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Lipolytic Enzymes Inhibitors: a new way for Antibacterial Drugs

Discovery

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Abstract

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M. tb*) still remains the deadliest infectious disease worldwide with 1.5 million deaths in 2018, of which about 15% are attributed to resistant strains. Another significant example is *Mycobacterium abscessus* (*M. abscessus*), a nontuberculous mycobacteria (NTM) responsible for cutaneous and pulmonary infections, representing up to 95% of NTM infections in cystic fibrosis (CF) patients. *M. abscessus* is a new clinically relevant pathogen and is considered one of the most drug-resistant mycobacteria for which standardized chemotherapeutic regimens are still lacking. Together the emergence of *M. tb* and *M. abscessus* multi-drug resistant strains with ineffective and expensive therapeutics, have paved the way to the development of new classes of anti-mycobacterial agents offering additional therapeutic options. In this context, specific inhibitors of mycobacterial lipolytic enzymes represent novel and promising antibacterial molecules to address this challenging issue. The results highlighted here include a complete overview of the antibacterial activities, either in broth medium or inside infected macrophages, of two families of promising and potent anti-mycobacterial multi-target agents, *i.e.* oxadiazolone-core compounds (*OX*) and Cyclophostin & Cyclipostins analogs (*CyC*); the identification and biochemical validation of their effective targets (*e.g.*, the antigen 85 complex and TesA playing key roles in mycolic acid metabolism) together with their respective crystal structures. To our knowledge, these are the first families of compounds able to target and impair replicating as well as intracellular bacteria. We are still impelled in deciphering their mode of action and finding new potential therapeutic targets against mycobacterial-related diseases.
Keywords: mycobacteria; tuberculosis; cystic fibrosis; antibiotics; oxadiazolone-core derivatives; Cyclipostins and Cyclophostin analogs
Abbreviations:

- ABP: activity-based probe
- ABPP: Activity-based protein profiling
- AChE: acetylcholinesterase
- CC_{50}: compound concentration leading to 50% host cell toxicity
- CF: cystic fibrosis
- CyC: Cyclophostin & Cyclipostins analogs
- FM: foamy macrophages
- GPL: glycopeptidolipids
- HSL: hormone-sensitive lipase
- IC_{50}: compound concentration leading to 50% enzyme activity inhibition
- ILI: intracytoplasmic lipid inclusions
- ILI^+3: foamy macrophages displaying many ILI occupying most of the mycobacterial cytoplasm
- LB: lipid bodies
- Lip-HSL: enzymes belonging to the hormone-sensitive lipase family members proteins
- mBMDM: murine bone-marrow-derived macrophages
- MIC: minimum inhibitory concentration
- MIC_{50}: compound concentration leading to 50% mycobacterial growth inhibition
- MIC_{50Raw}: compound concentration leading to 50% bacterial growth inhibition inside Raw264.7 macrophages as compared to untreated infected cells
- M. abscessus: Mycobacterium abscessus
- M. tb: Mycobacterium tuberculosis
- NTM: nontuberculous mycobacterium
- OX: Oxadiazolone-core derivatives
- PDIM: phthiocerol dimycolocerosate
- PGL: phenolic glycolipids
- RFU: relative fluorescence unit
- SI: Stereoselectivity Index
- TAG: triacylglycerols
- TB: tuberculosis
- TDM: trehalose dimycolate
- VLDL: Very Low-Density Lipoproteins
- x_{i}: inhibitor molar excess related to 1 mol of enzyme
- x_{150}: inhibitor molar excess leading to 50% lipase inhibition
1. Introduction

A hallmark of *Mycobacterium tuberculosis* (*M. tb*), the etiologic agent of tuberculosis (TB), is its ability to metabolize host lipids. *M. tb* hydrolyzes triacylglycerols (TAG) contained in host lipid bodies (LB) [1-3], and uses the released fatty acyl chains to resynthesize TAG which are stored in its own cytoplasm in the form of intracytoplasmic lipid inclusions (ILI) to serve as carbon source and energy. At this stage, lipid-loaded mycobacteria stop their replication and enter in a persistent and non-dividing state [1, 4, 5]. During the reactivation phase, these ILIs are hydrolyzed by *M. tb* and used to fuel the regrowth leading to its exit from the non-replicating state [4, 6].

These findings imply that assimilation of fatty acids from LB degradation, as well as TAG biosynthesis and hydrolysis are key aspects of mycobacterial metabolism. ILI formation has been described in many mycobacterial species like *M. tb* [1, 2], *M. bovis* BCG [6, 7], *M. leprae* [8], *M. abscessus* [9], and *M. smegmatis* [4]. However, their origin at the time of infection is poorly understood. It could result from the presence of intracellular and secreted/membrane-anchored mycobacterial lipolytic enzymes capable of degrading LBs, from the absorption of fatty acids available at the caseum center and originating from host cells degradation, or from *de novo* synthesis [1, 10-12].

Given the importance of lipid metabolism, the complete analysis of the *M. tb* genome revealed that this bacterium possesses 250 enzymes involved in lipid metabolism representing 6% of the full genome [13]. This characteristic strongly suggests that lipids and mycobacterial lipolytic enzymes play an essential role in the life cycle and the virulence of the tubercle bacilli.

Over the past decade, it has been well established by our group and others, that such enzymes, possessing a catalytic serine or cysteine residue in their catalytic site (*i.e.* (Ser/Cys)-based enzymes), are involved in the host-pathogen cross-talk [14] and play essential roles in the physiopathology of the disease [15]. These lipolytic enzymes are indeed involved in
bacterial growth [4, 16, 17], virulence (reactivation and propagation) [2, 7, 18, 19], dormancy [1, 6], cell wall biosynthesis [15, 16], and in lipid storage and degradation [1, 4, 5, 10].

More specifically, the physiological processes related to lipid accumulation/consumption are crucial to the *M. tb* infectious life-cycle for the propagation of the infection, the establishment of the dormancy state and the reactivation of the disease [2, 4]. Moreover, we have recently demonstrated that the presence of ILIs substantially enhanced bacterial burden and granulomas size in zebrafish embryos infected with *M. abscessus* lipid-rich vs. lipid-poor strains, suggesting that ILIs contribute actively to mycobacterial virulence and pathogenesis [11].

