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1 **Lipolytic Enzymes Inhibitors: a new way for Antibacterial Drugs**

2 **Discovery**

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19

20 **Abstract**

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

41

42 **Keywords:** mycobacteria; tuberculosis; cystic fibrosis; antibiotics; oxadiazolone-core
43 derivatives; Cyclipostins and Cyclophostin analogs
44

45 **Abbreviations:**

- 46 ABP: activity-based probe
- 47 ABPP: Activity-based protein profiling
- 48 AChE: acetylcholinesterase
- 49 CC₅₀: compound concentration leading to 50% host cell toxicity
- 50 CF: cystic fibrosis
- 51 CyC: Cyclophostin & Cyclipostins analogs
- 52 FM: foamy macrophages
- 53 GPL: glycopeptidolipids
- 54 HSL: hormone-sensitive lipase
- 55 IC₅₀: compound concentration leading to 50% enzyme activity inhibition
- 56 ILI: intracytoplasmic lipid inclusions
- 57 ILI⁺³: foamy macrophages displaying many ILI occupying most of the mycobacterial cytoplasm
- 58 LB: lipid bodies
- 59 Lip-HSL: enzymes belonging to the hormone-sensitive lipase family members proteins
- 60 mBMDM: murine bone-marrow-derived macrophages
- 61 MIC: minimum inhibitory concentration
- 62 MIC₅₀: compound concentration leading to 50% mycobacterial growth inhibition
- 63 MIC_{50Raw}: compound concentration leading to 50% bacterial growth inhibition inside Raw264.7
- 64 macrophages as compared to untreated infected cells
- 65 *M. abscessus*: *Mycobacterium abscessus*
- 66 *M. tb*: *Mycobacterium tuberculosis*
- 67 NTM: nontuberculous mycobacterium
- 68 OX: Oxadiazolone-core derivatives
- 69 PDIM: phthiocerol dimycocerosate
- 70 PGL: phenolic glycolipids
- 71 RFU: relative fluorescence unit
- 72 SI: Stereoselectivity Index
- 73 TAG: triacylglycerols
- 74 TB: tuberculosis
- 75 TDM: trehalose dimycolate
- 76 VLDL: Very Low-Density Lipoproteins
- 77 x_1 : inhibitor molar excess related to 1 mol of enzyme
- 78 x_{150} : inhibitor molar excess leading to 50% lipase inhibition

79 **1. Introduction**

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

88 These findings imply that assimilation of fatty acids from LB degradation, as well as TAG
89 biosynthesis and hydrolysis are key aspects of mycobacterial metabolism. ILI formation has
90 been described in many mycobacterial species like *M. tb* [1, 2], *M. bovis* BCG [6, 7], *M. leprae*
91 [8], *M. abscessus* [9], and *M. smegmatis* [4]. However, their origin at the time of infection is
92 poorly understood. It could result from the presence of intracellular and secreted/membrane-
93 anchored mycobacterial lipolytic enzymes capable of degrading LBs, from the absorption of
94 fatty acids available at the caseum center and originating from host cells degradation, or from
95 *de novo* synthesis [1, 10-12].

96 Given the importance of lipid metabolism, the complete analysis of the *M. tb* genome
97 revealed that this bacterium possesses 250 enzymes involved in lipid metabolism representing
98 6% of the full genome [13]. This characteristic strongly suggests that lipids and mycobacterial
99 lipolytic enzymes play an essential role in the life cycle and the virulence of the tubercle bacilli.

100 Over the past decade, it has been well established by our group and others, that such
101 enzymes, possessing a catalytic serine or cysteine residue in their catalytic site (*i.e.* (Ser/Cys)-
102 based enzymes), are involved in the host-pathogen cross-talk [14] and play essential roles in
103 the physiopathology of the disease [15]. These lipolytic enzymes are indeed involved in

104 bacterial growth [4, 16, 17], virulence (reactivation and propagation) [2, 7, 18, 19], dormancy
105 [1, 6], cell wall biosynthesis [15, 16], and in lipid storage and degradation [1, 4, 5, 10].

