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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 dimycocerosate

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_{I50}: 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].

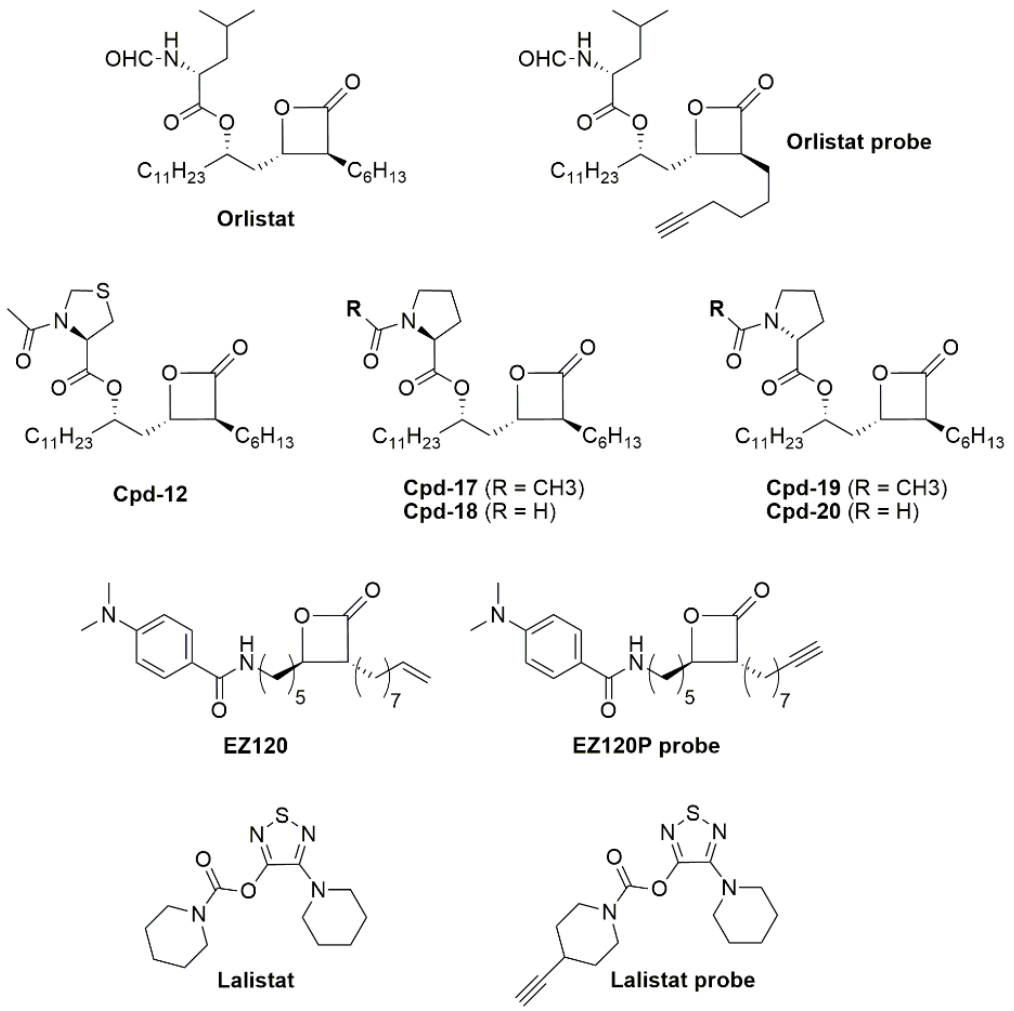
Therefore, finding ways to inhibit or control the activity of such mycobacterial lipolytic enzymes may open the way to new chemotherapeutic developments against pathogenic mycobacterial-related infections, especially against *M. tb* and *M. abscessus*, the two most drug-resistant and clinically relevant 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-17-2**0 [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(3*H*)-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 **M*m*PPOX** (**Figure 1**), was also able to inhibit *M. tb* growth on solid medium with moderated MIC values of 50-90 µM [28].

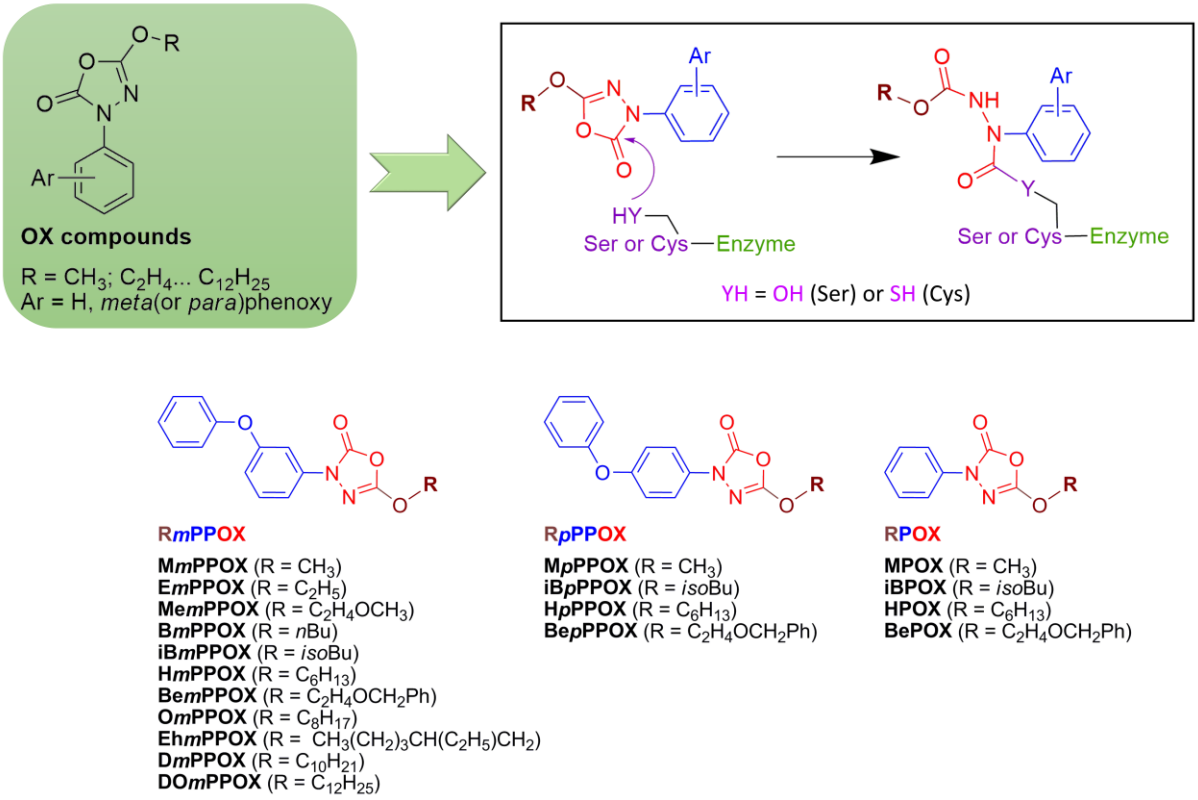


Figure 1. Chemical structure of Oxadiazolone-core derivatives (**OX**) as well as their mechanism of action on (Ser/Cys)-based enzymes in inset.

We further showed that **M*m*PPOX** 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 **M*m*PPOX** 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 **M*m*PPOX** 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, **M*m*PPOX** 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 **M*m*PPOX**, 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^{+3} profiles were retained in the presence *vs*. absence of **M*m*PPOX** (**Figure 2B**) [10]. These findings support **M*m*PPOX** 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 **M*m*PPOX** also resulted in nearly complete blockage of TAG lipolysis [11].

