and mycolyltransferase activity

CyC7₉, CyC₈₉, and CyC₁₇ were synthesized as described previously (31, 50). To study the inhibitory effect on DGAT activity, a 30 μM concentration of either Ag85A, Ag85B, Ag85C, or MPT51 was co-incubated with increasing concentrations of CyC₇₉, CyC₈₉, and CyC₁₇ for 1 h at room temperature in a reaction mixture containing 50 mM sodium phosphate buffer (pH 6.0), 10% DMSO, and 0.5 times the critical micelle concentration of n-dodecyl β-D-maltoside. Inhibition of the mycolyltransferase activity was determined using 55 μM of Ag85C co-incubated with increasing concentrations of CyC₇₉, CyC₈₉, and CyC₁₇ for 30 min at room temperature in 50 mM sodium phosphate buffer (pH 6.0), 10% DMSO, and 0.5 times the critical micelle concentration of n-dodecyl β-D-maltoside. Ag85C and Ag85C<sup>S124A</sup> pretreated or not with the CyC analogs were further incubated with 10 μM ActivX TAMRA-FP probe (Thermo Fisher Scientific) for 1 h at room temperature in the darkness. The reaction was stopped by adding 5× Laemmli reducing buffer followed by boiling, and proteins were separated by 12% SDS-PAGE. Subsequently, TAMRA FP-labeled proteins were detected by fluorescent gel scanning (TAMRA: λ<sub>ex</sub> 557 nm, λ<sub>em</sub> 583 nm) using the Cy<sub>3</sub> filter of a ChemiDoc MP Imager (Bio-Rad) before staining of the gels with Coomassie Brilliant Blue dye.

Overexpression of Ag85C variants in *M. tuberculosis*

The Rv0129c gene was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA using the forward primer 5′-CCC AGG TTG TTT ACA GGG TTC GTG-3′ and the reverse primer 5′-ACC ATG CAT CCC TAG GCG CCC CGG GCC GGG C-3′ (BamHI site in boldface type). After amplification, the PCR product was digested with BamHI (Promega) and cloned into pMV261-Ag85C<sup>S124A</sup> mutant plasmid was constructed by the QuikChange method using the pMV261-Ag85C<sup>S124A</sup> plasmid in the reaction was 3 μM (0.5 μM in the case of Tgs1). At the end of the assay, an equal volume of DTNB (360 μg/ml) was added to the reaction, and the absorbance was measured at 412 nm with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific), enabling the calculation of the specific activity of the enzymes (nmol of TNB produced × min⁻¹ × mg of protein⁻¹).
with Flexanalysis version 3.0 software (Bruker) with an adapted analysis method. To eliminate salts from the samples, 10 μl of each preparation was submitted to a desalting step on a C4 Zip-Tip μcolumn (Millipore). 1 μl of desalted sample was mixed with 1 μl of α-cyano-4-hydroxycinnamic acid matrix in a 50% acetonitrile, 0.3% TFA mixture (1:1, v/v). 1 μl was spotted on the target, dried, and analyzed with the LP_66 kDa method. Peak picking was performed with Flexanalysis version 3.0 software (Bruker) with an adapted analysis method. Parameters used were as follows: SNAP peak detection algorithm, S/N threshold fixed to 6, and a quality factor threshold of 30.

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Whole-cell radiolabeling experiments and lipid analysis