Therefore, finding ways to inhibit or control the activity of such mycobacterial lipolytic enzymes may open the way to new chemotherapeutic developments against pathogenic mycobacterial-related infections, especially against *M. tb* and *M. abscessus*, the two most drug-resistant and clinically relevant mycobacterial species.

2. Mycobacterial Lipolytic Enzyme Inhibitors are Promising Anti-tuberculous Candidates

2.1. Orlistat, β-lactones and related compounds. Among the potent lipolytic enzyme inhibitors, β-lactones bearing the strained 2-oxetanone 4-membered ring represent an important class of compounds that display potent inhibitory activity against (Ser/Cys)-based enzymes. The most representative member of this family of inhibitors, is the FDA-approved drug Orlistat (also known as Tetrahydrolipstatin, THL, Scheme 1). Orlistat is an active site-directed inhibitor that forms a stoichiometric but reversible long-lived acyl-enzyme complex with lipolytic enzymes as a result of nucleophilic attack of the catalytic serine (or cysteine) residue on the β-lactone ring [20-22]. Since 1997, Orlistat was known to inhibit microbial lipases [23]. Functioning as a versatile (Ser/Cys)-hydrolase inhibitor, Orlistat was indeed found to inhibit enzymes belonging to the Cutinase-like family proteins, including the essential *M. tb*
phospholipase/thioesterase Cut6 (Rv3802c) [24-26]; enzymes belonging to the hormone-sensitive lipase (HSL) family member proteins (i.e., Lip-HSL) [27, 28]; as well as the mycolyltransferase Antigen 85C [29, 30]. When tested as a possible anti-mycobacterial agent, Orlistat impaired *M. tb* growth with a minimum inhibitory concentration (MIC) of around 15-30 µM [26, 28, 31, 32], and displayed a strong synergistic effect with vancomycin resulting in a MIC drop of around 16-fold [31]. Lipids analysis confirmed that Orlistat destabilized the outer membrane of the cell envelope by reducing the amount of phthiocerol dimycocerosate (PDIM) content in the mycobacterial cell wall, therefore facilitating the action of vancomycin [31].

Similar to Orlistat, the human lysosomal acid lipase inhibitor Lalistat was found to not only inhibit *M. tb* growth with moderated MIC values of 25-50 µM, but to also act in synergy with vancomycin. Activity-based protein profiling (ABPP) approach using an alkyne-modified Lalistat probe allowed identification of a variety of hydrolases as molecular targets, including 8 Lip-HSL enzymes [33].

Various structural modifications based on the Orlistat pharmacophore have been further investigated in order to improve the specificity and antibacterial potency of the new synthesized analogs (Scheme 1) [26, 32, 34]. Of interest, compound Cpd-12, bearing an L-thiazolidyl ester side chain, and analogs Cpd-17 to Cpd-20 bearing L- and D-prolyl ester side chains displayed a 10-fold lower MIC against *M. tb* growth and also improved inhibitory concentrations, i.e., IC$_{50}$ values of 0.2-0.8 µM toward Cut6, compared with Orlistat (IC$_{50}$ = 3.8 µM) [26]. More recently, the β-lactone EZ120 was identified as hit compound inhibiting *M. tb* growth with bactericidal activity of 1.6 µM, and low cytotoxicity against mouse macrophages [34]. Chemical proteomics with the alkyne-modified EZ120P activity-based probe (ABP) identified the antigen 85 enzymes [35] and the thioesterase domain of Pks13 [36], which are essential enzymes involved in mycolic acid biosynthesis, as major targets of EZ120 [34].
Scheme 1. Chemical structures of Orlistat & Orlistat probe [27], and related β-lactones Cpd-12, Cpd-17-20 [26], EZ120 & EZ120P probe [34]; as well as the human lysosomal acid lipase inhibitor Lalistat and its corresponding alkyne-modified probe [33].

All these above-mentioned results, strongly support the therapeutic potential of lipolytic enzyme inhibitors targeting (Ser/Cys)-based hydrolases involved in the global mycobacterial lipid metabolism. Given such findings, this review will now focus on and discuss the development of two new families of promising anti-mycobacterial molecules exhibiting potent anti-lipolytic enzyme activity: Oxadiazolone (OX) derivatives (Figure 1) and the Cyclophostin & Cyclipostins (CyC) analogs (Figure 3).
2.2. Oxadiazolone-core derivatives (OX). 3,5-substituted 1,3,4-oxadiazol-2(3H)-one derivatives were first described in 1954 as active anti-TB molecules [37, 38]. Second generation compounds were further found to exhibit interesting anti-mycobacterial activity with MIC ranging from 8 to 50 µM [39, 40]. Few years later, we reported that a new substitutive OX derivative, the MmPPOX (Figure 1), was also able to inhibit M. tb growth on solid medium with moderated MIC values of 50-90 µM [28].

Figure 1. Chemical structure of Oxadiazolone-core derivatives (OX) as well as their mechanism of action on (Ser/Cys)-based enzymes in inset.

We further showed that MmPPOX efficiently inhibited pure recombinant M. tb enzymes belonging to the hormone-sensitive lipase (HSL) family member proteins (i.e., Lip-HSL) [28], including LipY (Rv3097c) the major M. tb Lip-HSL lipase involved in TAG acquisition from the host and in ILI breakdown [2, 10, 17, 41]. The mechanism of action of MmPPOX involving the formation of a covalent bond with the catalytic serine residue of the enzymes (Figure 1
inset) thus resulting in a total abolition of their activities was also confirmed [28]. Overall, the fact that MmPPOX alters mycobacterial growth and abolishes the activity of Lip-HSL proteins suggest that HSL family member proteins fulfill essential metabolic and/or physiologic functions in M. tb life cycle.