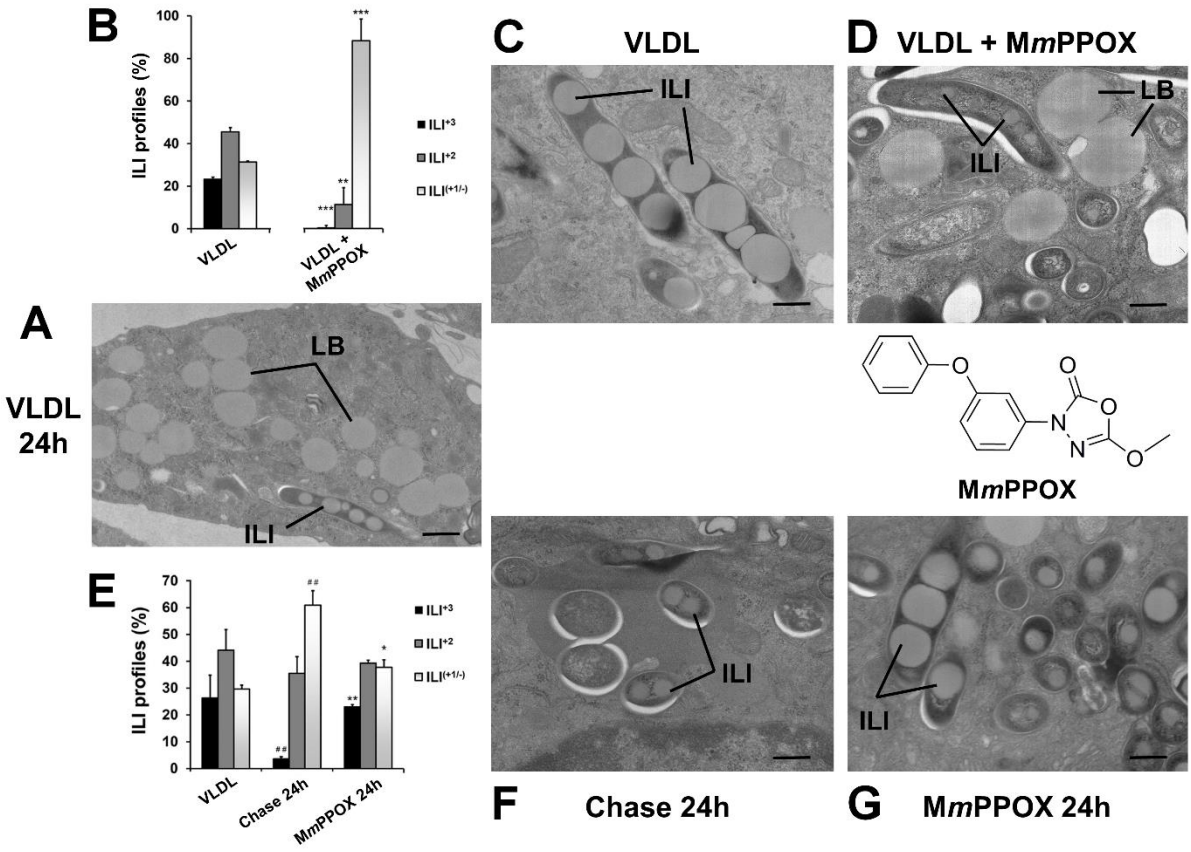


Figure 2. (A) Foamy cell after 24h-exposure to VLDL showing large amounts of LB and *M. bovis* BCG-containing ILI. **(B-D)** Effect of **M*m*PPOX** during exposure to VLDL on host LB formation and ILI accumulation. At day 6 post-infection with *M. bovis* BCG, mBMDM were exposed for 24h to VLDL in the absence or presence of **M*m*PPOX**. When exposed to VLDL only **(C)** many mycobacterial profiles are ILI^{+3}; whereas exposure to VLDL+**M*m*PPOX (D)** results in small ILI with no ILI^{+3} profiles. **(E-G)** Effect of **M*m*PPOX** 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 **M*m*PPOX** 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 **M*m*PPOX (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 **R*m***(or

p*)PPOX**; where ***m(or ***p*)PP** (= R^1) represents the *meta* (or *para*)-PhenoxyPhenyl group, when present; **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 **OXs** towards the TAG-lipase LipY. Their inhibitory power, defined here as the inhibitor molar excess leading to 50% lipase inhibition (x_{I50}), ranges from 0.59 to 0.93 (**Table 1**) implying that the best **OXs** react in close stoichiometry with this lipase, being thus more potent than **M*m*PPOX** (x_{I50} = 2.4), but also than Orlistat (x_{I50} = 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 *Streptomyces sp* [45, 46], on pure mycobacterial lipases and various mycobacterial strains.

Members of the Cyclipostins family (**Figure 3** – X=O, R^3=C_{15}-C_{18}) possess a core structure similar to that of Cyclophostin (**Figure 3** – X=O, R^3=CH_3) but are phosphate esters of long chain lipophilic alcohols. The Cyclipostins were described to inhibit the growth of various mycobacteria (including *M. smegmatis*, *M. phlei*, *Nocardia abscessus*, and *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].

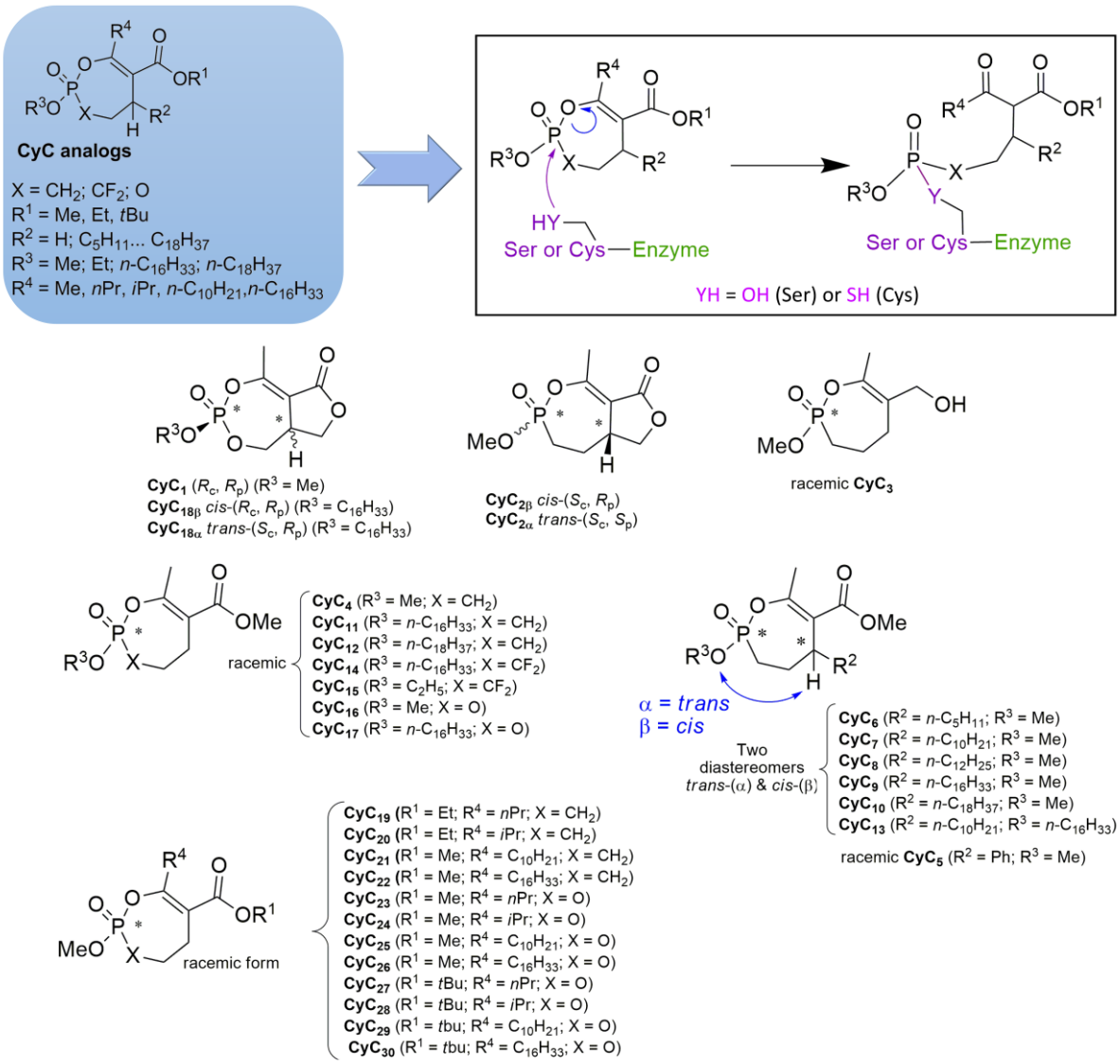


Figure 3. Chemical structure of Cyclipostins & Cyclophostin analogs (**CyC**), as well as their mechanism of action on (Ser/Cys)-based enzymes in inset. For a review on the **CyC** synthesis, please refer to [50].

From these findings, we first reported the total synthesis of natural Cyclophostin **CyC$_1$**, its phosphonate analogues **CyC$_{2\alpha,\beta}$** [48, 49], and related monocyclic enol-phosphonates **CyC$_{3\text{-}10,19\text{-}22}$** [51-53] and phosphates **CyC$_{23\text{-}30}$** [53]; as well as Cyclipostin P **CyC$_{18\beta}$**, its *trans*-(α) diastereoisomer **CyC$_{18\alpha}$** [49], and the corresponding monocyclic enol-phosphonates **CyC$_{11\text{-}13}$** [52, 54], difluorophosphonates **CyC$_{14\text{-}15}$** and phosphates **CyC$_{16\text{-}17}$** [55, 56] (**Figure 3**). Of particular importance, diastereomeric *cis*- and *trans*-monocyclic enolphosphonates **CyC$_{6\text{-}10}$**

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.*, R^2 group – **Figure 3**) or at the phosphorous center (*i.e.*, R^3 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, **CyC$_7$** 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 **CyC$_7$** 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 (S_p) configuration with a Stereoselectivity Index (SI) derived from x_{I50} values of 94.9% (**Table 2**) when using **CyC$_7$**-(S_c) inhibitors, whereas no obvious stereopreference at the phosphorus center was observed with the **CyC$_7$**-(R_c) inhibitors. On the contrary, Rv0183 strongly discriminated the (S_p) configuration (SI = 72-81.4%) independently of the absolute (R_c) or (S_c) 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 **CyC$_{17}$**) 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**).