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

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

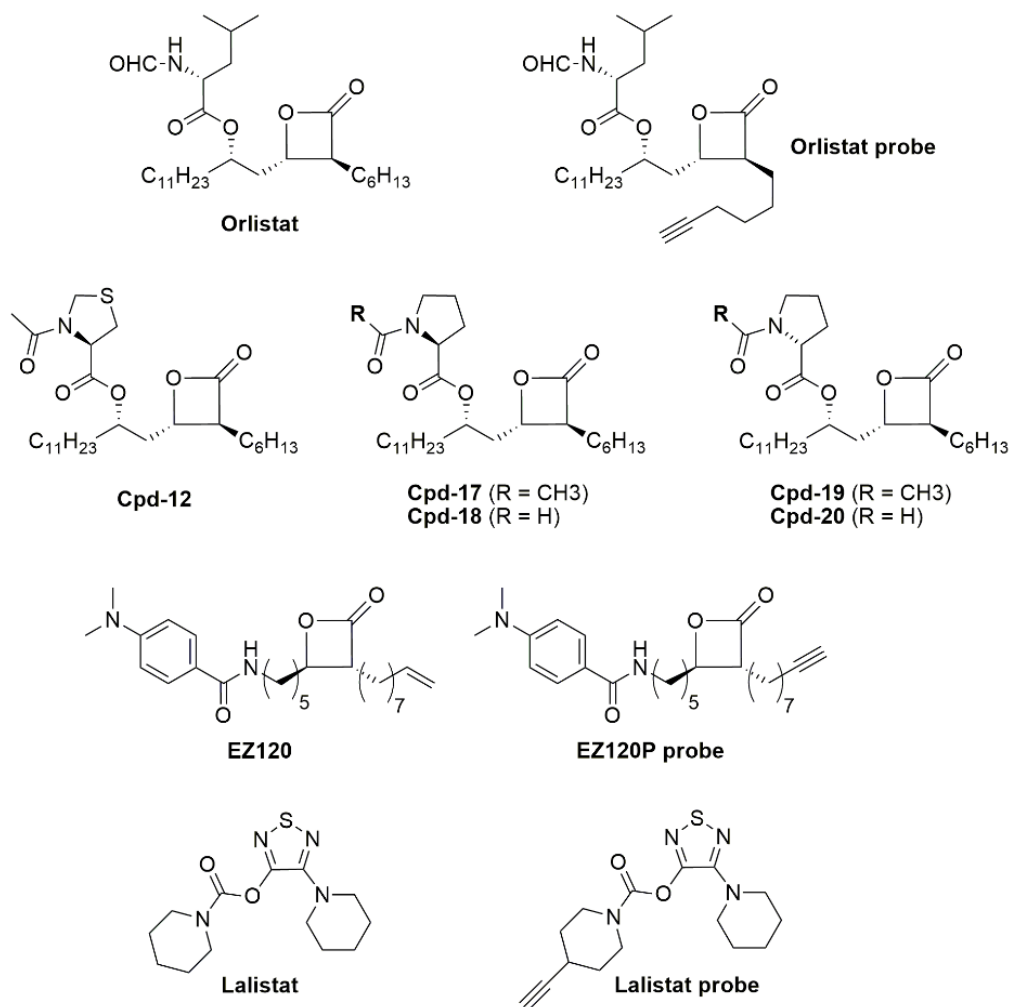
116

117 **2. Mycobacterial Lipolytic Enzyme Inhibitors are Promising Anti-** 118 **tuberculous Candidates**

119 **2.1. Orlistat, β -lactones and related compounds.** Among the potent lipolytic enzyme
120 inhibitors, β -lactones bearing the strained 2-oxetanone 4-membered ring represent an important
121 class of compounds that display potent inhibitory activity against (Ser/Cys)-based enzymes.
122 The most representative member of this family of inhibitors, is the FDA-approved drug Orlistat
123 (also known as Tetrahydrolipstatin, THL, **Scheme 1**). Orlistat is an active site-directed inhibitor
124 that forms a stoichiometric but reversible long-lived acyl-enzyme complex with lipolytic
125 enzymes as a result of nucleophilic attack of the catalytic serine (or cysteine) residue on the β -
126 lactone ring [20-22]. Since 1997, Orlistat was known to inhibit microbial lipases [23].
127 Functioning as a versatile (Ser/Cys)-hydrolase inhibitor, Orlistat was indeed found to inhibit
128 enzymes belonging to the Cutinase-like family proteins, including the essential *M. tb*