Interestingly, MmPPOX has also proven its ability to prevent ILI catabolism using the experimental ex vivo model of foamy macrophages developed in the lab [5, 10, 42]. Following M. bovis BCG infection, murine bone-marrow-derived macrophages (mBMDM) were fed with Very Low-Density Lipoproteins (VLDL), allowing the differentiation of these infected BMDM into foamy macrophages (FM) [5, 10, 42]. In this specific environment, the bacteria are able to i) accumulate lipids from host LBs to form ILIs; ii) enter in persistence phase depicted by a typical absence of septation; and iii) hydrolyze stored ILIs after VLDL removal from the culture medium thus mimicking reactivation and propagation of the disease. When infected cells were exposed to VLDL in the presence of 50 µM MmPPOX, macrophages still retained the ability to become foamy, but M. bovis BCG was unable to form ILIs (Figure 2A). Conversely, when VLDL-treated M. bovis BCG-infected cells were further incubated for 24h in fresh medium, nearly 90% of the ILI+ profiles were retained in the presence vs. absence of MmPPOX (Figure 2B) [10]. These findings support MmPPOX as effective inhibitor of mycobacterial lipolytic enzymes, including LipY (Rv3097c) [28], involved in LB and ILI degradation. Such results were further confirmed using an in vitro model of mycobacteria growing under carbon excess and nitrogen-deprived conditions allowing ILI biosynthesis and hydrolysis. Incubation of the resulting lipid-rich mycobacteria (i.e., M. smegmatis and M. abscessus) with MmPPOX also resulted in nearly complete blockage of TAG lipolysis [11].
Figure 2. (A) Foamy cell after 24h-exposure to VLDL showing large amounts of LB and *M. bovis* BCG-containing ILI. (B-D) Effect of *MmPPOX* during exposure to VLDL on host LB formation and ILI accumulation. At day 6 post-infection with *M. bovis* BCG, mBMDM were exposed for 24h to VLDL in the absence or presence of *MmPPOX*. When exposed to VLDL only (C) many mycobacterial profiles are ILI\(^+3\); whereas exposure to VLDL+*MmPPOX* (D) results in small ILI with no ILI\(^+3\) profiles. (E-G) Effect of *MmPPOX* during TAG consumption within ILIs. At day 6 post-infection with *M. bovis* BCG, mBMDM were exposed to VLDL and re-incubated in VLDL-free culture medium alone or with *MmPPOX* for 24h. After VLDL treatment followed by a 24-h chase in medium devoid of inhibitor, (F) cells contain few ILI\(^+3\) profiles. Conversely, in the presence of *MmPPOX* (G) cells still contain ILI\(^+3\) profiles. (B, E) Both bar graphs represent the quantitative evaluation of the percentage of each category of ILI profiles ±SD (\(*\), \(p<0.05\); **,\(\#\#\), \(p<0.01\); ***, \(p<0.001\)). Bars in panels A, 1 µm; C-D-E-F, 0.5 µm. Adapted from [10, 42].

Based on these data, a new series of 18 lipophilic OX derivatives were designed and synthesized (Figure 1) [43]. A specific nomenclature was set up for these OXs noted *Rm* (or
\( p \)PPOX; where \( m \) (or \( p \))PP (\( = R^1 \)) represents the meta (or para)-PhenoxyPhenyl group, when present; \( \text{OX} \) the oxadiazol-2(3\( H \))-one core; and \( R \) the alkyl chain (Figure 1) [43]. Data reported in Table 1 point out to the potent inhibitory activity of this new series of \( \text{OXs} \) towards the TAG-lipase LipY. Their inhibitory power, defined here as the inhibitor molar excess leading to 50\% lipase inhibition (\( x_{50} \)), ranges from 0.59 to 0.93 (Table 1) implying that the best \( \text{OXs} \) react in close stoichiometry with this lipase, being thus more potent than \( \text{MmPPOX} \) (\( x_{50} = 2.4 \)), but also than Orlistat (\( x_{50} = 7.1 \)) used as reference inhibitor [28, 44].

### 2.3. The Cyclipostins & Cyclophostin analogs (CyC)

In parallel, we investigated the inhibitory properties of new monocyclic analogs of Cyclophostin and Cyclipostins (CyC) (Figure 3), derived from phosphorus-containing natural products isolated from fermentation broths of \( \text{Streptomyces sp} \) [45, 46], on pure mycobacterial lipases and various mycobacterial strains.

Members of the Cyclipostins family (Figure 3 – \( X=O, R^3 = \text{C}_{15}-\text{C}_{18} \)) possess a core structure similar to that of Cyclophostin (Figure 3 – \( X=O, R^3 = \text{CH}_3 \)) but are phosphate esters of long chain lipophilic alcohols. The Cyclipostins were described to inhibit the growth of various mycobacteria (including \( \text{M. smegmatis}, \text{M. phlei}, \text{Nocardia abscessus}, \) and \( \text{Corynebacterium diphtheriae} \)) with MIC values similar to that of rifampicin and penicillin G [47]. These natural products were initially described as potent inhibitors of either human acetylcholinesterase (AChE) (\( i.e., \) Cyclophostin) [45, 48] or human and rat HSL (\( i.e., \) Cyclipostins) [46, 49].
Figure 3. Chemical structure of Cyclipostins & Cyclophostin analogs (CyC), as well as their mechanism of action on (Ser/Cys)-based enzymes in inset. For a review on the CyC synthesis, please refer to [50].

From these findings, we first reported the total synthesis of natural Cyclophostin CyC1, its phosphonate analogues CyC2α,β [48, 49], and related monocyclic enol-phosphonates CyC3-10,19-22 [51-53] and phosphates CyC23-30 [53]; as well as Cyclipostin P CyC18β, its trans-(α) diastereoisomer CyC18α [49], and the corresponding monocyclic enol-phosphonates CyC11-13 [52, 54], difluorophosphonates CyC14-15 and phosphates CyC16-17 [55, 56] (Figure 3).