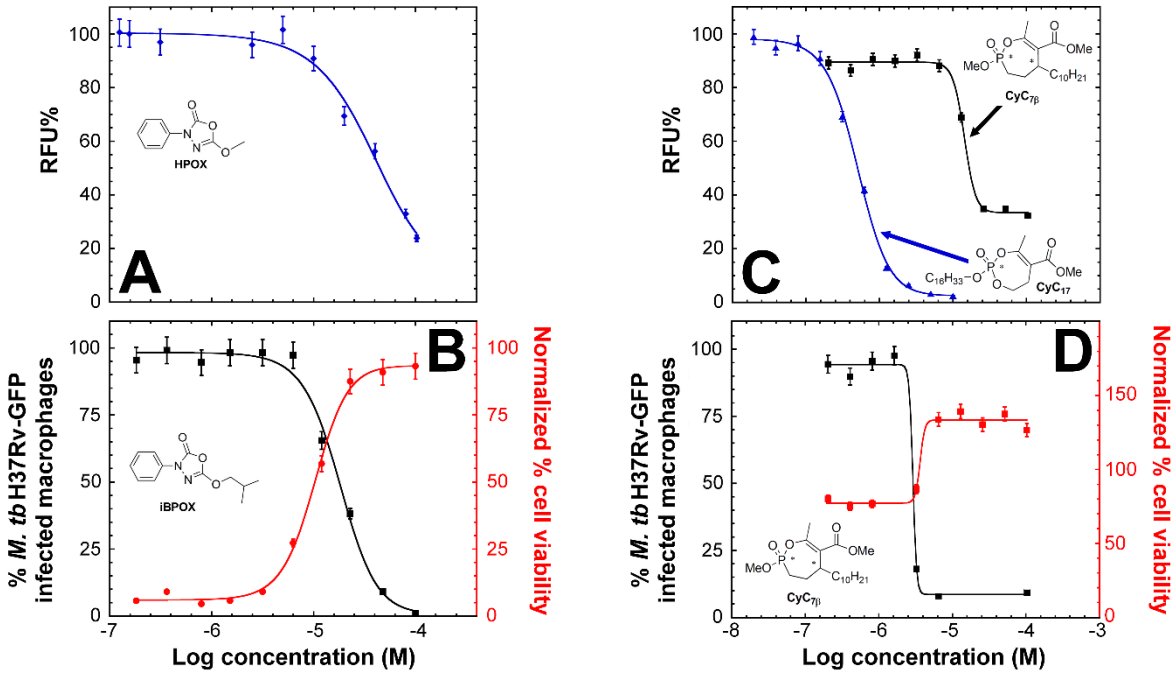


Figure 4. (A-C) Activity of **HPOX, CyC$_{7β}$** & **CyC$_{17}$** against GFP-labelled *M. tb* H37Rv replicating in culture medium expressed as normalized relative fluorescence units (RFU%). **(B-D)** Activity of **iBPOX** and **CyC$_{7β}$** against *M. tb* H37Rv-GFP replicating inside Raw264.7 macrophages. Adapted from [54, 61].

First, 9 out of 14 compounds exhibited higher activity against intracellular bacteria than against extracellular ones: *i.e.*, **iB*p*PPOX**, **iBPOX**, **H*p*PPOX**, **Be*p*PPOX**, **CyC$_{6β}$**, **CyC$_{7α,β}$** and **CyC$_{8α,β}$**. 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**, **CyC$_{17}$**, **CyC$_{18\alpha,\beta}$**; 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.

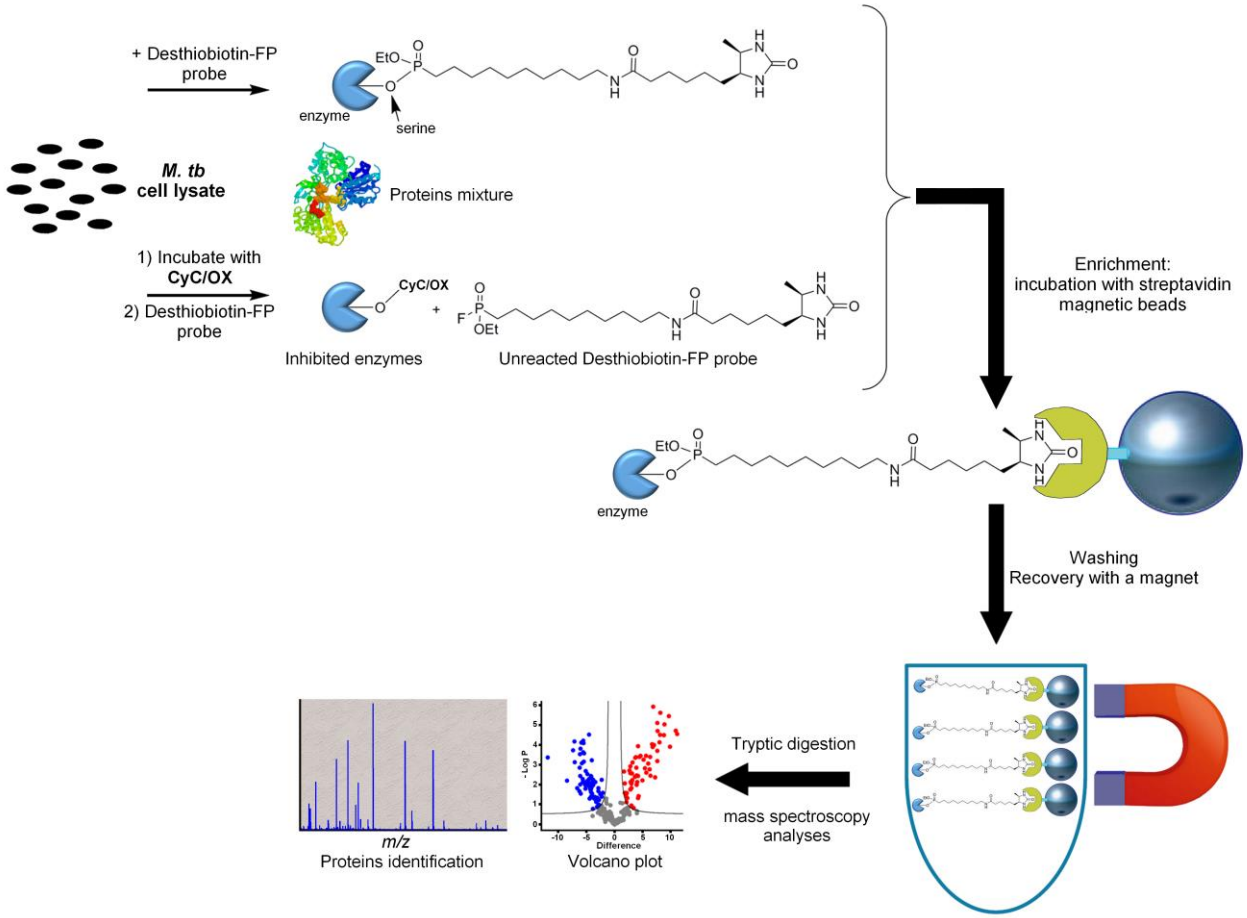


Figure 5. General scheme for the identification of the target enzymes of our inhibitors using ABPP approach.

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 **CyC$_{17}$**, 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 **CyC$_{17}$**-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 **CyC$_{17}$**, but also of **CyC$_{7\beta}$** and **CyC$_{8\beta}$** [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 **CyC17** (x_{I50} = 12.4) [79] and **HPOX** (x_{I50} = 0.59) [61].