129 phospholipase/thioesterase Cut6 (Rv3802c) [24-26]; enzymes belonging to the hormone-
130 sensitive lipase (HSL) family member proteins (*i.e.*, Lip-HSL) [27, 28]; as well as the
131 mycolyltransferase Antigen 85C [29, 30]. When tested as a possible anti-mycobacterial agent,
132 Orlistat impaired *M. tb* growth with a minimum inhibitory concentration (MIC) of around 15-
133 30 μM [26, 28, 31, 32], and displayed a strong synergistic effect with vancomycin resulting in
134 a MIC drop of around 16-fold [31]. Lipids analysis confirmed that Orlistat destabilized the outer
135 membrane of the cell envelope by reducing the amount of phthiocerol dimycocerosate (PDIM)
136 content in the mycobacterial cell wall, therefore facilitating the action of vancomycin [31].
137 Similar to Orlistat, the human lysosomal acid lipase inhibitor **Lalistat** was found to not only
138 inhibit *M. tb* growth with moderated MIC values of 25-50 μM , but to also act in synergy with
139 vancomycin. Activity-based protein profiling (ABPP) approach using an alkyne-modified
140 **Lalistat** probe allowed identification of a variety of hydrolases as molecular targets, including
141 8 Lip-HSL enzymes [33].

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



153

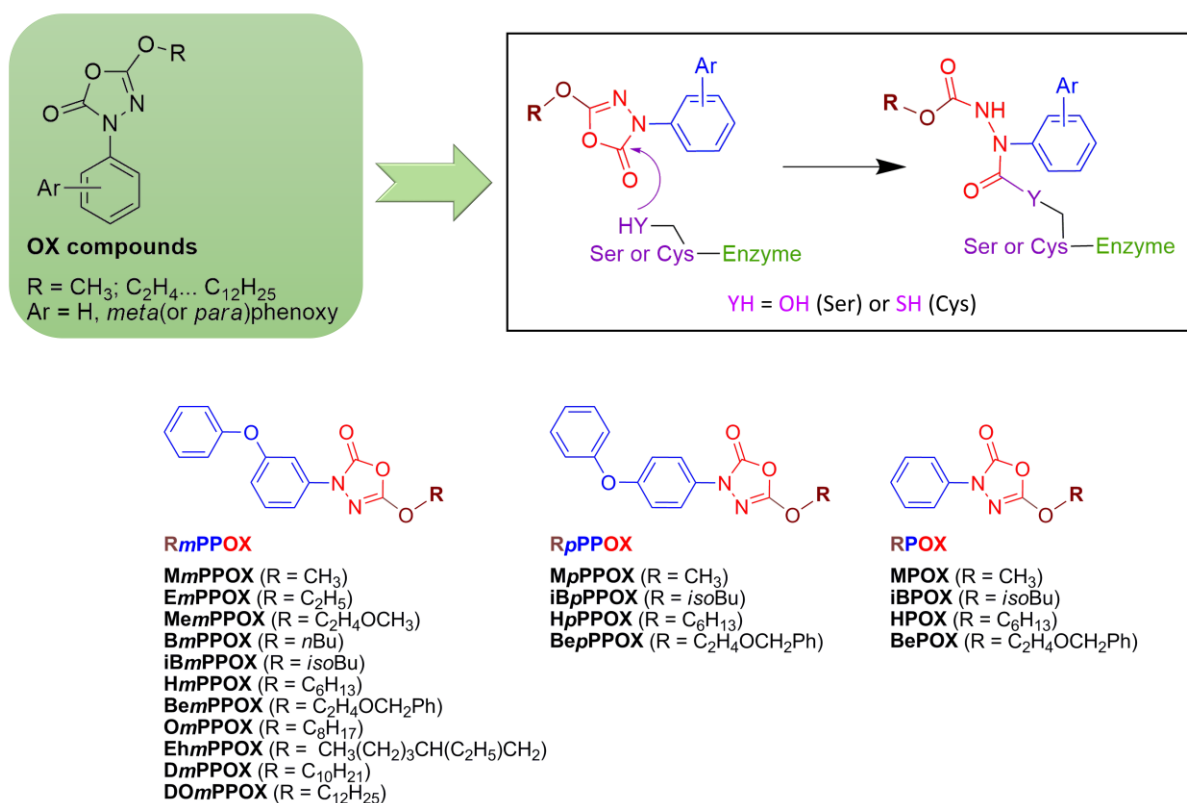
154 **Scheme 1.** Chemical structures of Orlistat & Orlistat probe [27], and related β-lactones **Cpd-**
 155 **12, Cpd-17-20** [26], **EZ120 & EZ120P** probe [34]; as well as the human lysosomal acid lipase
 156 inhibitor **Lalistat** and its corresponding alkyne-modified probe [33].