Of particular importance, diastereomeric cis- and trans-monocyclic enolphosphonates CyC6-10...
were screened against six representative Ser-based enzymes belonging to distinct lipolytic enzyme families [52]. None of these enolphosphonates inhibited the mammalian AChE [55], HSL [56], or gastric and pancreatic lipases [52]. The microbial enzymes; *i.e.*, *Fusarium solani* Cutinase [57] and lipolytic enzymes from *M. tb* (*i.e.*, Rv0183 [16, 58] and LipY); were, however, all fully inactivated by formation of a covalent and irreversible bond between the enol-phosphorous atom and the catalytic serine residue [51, 52]. Moreover, modulation of the lipophilicity by varying the nature of the alkyl group, either at the C-5 carbon atom (*i.e.*, R² group – Figure 3) or at the phosphorous center (*i.e.*, R³ group – Figure 3), strongly impacted the inhibitory efficiency of these CyCs [52]. This property has been exploited to significantly attenuate or increase the affinity of one inhibitor towards a specific enzyme [52, 59].

In order to shed more light on the influence of the chirality on enzyme inhibition, CyC₇ bearing a C10-side alkyl chain was chosen for its significant inhibitory potency towards the Cutinase, Rv0183 and LipY, but also for the high diastereoselectivity (51.9%-78.3%) exerted by these enzymes in favor of the *cis*(β)-isomer [52]. The four stereoisomers of CyC₇ were prepared by asymmetric synthesis, and the absolute configuration at both the phosphorus and C-5 carbon stereocenters were assigned unambiguously [59]. Pure compounds at phosphorus were obtained with a diastereoisomeric excess of around 95%, together with enantiomeric excess of >85% related to the cyclized C-5 carbon center [59].

Cutinase displayed a high diastereoselectivity for the (Sₚ) configuration with a Stereoselectivity Index (SI) derived from *x*₅₀ values of 94.9% (*Table 2*) when using CyC₇-(Sₑ) inhibitors, whereas no obvious stereopreference at the phosphorus center was observed with the CyC₇-(Rₑ) inhibitors. On the contrary, Rv0183 strongly discriminated the (Sₚ) configuration (SI = 72-81.4%) independently of the absolute (Rₑ) or (Sₑ) configuration on the asymmetric C-5 carbon atom; and thus, exhibited the classical enantiopreference of lipolytic enzymes [60]. The influence of chirality was much more pronounced in the case of LipY. Significantly, this lipase
discriminated only the unusual diastereoisomeric configuration \((R_c,R_p)\), which led to the most potent CyC\(_7\beta\)-(\(R_c,R_p\)) inhibitor (SI > 80.7% - Table 2). Modulation of the lipophilicity at the C-5 carbon atom combined with this unusual high enantiopreference displayed by LipY for the \((R_p)\) and \((R_c)\) associated absolute configurations, should open new prospects in the design of specific inhibitors of this mycobacterial lipase [59]. Overall, these results raised significant achievements in the understanding of the stereoselective relationships between pure non-racemic compounds and their inhibitory activity towards several microbial lipases of interest.

To summarize, these CyC derivatives have not only proved to be powerful bacterial (Ser/Cys)-based enzymes inhibitors [52, 59], but above all, they had lost their inhibitory activity towards the mammalian enzymes initially targeted by the natural parent molecules [52, 55, 56].

### 2.4. The OX and CyC derivatives are novel promising multi-target inhibitors of *M. tb* growth.

The set of 19 OX derivatives and 26 CyC analogues were further evaluated for their anti-mycobacterial activity on a *M. tb* H37Rv-GFP strain using a high-content screening assay based on the fluorescence measurement of GFP-expressing bacteria [54, 61]. *In vitro* growth of *M. tb* H37Rv-GFP was monitored by directly measuring GFP fluorescence after 5 days at 37 °C in presence of increasing concentrations of candidate inhibitors. Intracellular growth of *M. tb* H37Rv-GFP was also assessed following a 5-day exposure of infected Raw264.7 murine macrophage cell lines to the different compounds. In the latter case, the percentage of infected cells and the number of living host cells allowed determining the values of both MIC\(_{50}\) (compound concentration leading to 50% growth inhibition) and CC\(_{50}\) (compound concentration leading to 50% host cell toxicity) [62, 63].

Among all molecules tested, 14 potential candidates (6 OXs and 8 CyCs – Table 3) exhibited very promising anti-tubercular activities with moderate (15-50 µM) to good (3-4 µM)
and to excellent (500 nM for CyC17) MIC_{50} values. Of great importance, beside their inhibitory activity against bacterial growth, both set of compounds exhibited very low toxicity towards host macrophages (CC_{50} > 100 µM). Data show that both OXs and CyCs can be divided into two different classes based on their antibacterial activity (Figure 4 and Table 3).

Figure 4. (A-C) Activity of HPOX, CyC7β & CyC17 against GFP-labelled M. tb H37Rv replicating in culture medium expressed as normalized relative fluorescence units (RFU%). (B-D) Activity of iBPOX and CyC7β against M. tb H37Rv-GFP replicating inside Raw264.7 macrophages. Adapted from [54, 61].

First, 9 out of 14 compounds exhibited higher activity against intracellular bacteria than against extracellular ones: i.e., iBpPPOX, iBPOX, HpPPOX, BepPPOX, CyC6β, CyC7α,β and CyC8α,β. Such feature supposes that the intracellular mode of action of these inhibitors may differ from that of those acting on extracellularly-replicating bacilli. It can therefore be hypothesized that the vulnerability of the corresponding bacterial target(s) of these 4 OXs and 5 CyCs is higher and/or more critical during the intracellular life of M. tb than for
extracellularly-growing bacteria. Alternatively, a specific stringent response of the macrophage caused by the compounds and leading to bacterial death can, however, not be excluded. The second type of compounds are active against extracellular bacteria and display only poor or even no activity against intracellular *M. tb*: HPOX, BePOX, CyC17, CyC18α,β; a property already reported for 1,2,4-Oxadiazole EthR inhibitors [63]. The observed differences in the behavior of studied compounds, particularly the high ratio between the intracellular vs. extracellular antibacterial effects, may translate in the prevention of entry of actively replicating bacilli into the persistence phase and/or prevent reactivation of dormant bacilli within FM.