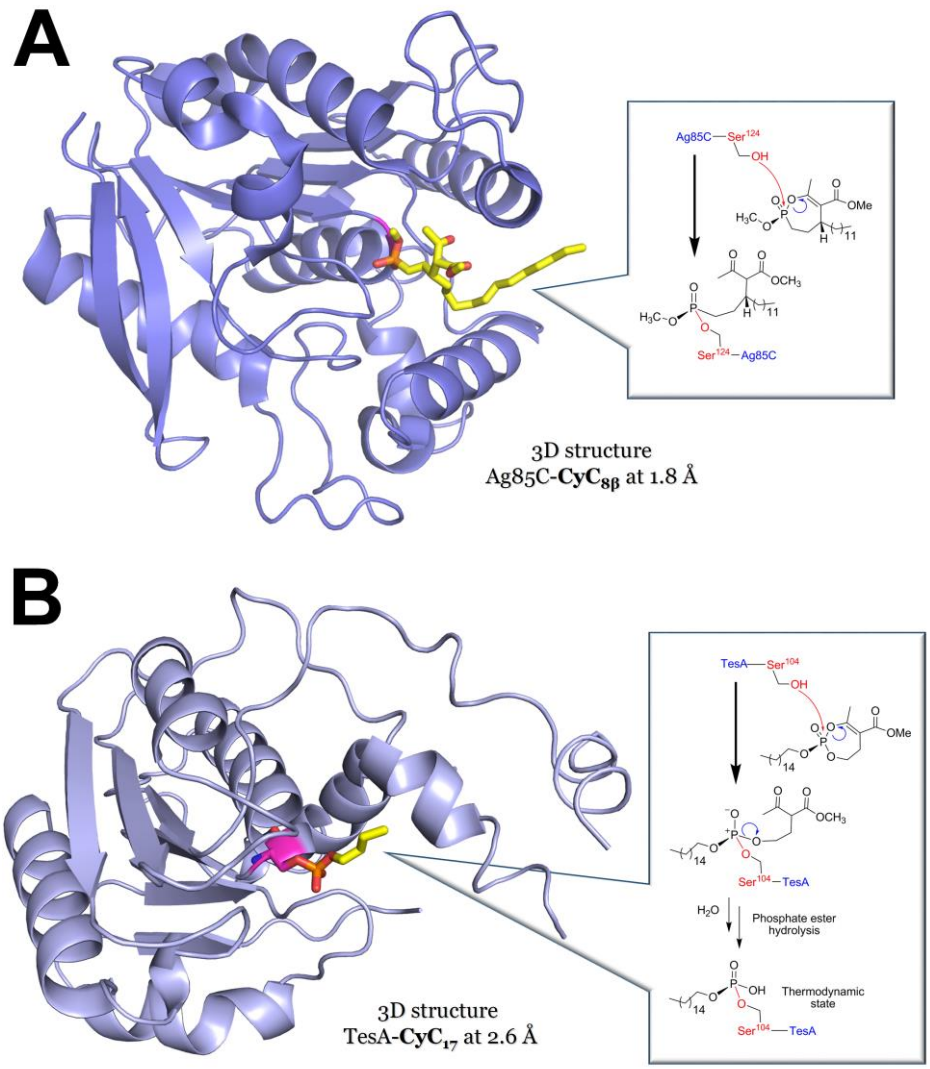


Figure 6. Crystal structures of **(A)** Ag85C in complex with **CyC8β** (PDB ID: 5OCJ; 1.8Å resolution) [78] and **(B)** TesA in complex with **CyC17** (PDB ID: 6FVJ; 2.6Å resolution) [79]. The mechanism of action of the phosphonate (**CyC8β**) and the phosphate (**CyC17**) analog based on mass spectrometry 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 **CyC$_{8\beta}$** (**Figure 6A**) [78] and TesA in complex with **CyC$_{17}$** (**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 **CyC$_{17}$** 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 **CyC$_{17}$** 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 **CyC$_{7\alpha}$** (x_{I50} = 5.0) and in near stoichiometry with **CyC$_{17}$** (x_{I50} = 0.98), but not with **CyC$_{7\beta}$** and **CyC$_{8\alpha,\beta}$** 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 **CyC$_{1\text{-}18}$** 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, **CyC$_{17}$** 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 (**CyC$_{17}$**) 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 (**CyC$_{19\text{-}30}$** – **Figure 3**) were synthesized by varying the R^4 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**, **CyC$_{25}$** and **CyC$_{26}$** exhibited very promising MIC_{50} 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 R^3 or R^4 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, **iB*p*PPOX** 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 **CyC$_{7\alpha,\beta}$** and **CyC$_{8\alpha,\beta}$** were found active against intracellularly replicating *M. abscessus* (**Table 4**). With MIC_{50Raw} values of 7.9 and 2.0 µM, respectively, **CyC$_{8\alpha}$** and **CyC$_{8\beta}$** 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 MIC_{50Raw} are of the same order of magnitude than those previously obtained with the same **CyC$_{8\alpha,\beta}$** against intracellular *M. tb* (4-12 µM – **Table 3**). Regarding the **OXs**, only **MPOX** and **M*p*PPOX** (inactive against extracellular bacterial growth) and **iB*p*PPOX** were able to block intracellularly-growing *M. abscessus*. As previously observed for *M. tb* [61], **iB*p*PPOX** impairs both extracellular (MIC_{50} = 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 **CyC$_{17}$** and **iB*p*PPOX**, 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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References

[1] J. Daniel, H. Maamar, C. Deb, T.D. Sirakova, P.E. Kolattukudy, Mycobacterium tuberculosis uses host triacylglycerol to accumulate lipid droplets and acquires a dormancy-like phenotype in lipid-loaded macrophages, PLoS Pathog, 7 (2011) e1002093.

[2] C. Deb, J. Daniel, T. Sirakova, B. Abomoelak, V. Dubey, P. Kolattukudy, A Novel Lipase Belonging to the Hormone-sensitive Lipase Family Induced under Starvation to Utilize Stored Triacylglycerol in Mycobacterium tuberculosis, J. Biol. Chem., 281 (2006) 3866-3875.

[3] D.G. Russell, P.J. Cardona, M.J. Kim, S. Allain, F. Altare, Foamy macrophages and the progression of the human tuberculosis granuloma, Nat Immunol, 10 (2009) 943-948.

[4] R. Dhouib, A. Ducret, P. Hubert, F. Carriere, S. Dukan, S. Canaan, Watching intracellular lipolysis in mycobacteria using time lapse fluorescence microscopy, Biochim Biophys Acta, 1811 (2011) 234-241.

[5] P. Santucci, F. Bouzid, N. Smichi, I. Poncin, L. Kremer, C. De Chastellier, M. Drancourt, S. Canaan, Experimental Models of Foamy Macrophages and Approaches for Dissecting the Mechanisms of Lipid Accumulation and Consumption during Dormancy and Reactivation of Tuberculosis, Front Cell Infect Microbiol., 6 (2016) 122.

[6] K.L. Low, P.S. Rao, G. Shui, A.K. Bendt, K. Pethe, T. Dick, M.R. Wenk, Triacylglycerol utilization is required for regrowth of in vitro hypoxic nonreplicating Mycobacterium bovis bacillus Calmette-Guerin, J Bacteriol, 191 (2009) 5037-5043.

[7] K.L. Low, G. Shui, K. Natter, W.K. Yeo, S.D. Kohlwein, T. Dick, S.P. Rao, M.R. Wenk, Lipid droplet-associated proteins are involved in the biosynthesis and hydrolysis of triacylglycerol in *Mycobacterium bovis* bacillus Calmette-Guerin, J Biol Chem, 285 (2010) 21662-21670.

[8] K.A. Mattos, F.A. Lara, V.G. Oliveira, L.S. Rodrigues, H. D'Avila, R.C. Melo, P.P. Manso, E.N. Sarno, P.T. Bozza, M.C. Pessolani, Modulation of lipid droplets by *Mycobacterium leprae* in Schwann cells: a putative mechanism for host lipid acquisition and bacterial survival in phagosomes, Cell Microbiol, 13 (2011) 259-273.

[9] A. Viljoen, M. Blaise, C. de Chastellier, L. Kremer, MAB_3551c encodes the primary triacylglycerol synthase involved in lipid accumulation in Mycobacterium abscessus, Mol Microbiol, 102 (2016) 611-627.

[10] P. Santucci, S. Diomande, I. Poncin, L. Alibaud, A. Viljoen, L. Kremer, C. de Chastellier, S. Canaan, Delineating the Physiological Roles of the PE and Catalytic Domains of LipY in

Lipid Consumption in Mycobacterium-Infected Foamy Macrophages, Infect Immun, 86 (2018) e00394-00318.

[11] P. Santucci, M.D. Johansen, V. Point, I. Poncin, A. Viljoen, J.-F. Cavalier, L. Kremer, S. Canaan, Nitrogen deprivation induces triacylglycerol accumulation, drug tolerance and hypervirulence in mycobacteria, Sci Rep, 9 (2019) 8667.

[12] J.G. Rodriguez, A.C. Hernandez, C. Helguera-Repetto, D. Aguilar Ayala, R. Guadarrama-Medina, J.M. Anzola, J.R. Bustos, M.M. Zambrano, Y.M.J. Gonzalez, M.J. Garcia, P. Del Portillo, Global adaptation to a lipid environment triggers the dormancy-related phenotype of Mycobacterium tuberculosis, MBio, 5 (2014) e01125-01114.

[13] J.C. Camus, M.J. Pryor, C. Medigue, S.T. Cole, Re-annotation of the genome sequence of Mycobacterium tuberculosis H37Rv, Microbiology, 148 (2002) 2967-2973.

[14] B. Brust, M. Lecoufle, E. Tuaillon, L. Dedieu, S. Canaan, V. Valverde, L. Kremer, Mycobacterium tuberculosis lipolytic enzymes as potential biomarkers for the diagnosis of active tuberculosis, PLoS ONE, 6 (2011) e25078.

[15] G. Johnson, The alpha/beta Hydrolase Fold Proteins of Mycobacterium tuberculosis, With Reference to their Contribution to Virulence, Curr Protein Pept Sci, 18 (2017) 190-210.

[16] K. Côtes, R. Dhouib, I. Douchet, H. Chahinian, A. De Caro, F. Carriere, S. Canaan, Characterization of an exported monoglyceride lipase from Mycobacterium tuberculosis possibly involved in the metabolism of host cell membrane lipids, Biochem J., 408 (2007) 417-427.

[17] K.C. Mishra, C. de Chastellier, Y. Narayana, P. Bifani, A.K. Brown, G.S. Besra, V.M. Katoch, B. Joshi, K.N. Balaji, L. Kremer, Functional role of the PE domain and immunogenicity of the Mycobacterium tuberculosis triacylglycerol hydrolase LipY, Infect Immun, 76 (2008) 127-140.