157

158 All these above-mentioned results, strongly support the therapeutic potential of lipolytic
 159 enzyme inhibitors targeting (Ser/Cys)-based hydrolases involved in the global mycobacterial
 160 lipid metabolism. Given such findings, this review will now focus on and discuss the
 161 development of two new families of promising anti-mycobacterial molecules exhibiting potent
 162 anti-lipolytic enzyme activity: Oxadiazolone (**OX**) derivatives (**Figure 1**) and the Cyclophostin
 163 & Cyclipostins (**CyC**) analogs (**Figure 3**).

164

165 **2.2. Oxadiazolone-core derivatives (OX).** 3,5-substituted 1,3,4-oxadiazol-2(3*H*)-one
 166 derivatives were first described in 1954 as active anti-TB molecules [37, 38]. Second generation
 167 compounds were further found to exhibit interesting anti-mycobacterial activity with MIC
 168 ranging from 8 to 50 μM [39, 40]. Few years later, we reported that a new substitutive **OX**
 169 derivative, the **MmPPOX** (**Figure 1**), was also able to inhibit *M. tb* growth on solid medium
 170 with moderated MIC values of 50-90 μM [28].



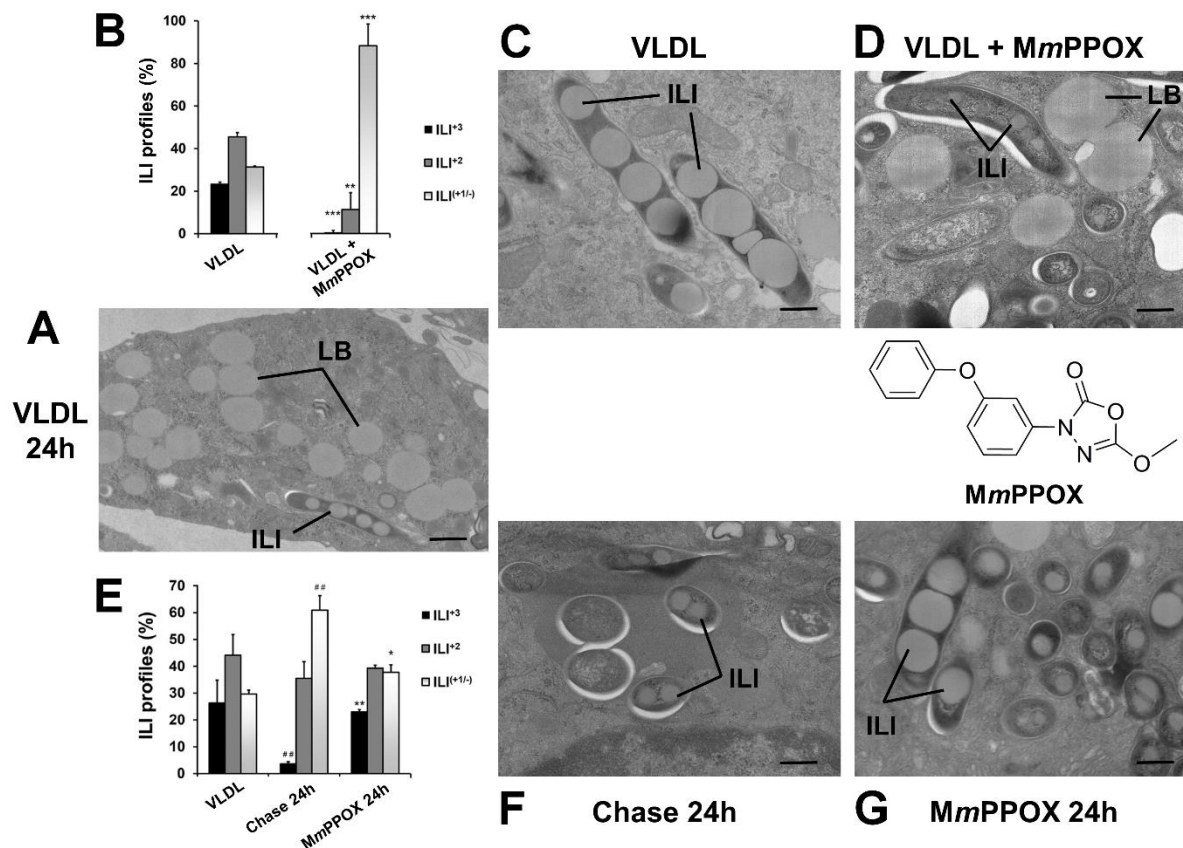
172 **Figure 1.** Chemical structure of Oxadiazolone-core derivatives (**OX**) as well as their
 173 mechanism of action on (Ser/Cys)-based enzymes in inset.