**Figure 5.** General scheme for the identification of the target enzymes of our inhibitors using ABPP approach.

Based on these aforementioned results, and given the strong affinity of our OXs and CyCs for *M. tb* lipolytic enzymes, these inhibitors might target and impair the activity of various
(Ser/Cys)-based enzymes involved in several processes of *M. tb* life cycle, thus resulting in bacterial death without any (or only very low) cytotoxicity towards host cells. Accordingly, target(s) identification experiments were next conducted by applying ABPP approach [27, 33, 34, 64-67] (Figure 5). Here, HPOX and CyC17, which selectively inhibit *M. tb* growth in culture broth medium only, were selected for such experiments [54, 61]. *M. tb* total cell lysate was incubated with each inhibitor and then subjected to competitive probe labelling/enrichment assays using an activity-based probe (ABP), *i.e.*, the ActivX Desthiobiotin-FP widely exploited to screen for reversible and irreversible inhibitors of drug targets [54, 61, 64, 68]. This resulted in the identification of a panel of 18 and 23 distinct proteins for the HPOX- and CyC17-pretreated lysate, respectively [54, 61]. Remarkably, these 41 identified proteins were all (Ser/Cys)-based enzymes, most of them participating in *M. tb* lipid metabolism and in cell wall biosynthesis. Among them, several are annotated as essential enzymes for the *in vitro* growth of *M. tb* and/or its survival of inside macrophages [69-71]. These included the antigen 85 complex, Ag85A (Rv3804c), Ag85B (Rv1886c) and Ag85C (Rv0129c) [35]; the thioesterase TesA (Rv2928) [72]; the carboxylesterase CaeA (Rv2224c) [73]; the β-ketoacyl synthase KasA (Rv2245) [74]; a bifunctional thioesterase-phospholipase enzyme LipG (Rv0646c) [75], and the hydrolase HsaD (Rv3569c) [76, 77].

Through biochemical and structural studies, the antigen 85 complex (*i.e.*, Ag85A/B/C) was validated as an effective target of CyC17, but also of CyCβ and CyC8β [78]. These three CyCs are indeed able to block the synthesis of trehalose dimycolate (TDM) as well as the mycolylation of arabinogalactan in *M. tb* which results in the disruption of the cell envelope integrity [78]. Among the targets of both the CyCs and OXs, TesA, a putative thioesterase involved in the synthesis of phthiocerol dimycocerosates (PDIM) and phenolic glycolipids (PGL), has been identified. These two lipids (PDIM and PGL), non-covalently linked to the outer cell wall of pathogenic mycobacteria such as *M. tb*, are important virulence factors. In
this context, TesA was validated as an effective pharmacological target of CyC$_{17}$ ($x_{150} = 12.4$) [79] and HPOX ($x_{150} = 0.59$) [61].

Figure 6. Crystal structures of (A) Ag85C in complex with CyC$_{8β}$ (PDB ID: 5OCJ; 1.8Å resolution) [78] and (B) TesA in complex with CyC$_{17}$ (PDB ID: 6FVJ; 2.6Å resolution) [79]. The mechanism of action of the phosphonate (CyC$_{8β}$) and the phosphate (CyC$_{17}$) analog based on mass spectrometry analyses are illustrated in inset. Each inhibitor is in stick representation, and the catalytic Serine residue is colored in magenta. Structures were redrawn as ribbon representations from their respective modified PDB IDs, with PyMOL Molecular Graphics System (Schrödinger, LLC).
From a molecular point of view, the obtained crystal structures of Ag85C inhibited by CyC8β (Figure 6A) [78] and TesA in complex with CyC17 (Figure 6B) [79], together with biochemical and mass spectrometry experiments, have clearly stated that the inhibition of these enzymes results from the phosphorylation of their respective catalytic Serine residue (Figure 6). Interestingly, following CyC17 phosphorylation of the TesA-Ser104 or Ag85C-Ser124, further rearrangement of the inner structure of the covalently bound inhibitor occurs resulting in the loss of the β-ketoester moiety (Figure 6B). Such chemical modification can therefore be considered as a signature of the CyC17 reactivity with Ser- and Cys-based enzymes [78, 79].

Finally, the bifunctional thioesterase-phospholipase enzyme LipG (Rv0646c), involved in the modification and remodeling of the mycobacterial envelope and described as essential for the survival and intracellular persistence of M. tb [75], has been biochemically characterized [80]. Inhibition kinetics indeed demonstrated that LipG was able to react with CyC7α ($x_{I50} = 5.0$) and in near stoichiometry with CyC17 ($x_{I50} = 0.98$), but not with CyC7β and CyC8α,β therefore exhibiting a certain selectivity of action.

3. The CyC And Ox Analogs are New Compounds for the Treatment of Mycobacterial-Related Infections

In view of the results obtained on M. tb, the specificity of our CyC compounds against various bacteria was further investigated. Susceptibility testing conducted on 7 bacterial strains, P. aeruginosa, E coli, B. subtilis, M. abscessus, M. marinum, and M. bovis BCG against the 26 available CyC1-18 surprisingly showed that these compounds block specifically the growth of the mycobacterial species without affecting the growth of Gram-positive and Gram-negative bacteria [81]. More particularly, CyC17 exhibited MIC$_{50}$ values of 12.7 µM and 0.37 µM towards the smooth and rough morphotype of M. abscessus, respectively; the latter value being
20- to 37-fold lower than that of reference antibiotics, amikacin (7.4 µM), imipenem (12 µM) or cefoxitin (27 µM) [81].

*M. abscessus*, a rapidly growing mycobacterium, is an opportunistic pathogen responsible for chronic lung infection in patients with respiratory diseases such as cystic fibrosis (CF) [82–84]. *M. abscessus* exists in two variants, a smooth (S) and a rough (R) one, the latter being considered the most virulent form in humans [85, 86]. Nicknamed the "antibiotics nightmare", this nontuberculous mycobacterium (NTM) is one of the most drug-resistant mycobacteria for which most standardized chemotherapeutic regimens are poorly effective, often leading to high treatment failures and death [86-88].