[18] G. Xu, H. Jia, Y. Li, X. Liu, M. Li, Y. Wang, Hemolytic phospholipase Rv0183 of Mycobacterium tuberculosis induces inflammatory response and apoptosis in alveolar macrophage RAW264.7 cells, Can J Microbiol, 56 (2010) 916-924.

[19] M. Schue, D. Maurin, R. Dhouib, J.C. Bakala N'Goma, V. Delorme, G. Lambeau, F. Carriere, S. Canaan, Two cutinase-like proteins secreted by Mycobacterium tuberculosis show very different lipolytic activities reflecting their physiological function, FASEB J, 24 (2010) 1893-1903.

[20] P. Hadvary, W. Sidler, W. Meister, W. Vetter, H. Wolfer, The lipase inhibitor tetrahydrolipstatin binds covalently to the putative active site serine of pancreatic lipase, J Biol Chem, 266 (1991) 2021-2027.

[21] H. Stalder, Oesterhelt, Borgström, Tetrahydrolipstatin: degradation products produced by human carboxyl-ester lipase, Helvetica Chimica Acta, 75 (1992) 11.

[22] A. Benarouche, V. Point, F. Carriere, J.F. Cavalier, Using the reversible inhibition of gastric lipase by Orlistat for investigating simultaneously lipase adsorption and substrate hydrolysis at the lipid-water interface, Biochimie, 101 (2014) 221-231.

[23] L. Haalck, F. Spener, On the inhibition of microbial lipases by tetrahydrolipstatin, Methods Enzymol, 286 (1997) 252-263.

[24] S.K. Parker, R.M. Barkley, J.G. Rino, M.L. Vasil, Mycobacterium tuberculosis Rv3802c encodes a phospholipase/thioesterase and is inhibited by the antimycobacterial agent tetrahydrolipstatin, PLoS One, 4 (2009) e4281.

[25] P.K. Crellin, J.P. Vivian, J. Scoble, F.M. Chow, N.P. West, R. Brammananth, N.I. Proellocks, A. Shahine, J. Le Nours, M.C.J. Wilce, W.J. Britton, R.L. Coppel, J. Rossjohn, T. Beddoe, Tetrahydrolipstatin Inhibition, Functional Analyses, and Three-dimensional Structure of a Lipase Essential for Mycobacterial Viability, J. Biol. Chem., 285 (2010) 30050-30060.

[26] N.P. West, K.M. Cergol, M. Xue, E.J. Randall, W.J. Britton, R.J. Payne, Inhibitors of an essential mycobacterial cell wall lipase (Rv3802c) as tuberculosis drug leads, Chem Commun (Camb), 47 (2011) 5166-5168.

[27] M.S. Ravindran, S.P. Rao, X. Cheng, A. Shukla, A. Cazenave-Gassiot, S.Q. Yao, M.R. Wenk, Targeting lipid esterases in mycobacteria grown under different physiological conditions using activity-based profiling with tetrahydrolipstatin (THL), Mol Cell Proteomics, 13 (2014) 435-448.

[28] V. Delorme, S.V. Diomande, L. Dedieu, J.-F. Cavalier, F. Carriere, L. Kremer, J. Leclaire, F. Fotiadu, S. Canaan, MmPPOX inhibits Mycobacterium tuberculosis lipolytic enzymes belonging to the hormone-sensitive lipase family and alters mycobacterial growth, PLoS ONE, 7 (2012) e46493.

[29] C.M. Goins, T.D. Sudasinghe, X. Liu, Y. Wang, G.A. O'Doherty, D.R. Ronning, Characterization of Tetrahydrolipstatin and Stereoderivatives on the Inhibition of Essential Mycobacterium tuberculosis Lipid Esterases, Biochemistry, 57 (2018) 2383-2393.

[30] C.M. Goins, S. Dajnowicz, M.D. Smith, J.M. Parks, D.R. Ronning, Mycolyltransferase from Mycobacterium tuberculosis in covalent complex with tetrahydrolipstatin provides insights into antigen 85 catalysis, J Biol Chem, 293 (2018) 3651-3662.

[31] C. Rens, F. Laval, M. Daffe, O. Denis, R. Frita, A. Baulard, R. Wattiez, P. Lefevre, V. Fontaine, Effects of lipid-lowering drugs on vancomycin susceptibility of mycobacteria, Antimicrob Agents Chemother, 60 (2016) 6193-6199.

[32] P. Santucci, C. Dedaki, A. Athanasoulis, L. Gallorini, A. Munoz, S. Canaan, J.-F. Cavalier, V. Magrioti, Synthesis of long chain β-lactones and their antibacterial activities against pathogenic mycobacteria, ChemMedChem, 14 (2019) 349-358.

[33] J. Lehmann, J. Vomacka, K. Esser, M. Nodwell, K. Kolbe, P. Ramer, U. Protzer, N. Reiling, S.A. Sieber, Human lysosomal acid lipase inhibitor lalistat impairs Mycobacterium tuberculosis growth by targeting bacterial hydrolases, MedChemComm 7(2016) 1797-1801.

[34] J. Lehmann, T.Y. Cheng, A. Aggarwal, A.S. Park, E. Zeiler, R.M. Raju, T. Akopian, O. Kandror, J.C. Sacchettini, D.B. Moody, E.J. Rubin, S.A. Sieber, An Antibacterial beta-Lactone Kills Mycobacterium tuberculosis by Disrupting Mycolic Acid Biosynthesis, Angew Chem Int Ed Engl, 57 (2018) 348-353.

[35] J.T. Belisle, V.D. Vissa, T. Sievert, K. Takayama, P.J. Brennan, G.S. Besra, Role of the major antigen of Mycobacterium tuberculosis in cell wall biogenesis, Science, 276 (1997) 1420-1422.

[36] A. Aggarwal, M.K. Parai, N. Shetty, D. Wallis, L. Woolhiser, C. Hastings, N.K. Dutta, S. Galaviz, R.C. Dhakal, R. Shrestha, S. Wakabayashi, C. Walpole, D. Matthews, D. Floyd, P. Scullion, J. Riley, O. Epemolu, S. Norval, T. Snavely, G.T. Robertson, E.J. Rubin, T.R. Ioerger, F.A. Sirgel, R. van der Merwe, P.D. van Helden, P. Keller, E.C. Böttger, P.C. Karakousis, A.J. Lenaerts, J.C. Sacchettini, Development of a Novel Lead that Targets M. tuberculosis Polyketide Synthase 13, Cell, 170 (2017) 249-259.e225.

[37] A.E. Wilder Smith, The Action of Phosgene on Acid Hydrazides to Give 1, 3, 4-Oxdiazolones of Interest in the Treatment of Tuberculosis, Science, 119 (1954) 514.

[38] A.E. Wilder Smith, H. Brodhage, Biological spectrum of some new tuberculostatic 1,3,4-oxadiazolones with special reference to cross-resistance and rates of emergence of resistance, Nature, 192 (1961) 1195.

[39] M.G. Mamolo, D. Zampieri, L. Vio, M. Fermeglia, M. Ferrone, S. Pricl, G. Scialino, E. Banfi, Antimycobacterial activity of new 3-substituted 5-(pyridin-4-yl)-3H-1,3,4-oxadiazol-2-

636 one and 2-thione derivatives. Preliminary molecular modeling investigations, Bioorg Med
637 Chem, 13 (2005) 3797-3809.

638 [40] D. Zampieri, M.G. Mamolo, E. Laurini, M. Fermeglia, P. Posocco, S. Pricl, E. Banfi, G.
639 Scialino, L. Vio, Antimycobacterial activity of new 3,5-disubstituted 1,3,4-oxadiazol-2(3H)-
640 one derivatives. Molecular modeling investigations, Bioorg Med Chem, 17 (2009) 4693-4707.

641 [41] P. Santucci, N. Smichi, S. Diomande, I. Poncin, V. Point, H. Gaussier, J.-F. Cavalier, L.
642 Kremer, S. Canaan, Dissecting the membrane lipid binding properties and lipase activity of
643 Mycobacterium tuberculosis LipY domains, The FEBS journal, 286 (2019) 3164-3181.

644 [42] I. Caire-Brandli, A. Papadopoulos, W. Malaga, D. Marais, S. Canaan, L. Thilo, C. de
645 Chastellier, Reversible lipid accumulation and associated division arrest of Mycobacterium
646 avium in lipoprotein-induced foamy macrophages may resemble key events during latency and
647 reactivation of tuberculosis, Infect Immun, 82 (2014) 476-490.

648 [43] V. Point, A. Benarouche, J. Zarrillo, A. Guy, R. Magnez, L. Fonseca, B. Raux, J. Leclaire,
649 G. Buono, F. Fotiadu, T. Durand, F. Carriere, C. Vaysse, L. Couedelo, J.-F. Cavalier, Slowing
650 down fat digestion and absorption by an oxadiazolone inhibitor targeting selectively gastric
651 lipolysis, Eur J Med Chem, 123 (2016) 834-848.