174

175 We further showed that **MmPPOX** efficiently inhibited pure recombinant *M. tb* enzymes
 176 belonging to the hormone-sensitive lipase (HSL) family member proteins (*i.e.*, Lip-HSL) [28],
 177 including LipY (Rv3097c) the major *M. tb* Lip-HSL lipase involved in TAG acquisition from
 178 the host and in ILI breakdown [2, 10, 17, 41]. The mechanism of action of **MmPPOX** involving
 179 the formation of a covalent bond with the catalytic serine residue of the enzymes (**Figure 1**

180 **inset**) thus resulting in a total abolition of their activities was also confirmed [28]. Overall, the
181 fact that **MmPPOX** alters mycobacterial growth and abolishes the activity of Lip-HSL proteins
182 suggest that HSL family member proteins fulfill essential metabolic and/or physiologic
183 functions in *M. tb* life cycle.

184 Interestingly, **MmPPOX** has also proven its ability to prevent ILI catabolism using the
185 experimental *ex vivo* model of foamy macrophages developed in the lab [5, 10, 42]. Following
186 *M. bovis* BCG infection, murine bone-marrow-derived macrophages (mBMDM) were fed with
187 Very Low-Density Lipoproteins (VLDL), allowing the differentiation of these infected BMDM
188 into foamy macrophages (FM) [5, 10, 42]. In this specific environment, the bacteria are able to
189 **i)** accumulate lipids from host LBs to form ILIs; **ii)** enter in persistence phase depicted by a
190 typical absence of septation; and **iii)** hydrolyze stored ILIs after VLDL removal from the culture
191 medium thus mimicking reactivation and propagation of the disease. When infected cells were
192 exposed to VLDL in the presence of 50 μ M **MmPPOX**, macrophages still retained the ability
193 to become foamy, but *M. bovis* BCG was unable to form ILIs (**Figure 2A**). Conversely, when
194 VLDL-treated *M. bovis* BCG-infected cells were further incubated for 24h in fresh medium,
195 nearly 90% of the ILI⁺³ profiles were retained in the presence *vs.* absence of **MmPPOX** (**Figure**
196 **2B**) [10]. These findings support **MmPPOX** as effective inhibitor of mycobacterial lipolytic
197 enzymes, including LipY (Rv3097c) [28]. involved in LB and ILI degradation. Such results
198 were further confirmed using an *in vitro* model of mycobacteria growing under carbon excess
199 and nitrogen-deprived conditions allowing ILI biosynthesis and hydrolysis. Incubation of the
200 resulting lipid-rich mycobacteria (*i.e.*, *M. smegmatis* and *M. abscessus*) with **MmPPOX** also
201 resulted in nearly complete blockage of TAG lipolysis [11].



202

203 **Figure 2.** (A) Foamy cell after 24h-exposure to VLDL showing large amounts of LB and *M.*
 204 *bovis* BCG-containing ILI. (B-D) Effect of **MmPPOX** during exposure to VLDL on host LB
 205 formation and ILI accumulation. At day 6 post-infection with *M. bovis* BCG, mBMDM were
 206 exposed for 24h to VLDL in the absence or presence of **MmPPOX**. When exposed to VLDL
 207 only (C) many mycobacterial profiles are ILI⁺³; whereas exposure to VLDL+**MmPPOX** (D)
 208 results in small ILI with no ILI⁺³ profiles. (E-G) Effect of **MmPPOX** during TAG consumption
 209 within ILIs. At day 6 post-infection with *M. bovis* BCG, mBMDM were exposed to VLDL and
 210 re-incubated in VLDL-free culture medium alone or with **MmPPOX** for 24h. After VLDL
 211 treatment followed by a 24-h chase in medium devoid of inhibitor, (F) cells contain few ILI⁺³
 212 profiles. Conversely, in the presence of **MmPPOX** (G) cells still contain ILI⁺³ profiles. (B, E)
 213 Both bar graphs represent the quantitative evaluation of the percentage of each category of ILI
 214 profiles \pm SD (*, $p < 0.05$; **,#, $p < 0.01$; ***, $p < 0.001$). Bars in panels A, 1 μ m; C-D-E-F, 0.5
 215 μ m. Adapted from [10, 42].