When tested on 37 bacterial strains isolated from CF patients, including 26 clinical isolates belonging to the *M. chelonae-abscessus* complex, the best candidate (CyC17) showed high selectivity for mycobacteria only, and MICs (<2-40 µg/mL) comparable with those of clinically used antibiotics [81]. To refine the structure-activity relationships of this family of compounds, 12 new CyC analogs (CyC19-30 – Figure 3) were synthesized by varying the R⁴ chain on the enolphosphorus ring [53]. Such structural modification was also guided by the isolation of the antimalarial Salinipostin A, a natural product produced by a *Salinispora sp.* bacterium, which is a Cyclipostin-like compound with variations in the alkyl enol substituent [89, 90]. Among these 12 new CyCs, CyC25 and CyC26 exhibited very promising MIC₅₀ values against *M. abscessus* R (Table 4) [53]. Remarkably, these latter three best inhibitors of *M. abscessus* growth were all phosphate esters bearing a long lipophilic C10/C16 alkyl chain either at the R³ or R⁴ position (Figure 3). We also demonstrated that the absence of surface exposed glycopeptidolipids (GPL) in the R variant was responsible for the higher susceptibility (up to 34×) to the CyCs as compared to the S strain. More recently, by combining fast quantitative imaging (QI) atomic force microscopy (AFM) with hydrophobic tips, Viljoen *et al.* demonstrated that GPL modulated the nanoscale surface hydrophobicity of *M. abscessus* [91].
While S morphotype displayed unusual variations of nanoscale hydrophobic properties, the R variant showed homogeneous surface hydrophobicity conferred by surface exposed mycolic acids when GPL were lacking [91]. Given together, the antibacterial activity of the CyCs against the R and S variants, respectively, can thus be directly correlated with the GPL-dependent variation in the nanoscale distribution of M. abscessus hydrophobicity.

This was, however, not the case when using the OX derivatives, for which M. abscessus R was nearly 1.6-times less sensitive to these compounds than the S morphotype, iBpPPOX being the best growth inhibitor of both strains (Table 4) [92]. Interestingly, M. abscessus S susceptibility to this latter inhibitor was similar to that of M. tb (Table 3) [61]. Such increased tolerance of virulent M. abscessus R variant towards the OX compounds is in line with its high resistance to classical antibiotics [86] compared to M. tb.

M. abscessus, like M. tb, is also able to survive and replicate inside macrophages [93, 94]. Altogether, high intrinsic resistance and intracellular forms of M. abscessus are thus mostly contributing to treatment failures since only few antimicrobials can penetrate the cell membrane of phagocytes [95]. In this context, the 38 CyCs and 19 OXs were tested for their capability to inhibit the intramacrophagic growth of M. abscessus inside Raw264.7 macrophages. Only CyC7α,β and CyC8α,β were found active against intracellularly replicating M. abscessus (Table 4). With MIC50Raw values of 7.9 and 2.0 μM, respectively, CyC8α and CyC8β were 3.6- and 14-fold more potent against intracellular M. abscessus than imipenem (28.3 μM), used as reference. It is noteworthy that such MIC50Raw are of the same order of magnitude than those previously obtained with the same CyC8α,β against intracellular M. tb (4-12 μM – Table 3). Regarding the OXs, only MPOX and MpPPOX (inactive against extracellular bacterial growth) and iBpPPOX were able to block intracellularly-growing M. abscessus. As previously observed for M. tb [61], iBpPPOX impairs both extracellular (MIC50 = 33.0 μM) and intracellular (~59%
bacterial clearance at 30 μM) replicating bacteria with similar potency/efficiency to that of imipenem (Table 4).

By using similar strategy applied for M. tb (Figure 5), 39 and 21 potential target enzymes of CyC17 and iBpPPOX, respectively, have been identified from a M. abscessus R cell culture. Interestingly, 11 out of the 60 identified proteins possess orthologs annotated as essential enzymes in the M. tb genome. Moreover, consistent with our previous works [54, 61], most of them are (Ser/Cys)-based enzymes mainly involved in M. abscessus lipid metabolism or cell-wall synthesis. These include several Lip-HSL enzymes, some Cutinase-like family proteins, and the members of the antigen 85 (Ag85) complex which play a central role in the mycobacterial cell wall biosynthesis and in the survival of mycobacteria [96, 97].

The fact that Ag85A/B/C proteins have been identified and validated as effective targets of both the CyC and OX compounds in M. tb [61, 78] and M. abscessus [53, 92], but also of Orlistat and related analogs [25, 30, 34], suggest that these mycolyltransferases represent a common primary target of such inhibitors, regardless of the mycobacterial strain tested. Indeed, as Ag85A/B/C proteins are secreted enzymes, they may be easily accessible to the CyCs and OXs. Due to their importance in mycolic acid metabolism, the Ag85 enzymes have often been proposed as attractive targets for future chemotherapeutic developments against mycobacteria [35, 78]. Overall, our studies provide compelling evidence that both the CyC analogs and the OX compounds inhibit the activity of the Ag85 complex in vitro and in mycobacteria [78, 92], therefore opening the way to a new strategy to fight against pathogenic mycobacteria.

4. Conclusion and future perspectives

Collectively all our results strengthen the fact that the OX and the CyC inhibitors are not only non-toxic for mammalian cells, but above all represent a novel class of selective and efficient multi-target inhibitors [98] leading to the arrest of M. tb and M. abscessus, two major infectious
pathogens. By impairing simultaneously, the activity of various (Ser/Cys)-base enzymes participating in important physiological processes related to the whole bacterial lipid metabolism, including the Ag85 complex proteins, both the CyCs and the OXs will inhibit extracellular and/or intracellular *M. tb* and *M. abscessus* growth, therefore leading to bacterial death. Consequently, the probability that a strain would develop resistance to such inhibitors would be very low, because resistant mutants would require the simultaneous acquisition of several mutations in the same bacterial genome; thus, making it difficult or impossible for the bacteria to adapt themselves and survive.