652 [44] S. Ulker, C. Placidi, V. Point, B. Gadenne, C. Serveau-Avesque, S. Canaan, F. Carriere,
653 J.-F. Cavalier, New lipase assay using Pomegranate oil coating in microtiter plates, Biochimie,
654 120 (2016) 110-118.

655 [45] T. Kurokawa, K. Suzuki, T. Hayaoka, T. Nakagawa, T. Izawa, M. Kobayashi, N. Harada,
656 Cyclophostin, acetylcholinesterase inhibitor from Streptomyces lavendulae, J Antibiot
657 (Tokyo), 46 (1993) 1315-1318.

658 [46] L. Vertesy, B. Beck, M. Bronstrup, K. Ehrlich, M. Kurz, G. Muller, D. Schummer, G.
659 Seibert, Cyclipostins, novel hormone-sensitive lipase inhibitors from Streptomyces sp. DSM
660 13381. II. Isolation, structure elucidation and biological properties, J Antibiot (Tokyo), 55
661 (2002) 480-494.

662 [47] G. Seibert, L. Toti, J. Wink, Treating mycobacterial infections with cyclipostins., in:
663 Patent, 2008.

664 [48] S. Bandyopadhyay, S. Dutta, C.D. Spilling, C.M. Dupureur, N.P. Rath, Synthesis and
665 biological evaluation of a phosphonate analog of the natural acetyl cholinesterase inhibitor
666 cyclophostin, J Org Chem, 73 (2008) 8386-8391.

[49] R.K. Malla, S. Bandyopadhyay, C.D. Spilling, S. Dutta, C.M. Dupureur, The first total synthesis of (+/-)-cyclophostin and (+/-)-cyclipostin P: inhibitors of the serine hydrolases acetyl cholinesterase and hormone sensitive lipase, Org Lett, 13 (2011) 3094-3097.

[50] C.D. Spilling, The Chemistry and Biology of Cyclophostin, the Cyclipostins and Related Compounds, Molecules, 24 (2019) 2579.

[51] S. Dutta, R.K. Malla, S. Bandyopadhyay, C.D. Spilling, C.M. Dupureur, Synthesis and kinetic analysis of some phosphonate analogs of cyclophostin as inhibitors of human acetylcholinesterase, Bioorg Med Chem, 18 (2010) 2265-2274.

[52] V. Point, R.K. Malla, S. Diomande, B.P. Martin, V. Delorme, F. Carriere, S. Canaan, N.P. Rath, C.D. Spilling, J.-F. Cavalier, Synthesis and kinetic evaluation of cyclophostin and cyclipostins phosphonate analogs as selective and potent inhibitors of microbial lipases, J Med Chem, 55 (2012) 10204-10219.

[53] A. Madani, J.N. Ridenour, B.P. Martin, R.R. Paudel, A. Abdul Basir, V. Le Moigne, J.L. Herrmann, S. Audebert, L. Camoin, L. Kremer, C.D. Spilling, S. Canaan, J.-F. Cavalier, Cyclipostins and Cyclophostin Analogues as Multitarget Inhibitors That Impair Growth of *Mycobacterium abscessus*, ACS Infect Dis, 5 (2019) 1597-1608.

[54] P.C. Nguyen, V. Delorme, A. Benarouche, B.P. Martin, R. Paudel, G.R. Gnawali, A. Madani, R. Puppo, V. Landry, L. Kremer, P. Brodin, C.D. Spilling, J.-F. Cavalier, S. Canaan, Cyclipostins and Cyclophostin analogs as promising compounds in the fight against tuberculosis, Sci Rep, 7 (2017) 11751.

[55] B.P. Martin, E. Vasilieva, C.M. Dupureur, C.D. Spilling, Synthesis and comparison of the biological activity of monocyclic phosphonate, difluorophosphonate and phosphate analogs of the natural AChE inhibitor cyclophostin, Bioorg Med Chem, 23 (2015) 7529-7534.

[56] E. Vasilieva, S. Dutta, R.K. Malla, B.P. Martin, C.D. Spilling, C.M. Dupureur, Rat hormone sensitive lipase inhibition by cyclipostins and their analogs, Bioorg Med Chem, 23 (2015) 944-952.

[57] S. Longhi, M. Mannesse, H.M. Verheij, G.H. De Haas, M. Egmond, E. Knoops-Mouthuy, C. Cambillau, Crystal structure of cutinase covalently inhibited by a triglyceride analogue, Protein Sci, 6 (1997) 275-286.

[58] P. Aschauer, R. Zimmermann, R. Breinbauer, T. Pavkov-Keller, M. Oberer, The crystal structure of monoacylglycerol lipase from M. tuberculosis reveals the basis for specific inhibition, Sci Rep, 8 (2018) 8948.

[59] V. Point, R.K. Malla, F. Carriere, S. Canaan, C.D. Spilling, J.-F. Cavalier, Enantioselective inhibition of microbial lipolytic enzymes by nonracemic monocyclic enolphosphonate analogues of cyclophostin, J Med Chem, 56 (2013) 4393-4401.

[60] J.-F. Cavalier, G. Buono, R. Verger, Covalent inhibition of digestive lipases by chiral phosphonates, Acc. Chem. Res., 33 (2000) 579-589.

[61] P.C. Nguyen, V. Delorme, A. Benarouche, A. Guy, V. Landry, S. Audebert, M. Pophillat, L. Camoin, C. Crauste, J.M. Galano, T. Durand, P. Brodin, S. Canaan, J.-F. Cavalier, Oxadiazolone derivatives, new promising multi-target inhibitors against *M. tuberculosis*, Bioorg Chem, 81 (2018) 414-424.

[62] T. Christophe, M. Jackson, H.K. Jeon, D. Fenistein, M. Contreras-Dominguez, J. Kim, A. Genovesio, J.P. Carralot, F. Ewann, E.H. Kim, S.Y. Lee, S. Kang, M.J. Seo, E.J. Park, H. Skovierova, H. Pham, G. Riccardi, J.Y. Nam, L. Marsollier, M. Kempf, M.L. Joly-Guillou, T. Oh, W.K. Shin, Z. No, U. Nehrbass, R. Brosch, S.T. Cole, P. Brodin, High content screening identifies decaprenyl-phosphoribose 2' epimerase as a target for intracellular antimycobacterial inhibitors, PLoS Pathog, 5 (2009) e1000645.

[63] M. Flipo, M. Desroses, N. Lecat-Guillet, B. Dirie, X. Carette, F. Leroux, C. Piveteau, F. Demirkaya, Z. Lens, P. Rucktooa, V. Villeret, T. Christophe, H.K. Jeon, C. Locht, P. Brodin, B. Deprez, A.R. Baulard, N. Willand, Ethionamide boosters: synthesis, biological activity, and structure-activity relationships of a series of 1,2,4-oxadiazole EthR inhibitors, J Med Chem., 54 (2011) 2994-3010.

[64] C. Ortega, L.N. Anderson, A. Frando, N.C. Sadler, R.W. Brown, R.D. Smith, A.T. Wright, C. Grundner, Systematic Survey of Serine Hydrolase Activity in Mycobacterium tuberculosis Defines Changes Associated with Persistence, Cell Chem Biol., 23 (2016) 290-298.

[65] K.R. Tallman, S.R. Levine, K.E. Beatty, Small Molecule Probes Reveal Esterases with Persistent Activity in Dormant and Reactivating Mycobacterium tuberculosis, ACS Infect. Dis., 2 (2016) 936-944.

[66] F. Faucher, J.M. Bennett, M. Bogyo, S. Lovell, Strategies for Tuning the Selectivity of Chemical Probes that Target Serine Hydrolases, Cell Chem Biol, 27 (2020) 937-952.

[67] L.J. Keller, B.M. Babin, M. Lakemeyer, M. Bogyo, Activity-based protein profiling in bacteria: Applications for identification of therapeutic targets and characterization of microbial communities, Curr Opin Chem Biol, 54 (2019) 45-53.