216

217 Based on these data, a new series of 18 lipophilic **OX** derivatives were designed and
 218 synthesized (Figure 1) [43]. A specific nomenclature was set up for these **OXs** noted **Rm**(or

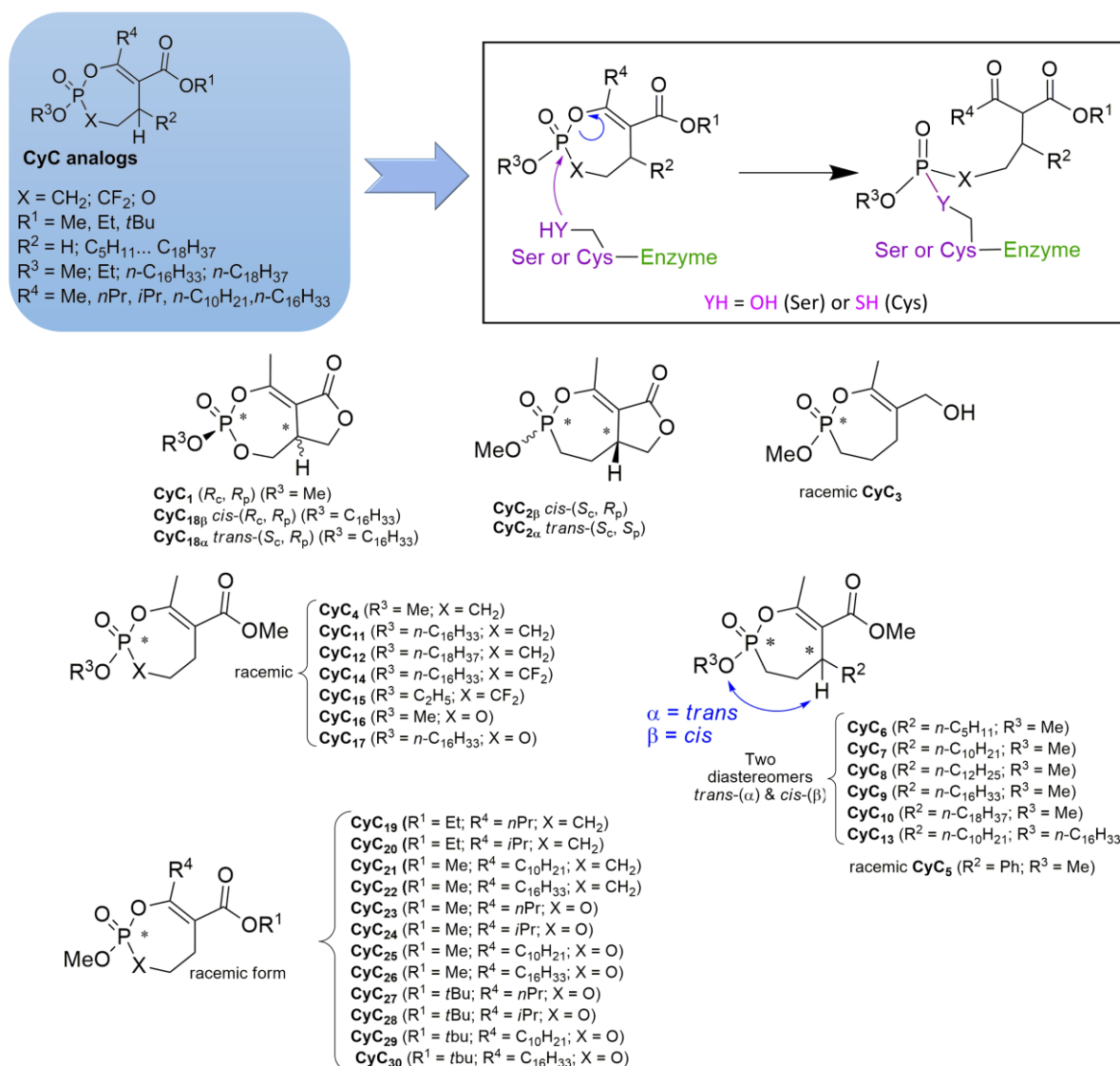
219 **p)PPOX**; where **m(or p)PP** (= R¹) represents the *meta* (or *para*)-PhenoxyPhenyl group, when
220 present; **OX** the oxadiazol-2(3*H*)-one core; and **R** the alkyl chain (**Figure 1**) [43]. Data reported
221 in **Table 1** point out to the potent inhibitory activity of this new series of **OXs** towards the
222 TAG-lipase LipY. Their inhibitory power, defined here as the inhibitor molar excess leading to
223 50% lipase inhibition (x_{150}), ranges from 0.59 to 0.93 (**Table 1**) implying that the best **OXs** react
224 in close stoichiometry with this lipase, being thus more potent than **MmPPOX** ($x_{150} = 2.4$), but
225 also than Orlistat ($x_{150} = 7.1$) used as reference inhibitor [28, 44].