These data also demonstrated that our inhibitors are able to penetrate both the bacteria and the host macrophage (without any cytotoxic effect). Such a dual activity of the CyCs and OXs is a key point as it may affect the different stages of the infection process: *i.e.*, entry of bacilli into the persistence phase, and/or interfere by blocking the lipid accumulation from foamy macrophages or the lipid consumption during the reactivation of dormant bacilli.

Given these promising properties, these two families of inhibitors will be chemically modified to allow, in living cells and *via* click chemistry, the direct capture of specific proteins that lead to *M. tb* or *M. abscessus* growth arrest during the mycobacterial phases of active replication, latency and/or reactivation of the bacilli in infected foamy as well as non-foamy macrophages [11, 42]. Accordingly, the *in vivo* identification of the proteins inactivated by our potent antibacterial activity-based probes will reveal new potential targets for treating mycobacterial-related diseases, and contribute to background information for the development of new therapeutic strategies for elimination of either actively replicating or latent bacilli from infected individuals. Deciphering the physiological role of mycobacterial lipolytic enzymes in lipid metabolism, notably focusing on the accumulation and consumption of lipids at key stages of the bacterial development will generate results that are directly relevant for deciphering the pathogenesis of actively replicating and latent bacilli.
Declaration of competing interest

The authors report no declarations of interest.

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Fast chemical force microscopy demonstrates that glycopeptidolipids define nanodomains of varying hydrophobicity on mycobacteria, Nanoscale Horiz, 5 (2020) 944-953.


Table 1. Inhibition of the TAG-lipase LipY from *M. tb* after a 30-min incubation period with each OX compound

<table>
<thead>
<tr>
<th>Compounds</th>
<th>%inhibition&lt;sup&gt;b&lt;/sup&gt;</th>
<th>x&lt;sub&gt;i&lt;/sub&gt; = 4</th>
<th>x&lt;sub&gt;i&lt;/sub&gt; = 20</th>
<th>I&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orlistat</td>
<td>26.8±1.5</td>
<td>87.6±4.8</td>
<td>7.1</td>
<td></td>
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<tr>
<td><strong>MmPPOX</strong></td>
<td>63.6±4.6</td>
<td>85.9±4.9</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td><strong>MpPPOX</strong></td>
<td>60.8±2.1</td>
<td>79.4±3.3</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td><strong>MPOX</strong></td>
<td>34.4±2.4</td>
<td>81.8±3.3</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td><strong>EmPPOX</strong></td>
<td>21.7±1.4</td>
<td>53.8±3.3</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td><strong>MemPPOX</strong></td>
<td>92.4±0.30</td>
<td>93.7±1.3</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td><strong>BmPPOX</strong></td>
<td>15.6±0.50</td>
<td>36.7±1.8</td>
<td>&gt;40</td>
<td></td>
</tr>
<tr>
<td><strong>iBmPPOX</strong></td>
<td>37.4±2.4</td>
<td>49.4±1.5</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td><strong>iBpPPOX</strong></td>
<td>33.8±1.5</td>
<td>41.6±0.40</td>
<td>&gt;40</td>
<td></td>
</tr>
<tr>
<td><strong>iBPOX</strong></td>
<td>31.6±1.0</td>
<td>47.9±2.1</td>
<td>9.2</td>
<td></td>
</tr>
<tr>
<td><strong>HmPPOX</strong></td>
<td>39.9±1.2</td>
<td>76.5±1.9</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td><strong>HpPPOX</strong></td>
<td>38.7±1.7</td>
<td>77.2±2.7</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td><strong>HPOX</strong></td>
<td>35.0±0.27</td>
<td>38.8±0.11</td>
<td>&gt;40</td>
<td></td>
</tr>
<tr>
<td><strong>BemPPOX</strong></td>
<td>88.0±0.70</td>
<td>90.2±0.91</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td><strong>BepPPOX</strong></td>
<td>97.1±3.9</td>
<td>98.4±2.2</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td><strong>BePPOX</strong></td>
<td>89.0±1.9</td>
<td>92.2±0.20</td>
<td>0.61</td>
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<tr>
<td><strong>OmpPPOX</strong></td>
<td>83.2±5.8</td>
<td>86.6±2.2</td>
<td>0.71</td>
<td></td>
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<tr>
<td><strong>EhmPPOX</strong></td>
<td>89.6±1.1</td>
<td>94.8±2.7</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td><strong>DmPPOX</strong></td>
<td>91.5±2.8</td>
<td>94.2±3.1</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td><strong>DomPPOX</strong></td>
<td>88.7±4.2</td>
<td>90.4±2.2</td>
<td>0.77</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Inhibition kinetics were performed using the microtiter plate assay with coated Pomegranate oil [44].

<sup>b</sup> Inhibition data (% of initial enzyme activity), at an inhibitor molar excess (x<sub>i</sub>) of 4 or 20 related to 1 mole of enzyme. Values are means of at least three independent assays. <sup>c</sup> I<sub>50</sub>: inhibitor molar excess leading to 50% lipase inhibition [28, 44].
### Table 2. Stereoselective inhibition of Cutinase, Rv0183, and LipY by enantiopure monocyclic enolphosphonates CyC7<sup>a</sup>