730 [68] D.A. Bachovchin, S.J. Brown, H. Rosen, B.F. Cravatt, Identification of selective inhibitors
731 of uncharacterized enzymes by high-throughput screening with fluorescent activity-based
732 probes, Nat Biotechnol, 27 (2009) 387-394.
733 [69] C.M. Sassetti, D.H. Boyd, E.J. Rubin, Genes required for mycobacterial growth defined
734 by high density mutagenesis, Mol Microbiol, 48 (2003) 77-84.
735 [70] C.M. Sassetti, E.J. Rubin, Genetic requirements for mycobacterial survival during
736 infection, Proc Natl Acad Sci U S A, 100 (2003) 12989-12994.
737 [71] J.E. Griffin, J.D. Gawronski, M.A. Dejesus, T.R. Ioerger, B.J. Akerley, C.M. Sassetti,
738 High-resolution phenotypic profiling defines genes essential for mycobacterial growth and
739 cholesterol catabolism, PLoS Pathog, 7 (2011) e1002251.
740 [72] L. Alibaud, Y. Rombouts, X. Trivelli, A. Burguiere, S.L. Cirillo, J.D. Cirillo, J.F.
741 Dubremetz, Y. Guerardel, G. Lutfalla, L. Kremer, A Mycobacterium marinum TesA mutant
742 defective for major cell wall-associated lipids is highly attenuated in Dictyostelium discoideum
743 and zebrafish embryos, Molecular Microbiology, 80 (2011) 919-934.
744 [73] S. Lun, W.R. Bishai, Characterization of a Novel Cell Wall-anchored Protein with
745 Carboxylesterase Activity Required for Virulence in Mycobacterium tuberculosis, J. Biol.
746 Chem., 282 (2007) 18348-18356.
747 [74] R.A. Slayden, C.E. Barry, 3rd, The role of KasA and KasB in the biosynthesis of
748 meromycolic acids and isoniazid resistance in Mycobacterium tuberculosis, Tuberculosis
749 (Edinb), 82 (2002) 149-160.
750 [75] J. Rengarajan, B.R. Bloom, E.J. Rubin, Genome-wide requirements for Mycobacterium
751 tuberculosis adaptation and survival in macrophages, Proc Natl Acad Sci U S A, 102 (2005)
752 8327-8332.
753 [76] N.A. Lack, K.C. Yam, E.D. Lowe, G.P. Horsman, R.L. Owen, E. Sim, L.D. Eltis,
754 Characterization of a carbon-carbon hydrolase from Mycobacterium tuberculosis involved in
755 cholesterol metabolism, J Biol Chem., 285 (2010) 434-443.
756 [77] A. Ryan, E. Polycarpou, N.A. Lack, D. Evangelopoulos, C. Sieg, A. Halman, S. Bhakta,
757 O. Eleftheriadou, T.D. McHugh, S. Keany, E.D. Lowe, R. Ballet, A. Abuhammad, W.R. Jacobs,
758 A. Ciulli, E. Sim, Investigation of the mycobacterial enzyme HsaD as a potential novel target
759 for anti-tubercular agents using a fragment-based drug design approach, British J Pharmacol,
760 174 (2017) 2209-2224.

761 [78] A. Viljoen, M. Richard, P.C. Nguyen, P. Fourquet, L. Camoin, R.R. Paudal, G.R. Gnawali,
762 C.D. Spilling, J.-F. Cavalier, S. Canaan, M. Blaise, L. Kremer, Cyclipostins and cyclophostin
763 analogs inhibit the antigen 85C from Mycobacterium tuberculosis both in vitro and in vivo, J
764 Biol Chem, 293 (2018) 2755-2769.
765 [79] P.C. Nguyen, V.S. Nguyen, B.P. Martin, P. Fourquet, L. Camoin, C.D. Spilling, J.-F.
766 Cavalier, C. Cambillau, S. Canaan, Biochemical and Structural Characterization of TesA, a
767 Major Thioesterase Required for Outer-Envelope Lipid Biosynthesis in Mycobacterium
768 tuberculosis, J Mol Biol, 430 (2018) 5120-5136.
769 [80] P. Santucci, V. Point, I. Poncin, A. Guy, C. Crauste, C. Serveau-Avesque, J.M. Galano,
770 C.D. Spilling, J.-F. Cavalier, S. Canaan, LipG a bifunctional phospholipase/thioesterase
771 involved in mycobacterial envelope remodeling, Biosci Rep, 38 (2018) BSR20181953.
772 [81] P.C. Nguyen, A. Madani, P. Santucci, B.P. Martin, R.R. Paudel, S. Delattre, J.L. Herrmann,
773 C.D. Spilling, L. Kremer, S. Canaan, J.-F. Cavalier, Cyclophostin and Cyclipostins analogues,
774 new promising molecules to treat mycobacterial-related diseases, Int J Antimicrob Agents, 51
775 (2018) 651-654.
776 [82] M. Osmani, D. Sotello, S. Alvarez, J.A. Odell, M. Thomas, Mycobacterium abscessus
777 infections in lung transplant recipients: 15-year experience from a single institution, Transpl
778 Infect Dis, 20 (2018) e12835.
779 [83] M.-L. Wu, D.B. Aziz, V. Dartois, T. Dick, NTM drug discovery: status, gaps and the way
780 forward, Drug Discov Today, 23 (2018) 1502-1519.
781 [84] M.D. Johansen, J.L. Herrmann, L. Kremer, Non-tuberculous mycobacteria and the rise of
782 Mycobacterium abscessus, Nat Rev Microbiol, 18 (2020) 392-407.
783 [85] E. Catherinot, J. Clarissou, G. Etienne, F. Ripoll, J.F. Emile, M. Daffe, C. Perronne, C.
784 Soudais, J.L. Gaillard, M. Rottman, Hypervirulence of a rough variant of the Mycobacterium
785 abscessus type strain, Infect Immun, 75 (2007) 1055-1058.
786 [86] R. Nessar, E. Cambau, J.M. Reyrat, A. Murray, B. Gicquel, Mycobacterium abscessus: a
787 new antibiotic nightmare, J Antimicrob Chemother, 67 (2012) 810-818.
788 [87] P.H. Candido, S. Nunes Lde, E.A. Marques, T.W. Folescu, F.S. Coelho, V.C. de Moura,
789 M.G. da Silva, K.M. Gomes, M.C. Lourenco, F.S. Aguiar, F. Chitolina, D.T. Armstrong, S.C.
790 Leao, F.P. Neves, F.C. Mello, R.S. Duarte, Multidrug-resistant nontuberculous mycobacteria
791 isolated from cystic fibrosis patients, J Clin Microbiol, 52 (2014) 2990-2997.

[88] F. Mougari, L. Guglielmetti, L. Raskine, I. Sermet-Gaudelus, N. Veziris, E. Cambau, Infections caused by Mycobacterium abscessus: epidemiology, diagnostic tools and treatment, Expert Rev Anti Infect Ther, 14 (2016) 1139-1154.

[89] C.J. Schulze, G. Navarro, D. Ebert, J. DeRisi, R.G. Linington, Salinipostins A-K, long-chain bicyclic phosphotriesters as a potent and selective antimalarial chemotype, J Org Chem., 80 (2015) 1312-1320.

[90] E. Yoo, C.J. Schulze, B.H. Stokes, O. Onguka, T. Yeo, S. Mok, N.F. Gnadig, Y. Zhou, K. Kurita, I.T. Foe, S.M. Terrell, M.J. Boucher, P. Cieplak, K. Kumpornsin, M.C.S. Lee, R.G. Linington, J.Z. Long, A.C. Uhlemann, E. Weerapana, D.A. Fidock, M. Bogyo, The Antimalarial Natural Product Salinipostin A Identifies Essential alpha/beta Serine Hydrolases Involved in Lipid Metabolism in *P. falciparum* Parasites, Cell Chem Biol, 27 (2020) 143-157 e145.

[91] A. Viljoen, F. Viela, L. Kremer, Y.F. Dufrene, Fast chemical force microscopy demonstrates that glycopeptidolipids define nanodomains of varying hydrophobicity on mycobacteria, Nanoscale Horiz, 5 (2020) 944-953.

[92] A. Madani, Y. Mallick, A. Guy, C. Crauste, T. Durand, P. Fourquet, S. Audebert, L. Camoin, S. Canaan, J.-F. Cavalier, Dissecting the antibacterial activity of Oxadiazolone-core derivatives against *Mycobacterium abscessus*, PLoS One, (2020).

[93] A.L. Roux, A. Viljoen, A. Bah, R. Simeone, A. Bernut, L. Laencina, T. Deramaudt, M. Rottman, J.L. Gaillard, L. Majlessi, R. Brosch, F. Girard-Misguich, I. Vergne, C. de Chastellier, L. Kremer, J.L. Herrmann, The distinct fate of smooth and rough Mycobacterium abscessus variants inside macrophages, Open Biol, 6 (2016) 160185.

[94] L. Laencina, V. Dubois, V. Le Moigne, A. Viljoen, L. Majlessi, J. Pritchard, A. Bernut, L. Piel, A.L. Roux, J.L. Gaillard, B. Lombard, D. Loew, E.J. Rubin, R. Brosch, L. Kremer, J.L. Herrmann, F. Girard-Misguich, Identification of genes required for Mycobacterium abscessus growth in vivo with a prominent role of the ESX-4 locus, Proc Natl Acad Sci U S A, 115 (2018) E1002-E1011.

[95] C.A. Molina-Torres, L. Tamez-Pena, J. Castro-Garza, J. Ocampo-Candiani, L. Vera-Cabrera, Evaluation of the intracellular activity of drugs against Mycobacterium abscessus using a THP-1 macrophage model, J Microbiol Methods, 148 (2018) 29-32.