226

227 **2.3. The Cyclipostins & Cyclophostin analogs (CyC).** In parallel, we investigated the
228 inhibitory properties of new monocyclic analogs of Cyclophostin and Cyclipostins (**CyC**)
229 (**Figure 3**), derived from phosphorus-containing natural products isolated from fermentation
230 broths of *Streptomyces sp* [45, 46], on pure mycobacterial lipases and various mycobacterial
231 strains.

232 Members of the Cyclipostins family (**Figure 3** – X=O, R³=C₁₅-C₁₈) possess a core structure
233 similar to that of Cyclophostin (**Figure 3** – X=O, R³=CH₃) but are phosphate esters of long
234 chain lipophilic alcohols. The Cyclipostins were described to inhibit the growth of various
235 mycobacteria (including *M. smegmatis*, *M. phlei*, *Nocardia abscessus*, and *Corynebacterium*
236 *diphtheriae*) with MIC values similar to that of rifampicin and penicillin G [47]. These natural
237 products were initially described as potent inhibitors of either human acetylcholinesterase
238 (AChE) (*i.e.*, Cyclophostin) [45, 48] or human and rat HSL (*i.e.*, Cyclipostins) [46, 49].

239



240

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

244

245 From these findings, we first reported the total synthesis of natural Cyclophostin **CyC₁**, its
 246 phosphonate analogues **CyC_{2 α,β}** [48, 49], and related monocyclic enol-phosphonates
 247 **CyC_{3-10,19-22}** [51-53] and phosphates **CyC₂₃₋₃₀** [53]; as well as Cyclophostin P **CyC_{18 β}** , its *trans*-
 248 (α) diastereoisomer **CyC_{18 α}** [49], and the corresponding monocyclic enol-phosphonates
 249 **CyC₁₁₋₁₃** [52, 54], difluorophosphonates **CyC₁₄₋₁₅** and phosphates **CyC₁₆₋₁₇** [55, 56] (**Figure 3**).
 250 Of particular importance, diastereomeric *cis*- and *trans*-monocyclic enolphosphonates **CyC₆₋₁₀**

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

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

269 Cutinase displayed a high diastereoselectivity for the (*S_p*) configuration with a
270 Stereoselectivity Index (SI) derived from α_{150} values of 94.9% (**Table 2**) when using **CyC₇**-(*S_c*)
271 inhibitors, whereas no obvious stereopreference at the phosphorus center was observed with the
272 **CyC₇**-(*R_c*) inhibitors. On the contrary, Rv0183 strongly discriminated the (*S_p*) configuration (SI
273 = 72-81.4%) independently of the absolute (*R_c*) or (*S_c*) configuration on the asymmetric C-5
274 carbon atom; and thus, exhibited the classical enantiopreference of lipolytic enzymes [60]. The
275 influence of chirality was much more pronounced in the case of LipY. Significantly, this lipase

276 discriminated only the unusual diastereoisomeric configuration (R_c, R_p), which led to the most
277 potent **CyC** $_{7\beta}$ -(R_c, R_p) inhibitor (SI > 80.7% - **Table 2**). Modulation of the lipophilicity at the C-
278 5 carbon atom combined with this unusual high enantioselectivity displayed by LipY for the
279 (R_p) and (R_c) associated absolute configurations, should open new prospects in the design of
280 specific inhibitors of this mycobacterial lipase [59]. Overall, these results raised significant
281 achievements in the understanding of the stereoselective relationships between pure non-
282 racemic compounds and their inhibitory activity towards several microbial lipases of interest.