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>SI (%)</th>
<th>x&lt;sub&gt;150&lt;/sub&gt;</th>
<th>SI (%)</th>
<th>x&lt;sub&gt;150&lt;/sub&gt;</th>
<th>SI (%)</th>
<th>x&lt;sub&gt;150&lt;/sub&gt;</th>
<th>SI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cutinase</td>
<td>97.3</td>
<td>2.52</td>
<td>4.07</td>
<td>3.38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rv0183</td>
<td>11.3</td>
<td>1.16</td>
<td>1.24</td>
<td>7.61</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LipY</td>
<td>25.9</td>
<td>15.4</td>
<td>1.64</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> x<sub>150</sub>, inhibitor molar excess leading to 50% lipase inhibition. SI, Stereoselectivity Index; SI = 100 × [(x<sub>150</sub>)<sub>β</sub> isomer − (x<sub>150</sub>)<sub>α</sub> isomer]/[(x<sub>150</sub>)<sub>β</sub> isomer + (x<sub>150</sub>)<sub>α</sub> isomer]. Adapted from [59].
Table 3. Activities of the most active OX derivatives and CyC analogs against *M. tb*.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Extracellular growth(^a)</th>
<th>Intracellular macrophage growth (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC(_{50}) [µM]</td>
<td>MIC(_{50\text{Raw}}) [µM]</td>
</tr>
<tr>
<td>Isoniazid (INH)</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Rifampicin (RIF)</td>
<td>0.01</td>
<td>2.9</td>
</tr>
<tr>
<td><strong>OX derivatives</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iBpPPOX</td>
<td>32.0 ±0.89</td>
<td>8.5 ±0.30</td>
</tr>
<tr>
<td>iBPOX</td>
<td>&gt;50</td>
<td>17.1 ±1.10</td>
</tr>
<tr>
<td>HpPPOX</td>
<td>&gt;50</td>
<td>9.5 ±0.49</td>
</tr>
<tr>
<td>HPOX</td>
<td>44.6 ±2.50</td>
<td>No effect</td>
</tr>
<tr>
<td>BepPPOX</td>
<td>&gt;50</td>
<td>3.5 ±0.28</td>
</tr>
<tr>
<td>BePOX</td>
<td>30.8 ±2.40</td>
<td>No effect</td>
</tr>
<tr>
<td><strong>CyC analogs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CyC(_{65})</td>
<td>NE</td>
<td>12.6 ±0.90</td>
</tr>
<tr>
<td>CyC(_{7a})</td>
<td>92.6 ±6.5</td>
<td>4.5 ±0.39</td>
</tr>
<tr>
<td>CyC(_{7b})</td>
<td>16.6 ±0.78</td>
<td>3.1 ±0.11</td>
</tr>
<tr>
<td>CyC(_{8a})</td>
<td>40.4 ±2.5</td>
<td>4.0 ±0.30</td>
</tr>
<tr>
<td>CyC(_{8p})</td>
<td>&gt;100</td>
<td>11.7 ±0.26</td>
</tr>
<tr>
<td>CyC(_{17})</td>
<td>0.50 ±0.02</td>
<td>No effect</td>
</tr>
<tr>
<td>CyC(_{18a})</td>
<td>24.4 ±2.14</td>
<td>No effect</td>
</tr>
<tr>
<td>CyC(_{18p})</td>
<td>1.7 ±0.03</td>
<td>No effect</td>
</tr>
</tbody>
</table>

\(^a\) MIC\(_{50}\), compound concentration leading to 50% bacterial growth inhibition in culture medium. \(^b\) CC\(_{50}\), compound concentration leading to 50% Raw264.7 macrophages toxicity. MIC\(_{50\text{Raw}}\), compound concentration leading to 50% bacterial growth inhibition inside Raw264.7 as compared to untreated infected cells. Raw264.7 cells were infected with *M. tb* H37Rv-GFP at a multiplicity of infection of 2. Adapted from [54, 61].
Table 4. Activities of the most active OX derivatives and CyC analogs against *M. abscessus*.

<table>
<thead>
<tr>
<th>Compounds</th>
<th><em>M. abscessus</em> CIP 104536&lt;sup&gt;†&lt;/sup&gt;</th>
<th></th>
<th></th>
<th>MIC&lt;sub&gt;50Raw&lt;/sub&gt; [µM]&lt;sup&gt;‡&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S variant</td>
<td>R variant</td>
<td></td>
</tr>
<tr>
<td>Amikacin (AMK)</td>
<td>3.9 ±0.19</td>
<td>7.4 ±0.26</td>
<td>Not determined</td>
<td></td>
</tr>
<tr>
<td>Imipenem (IMP)</td>
<td>11.3 ±0.50</td>
<td>29.9 ±1.6</td>
<td>28.3 ±4.7</td>
<td></td>
</tr>
<tr>
<td><strong>OX derivatives</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iBpPPOX</td>
<td>33.0 ±2.0</td>
<td>53.2 ±1.8</td>
<td>&lt; 30µM</td>
<td></td>
</tr>
<tr>
<td>MpPPOX</td>
<td>88.2 ±7.3</td>
<td>&gt;200</td>
<td>75.2 ±5.1</td>
<td></td>
</tr>
<tr>
<td>MPOX</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>36.5 ±2.6</td>
<td></td>
</tr>
<tr>
<td><strong>CyC analogs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CyC&lt;sub&gt;7α&lt;/sub&gt;</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>29.3 ±2.9</td>
<td></td>
</tr>
<tr>
<td>CyC&lt;sub&gt;7β&lt;/sub&gt;</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>65.3 ±6.2</td>
<td></td>
</tr>
<tr>
<td>CyC&lt;sub&gt;8α&lt;/sub&gt;</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>7.9 ±0.23</td>
<td></td>
</tr>
<tr>
<td>CyC&lt;sub&gt;8β&lt;/sub&gt;</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>2.0 ±0.91</td>
<td></td>
</tr>
<tr>
<td>CyC&lt;sub&gt;17&lt;/sub&gt;</td>
<td>12.7 ±0.26</td>
<td>0.37 ±0.01</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>CyC&lt;sub&gt;25&lt;/sub&gt;</td>
<td>85.0 ±0.86</td>
<td>13.9 ±0.56</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>CyC&lt;sub&gt;26&lt;/sub&gt;</td>
<td>60.0 ±2.50</td>
<td>6.9 ±0.16</td>
<td>No effect</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> MIC<sub>50</sub>, compound concentration leading to 50% bacterial growth inhibition in culture medium.  
<sup>b</sup> MIC<sub>50Raw</sub>, compound concentration leading to a 50% decrease in CFU count as compared to untreated cells. Raw264.7 cells were infected with *M. abscessus* S at a multiplicity of infection of 10. Adapted from [53, 92].