To investigate the CyC-induced changes in the lipid profile, increasing drug concentrations were added to exponentially growing *M. tuberculosis* mc²620 cultures grown in Middlebrook 7H9 supplemented with OADC enrichment and Tween 80 and 20 μg/ml pantothenate for 1 h. Subsequently, metabolic labeling of lipids was performed by adding 1 μCi/ml sodium [2-14C]acetate (56 mCi/mmol; American Radio Chemicals) for an additional 6 h at 37 °C. Cells were harvested and delipidated, as described previously (51). The apolar lipid fraction containing TMM and TDM was separated on a 1D TLC plate using the solvent system petroleum ether/diethyl ether (90:10, v/v) and revealed after exposure to film. Delipidated bacteria were washed twice with PBS-Tween 0.05%, and resuspended in 300 μl of PBS. Nile red fluorescent probe (Interchim), as described previously (40). Approximately 7.5 × 10⁷ cells (OD₁₅₀) were stained with Nile Red fluorescent probe (Interchim), as described previously (40). Approximately 7.5 × 10⁷ cells (OD₁₅₀) were collected at 9,000 × g for 3 min, washed twice with 500 μl of PBS-Tween 0.05%, and resuspended in 300 μl of PBS. Nile Red (15 μl of a solution at 0.5 mg/ml solubilized in ethanol) was added to the bacterial suspension, which was further incubated for 30 min at 37 °C in the darkness. Cells were then centrifuged, washed twice with PBS-Tween 0.05%, and resuspended in 300 μl of PBS. Bacteria were spotted between a 170-μm-thick coverslip and a 1.5% agarose-PBS pad. Image acquisition was performed with an OLYMPUS FV1000 confocal microscope at λₘₐₓ=λₑₐₜ = 530/590 ± 10 nm, and images were processed and analyzed using ImageJ.

Mass spectrometry

Mass analyses were performed on a MALDI-TOF-TOF Bruker Ultraflex III spectrometer (Bruker Daltonics, Wissembourg, France) controlled by the Flexcontrol version 3.0 package (Build 51). This instrument was used at a maximum accelerating potential of 25 kV and was operated in linear mode using the m/z range from 20,000 to 100,000 (LP_66 kDa method). Five external standards (Protein Calibration Standard II, Bruker Daltonics) were used to calibrate each spectrum to a mass accuracy within 200 ppm. Peak picking was performed with Flexanalysis version 3.0 software (Bruker) with an adapted analysis method. To eliminate salts from the samples, 10 μl of each preparation was submitted to a desalting step on a C4 Zip-Tip μcolumn (Millipore). 1 μl of desalted sample was mixed with 1 μl of α-cyano-4-hydroxycinnamic acid matrix in a 50% acetonitrile, 0.3% TFA mixture (1:1, v/v). 1 μl was spotted on the target, dried, and analyzed with the LP_66 kDa method. Peak picking was performed with Flexanalysis version 3.0 software (Bruker) with an adapted analysis method. Parameters used were as follows: SNAP peak detection algorithm, S/N threshold fixed to 6, and a quality factor threshold of 30.

**Crystallization, data collection, structure determination, and refinement**

Crystals were grown in sitting drops at 18 °C by mixing 0.8 μl of protein (in 50 mM Tris-HCl, pH 8.0, and 200 mM NaCl) at a concentration of 8 mg/ml with 0.8 μl of reservoir solution consisting of 0.2 m magnesium chloride hexahydrate, 0.1 m sodium citrate tris dihydrate, pH 5.0, and 10% (w/v) polyethylene glycol 20,000. 1-month-old crystals were then soaked for 24 h with a final concentration in the drop of 5 mM CyC₉₆. Crystals were fished with a latholoop and flash-cooled in liquid nitrogen without any cryoprotection. Data collection was performed at the ID-23.1 beamline at the ESRF synchrotron (Grenoble, France). Data were processed with XDS (53), and the structure was solved by molecular replacement with the structure of Ag85C as search model (PDB code 3HRH (54)) and using Phaser from the PHENIX software suite (55). Manual adjustments of the model were performed with Coot (56), and the structure was refined to 1.8 Å with PHENIX. PDB coordinates and structure factors were deposited in the Protein Data Bank under accession number 5OCJ. Data collection and refinement statistics are displayed in Table 1.

**References**

Inhibition of Ag85C by cyclipostins and cyclophostin
Inhibition of Ag85C by cyclipostins and cyclophostin

Cyclipostins and cyclophostin analogs inhibit the antigen 85C from Mycobacterium tuberculosis both in vitro and in vivo

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