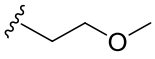
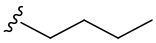
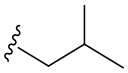
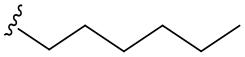
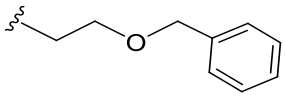
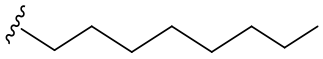
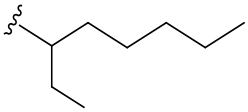
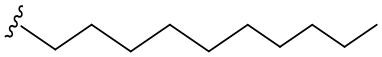
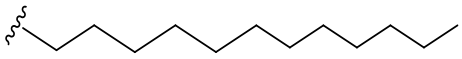
[96] M. Jackson, C. Raynaud, M.A. Laneelle, C. Guilhot, C. Laurent-Winter, D. Ensergueix, B. Gicquel, M. Daffe, Inactivation of the antigen 85C gene profoundly affects the mycolate

content and alters the permeability of the Mycobacterium tuberculosis cell envelope, Mol. Microbiol., 31 (1999) 1573-1587.

[97] T. Warrier, M. Tropis, J. Werngren, A. Diehl, M. Gengenbacher, B. Schlegel, M. Schade, H. Oschkinat, M. Daffe, S. Hoffner, A.N. Eddine, S.H. Kaufmann, Antigen 85C inhibition restricts Mycobacterium tuberculosis growth through disruption of cord factor biosynthesis, Antimicrob Agents Chemother., 56 (2012) 1735-1743.

[98] G. Stelitano, J.C. Sammartino, L.R. Chiarelli, Multitargeting Compounds: A Promising Strategy to Overcome Multi-Drug Resistant Tuberculosis, Molecules, 25 (2020) 1239.

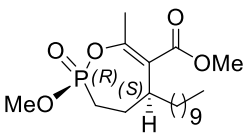
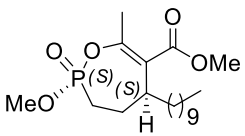
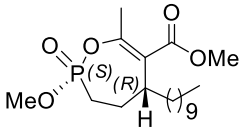
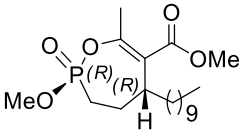
Table 1. Inhibition of the TAG-lipase LipY from *M. tb* after a 30-min incubation period with each **OX** compound [a]

Compounds		LipY		
		%inhibition [b]		
		$x_I = 4$	$x_I = 20$	x_{I50} [c]
Orlistat		26.8±1.5	87.6±4.8	7.1
M*m*PPOX		63.6±4.6	85.9±4.9	2.4
M*p*PPOX		60.8±2.1	79.4±3.3	0.93
MPOX		34.4±2.4	81.8±3.3	10.9
E*m*PPOX		21.7±1.4	53.8±3.3	6.5
Me*m*PPOX		92.4±0.30	93.7±1.3	0.69
B*m*PPOX		15.6±0.50	36.7±1.8	>40
iB*m*PPOX		37.4±2.4	49.4±1.5	3.9
iB*p*PPOX		33.8±1.5	41.6±0.40	>40
iBPOX		31.6±1.0	47.9±2.1	9.2
H*m*PPOX		39.9±1.2	76.5±1.9	3.3
H*p*PPOX		38.7±1.7	77.2±2.7	2.9
HPOX		35.0±0.27	38.8±0.11	>40
Be*m*PPOX		88.0±0.70	90.2±0.91	0.72
Be*p*PPOX		97.1±3.9	98.4±2.2	0.59
BePOX		89.0±1.9	92.2±0.20	0.61
O*m*PPOX		83.2±5.8	86.6±2.2	0.71
Eh*m*PPOX		89.6±1.1	94.8±2.7	0.73
D*m*PPOX		91.5±2.8	94.2±3.1	0.67
Do*m*PPOX		88.7±4.2	90.4±2.2	0.77

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

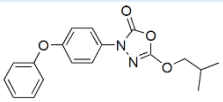
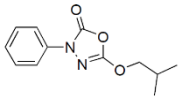
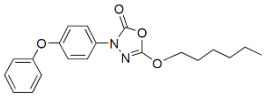
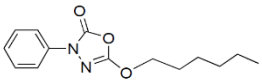
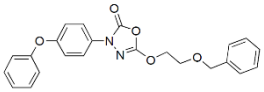
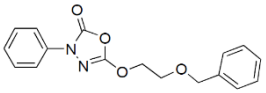
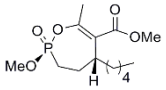
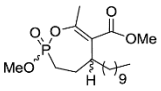
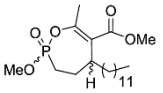
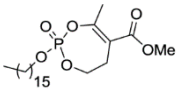
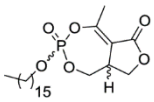
[b] Inhibition data (% of initial enzyme activity), at an inhibitor molar excess (x_I) of 4 or 20 related to 1 mole of enzyme. Values are means of at least three independent assays. [c] x_{I50}: inhibitor molar excess leading to 50% lipase inhibition [28, 44].

Table 2. Stereoselective inhibition of Cutinase, Rv0183, and LipY by enantiopure monocyclic enolphosphonates **CyC$_7$** [a]

	x_{I50}								
	 **CyC$_{7\alpha}$-(S_c,R_p)**			 **CyC$_{7\beta}$-(S_c,S_p)**		 **CyC$_{7\alpha}$-(R_c,S_p)**		 **CyC$_{7\beta}$-(R_c,R_p)**
Cutinase	97.3	←	**SI** = 94.9%	→	**2.52**	4.07		3.38
Rv0183	11.3	←	**SI** = 81.4%	→	**1.16**	**1.24**	← **SI** = 72.0%	→	7.61
LipY	25.9				20.0	15.4	← **SI** = 80.7%	→	**1.64**

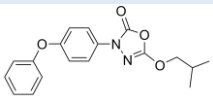
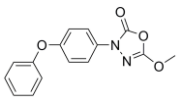
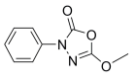
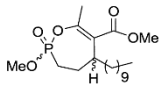
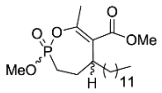
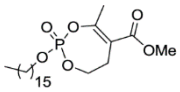
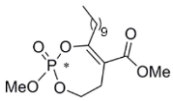
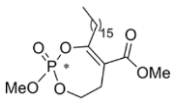
[a] x_{I50}, inhibitor molar excess leading to 50% lipase inhibition. SI, Stereoselectivity Index; SI = 100 × [|$(x_{I50})_{\beta\ isomer} - (x_{I50})_{\alpha\ isomer}$|]/ [$(x_{I50})_{\beta\ isomer} + (x_{I50})_{\alpha\ isomer}$]. Adapted from [59].

Table 3. Activities of the most active **OX** derivatives and **CyC** analogs against *M. tb*.

Compounds		*M. tb* H37Rv-GFP		
		Extracellular growth [a]	Intracellular macrophage growth [b]	
		MIC_{50} [µM]	MIC_{50Raw} [µM]	CC_{50} (µM)
Isoniazid (INH)		1.2	1.2	>150
Rifampicin (RIF)		0.01	2.9	24
OX derivatives				
iB*p*PPOX		32.0 ±0.89	8.5 ±0.30	>100
iBPOX		>50	17.1 ±1.10	>100
H*p*PPOX		>50	9.5 ±0.49	>100
HPOX		44.6 ±2.50	*No effect*	>100
Be*p*PPOX		>50	3.5 ±0.28	>100
BePOX		30.8 ±2.40	*No effect*	>100
CyC analogs				
CyC$_{6\beta}$		*NE*	12.6 ±0.90	>100
CyC$_{7\alpha}$		92.6 ±6.5	4.5 ±0.39	>100
CyC$_{7\beta}$		16.6 ±0.78	3.1 ±0.11	>100
CyC$_{8\alpha}$		40.4 ±2.5	4.0 ±0.30	>100
CyC$_{8\beta}$		>100	11.7 ±0.26	>20
CyC$_{17}$		0.50 ±0.02	*No effect*	>100
CyC$_{18\alpha}$		24.4 ±2.14	*No effect*	>100
CyC$_{18\beta}$		1.7 ±0.03	*No effect*	>100

[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_{50Raw}, 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*.

Compounds		***M. abscessus* CIP 104536^T**		
		MIC_{50} [µM] [a]		MIC_{50Raw} [µM] [b]
		S variant	R variant	
Amikacin (AMK)		3.9 ±0.19	7.4 ±0.26	*Not determined*
Imipenem (IMP)		11.3 ±0.50	29.9 ±1.6	28.3 ±4.7
OX derivatives				
iB*p*PPOX		33.0 ±2.0	53.2 ±1.8	< 30µM
M*p*PPOX		88.2 ±7.3	>200	75.2 ±5.1
MPOX		>200	>200	36.5 ±2.6
CyC analogs				
CyC$_{7\alpha}$		>100	>100	29.3 ±2.9
CyC$_{7\beta}$		>100	>100	65.3 ±6.2
CyC$_{8\alpha}$		>100	>100	7.9 ±0.23
CyC$_{8\beta}$		>100	>100	2.0 ±0.91
CyC$_{17}$		12.7 ±0.26	0.37 ±0.01	*No effect*
CyC$_{25}$		85.0 ±0.86	13.9 ±0.56	*No effect*
CyC$_{26}$		60.0 ±2.50	6.9 ±0.16	*No effect*

[a] MIC_{50}, compound concentration leading to 50% bacterial growth inhibition in culture medium. [b] MIC_{50Raw}, 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].