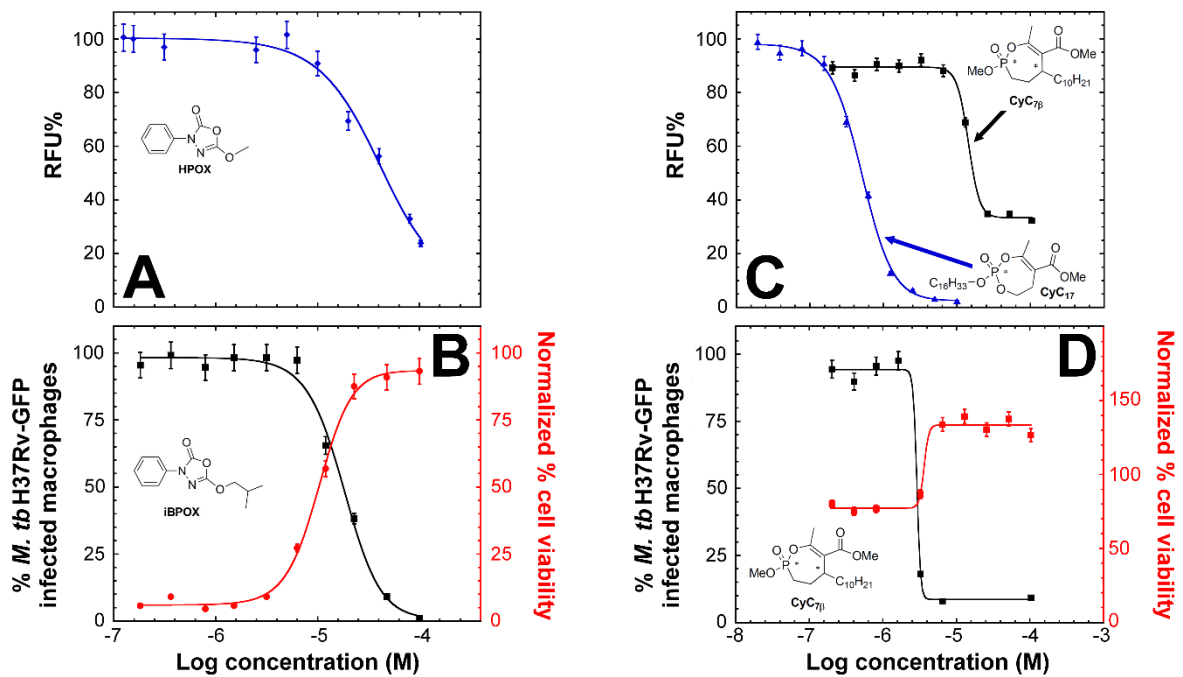
283 To summarize, these **CyC** derivatives have not only proved to be powerful bacterial
284 (Ser/Cys)-based enzymes inhibitors [52, 59], but above all, they had **lost their inhibitory**
285 **activity towards** the mammalian enzymes initially targeted by the natural parent molecules
286 [52, 55, 56].

287

288 **2.4. The OX and CyC derivatives are novel promising multi-target inhibitors of *M. tb***
289 **growth.** The set of 19 **OX** derivatives and 26 **CyC** analogues were further evaluated for their
290 anti-mycobacterial activity on a *M. tb* H37Rv-GFP strain using a high-content screening assay
291 based on the fluorescence measurement of GFP-expressing bacteria [54, 61]. *In vitro* growth of
292 *M. tb* H37Rv-GFP was monitored by directly measuring GFP fluorescence after 5 days at 37 °C
293 in presence of increasing concentrations of candidate inhibitors. Intracellular growth of *M. tb*
294 H37Rv-GFP was also assessed following a 5-day exposure of infected Raw264.7 murine
295 macrophage cell lines to the different compounds. In the latter case, the percentage of infected
296 cells and the number of living host cells allowed determining the values of both MIC₅₀
297 (compound concentration leading to 50% growth inhibition) and CC₅₀ (compound
298 concentration leading to 50% host cell toxicity) [62, 63].

299 Among all molecules tested, **14** potential candidates (**6 OXs** and **8 CyCs** – **Table 3**)
300 exhibited very promising anti-tubercular activities with moderate (15-50 μ M) to good (3-4 μ M)

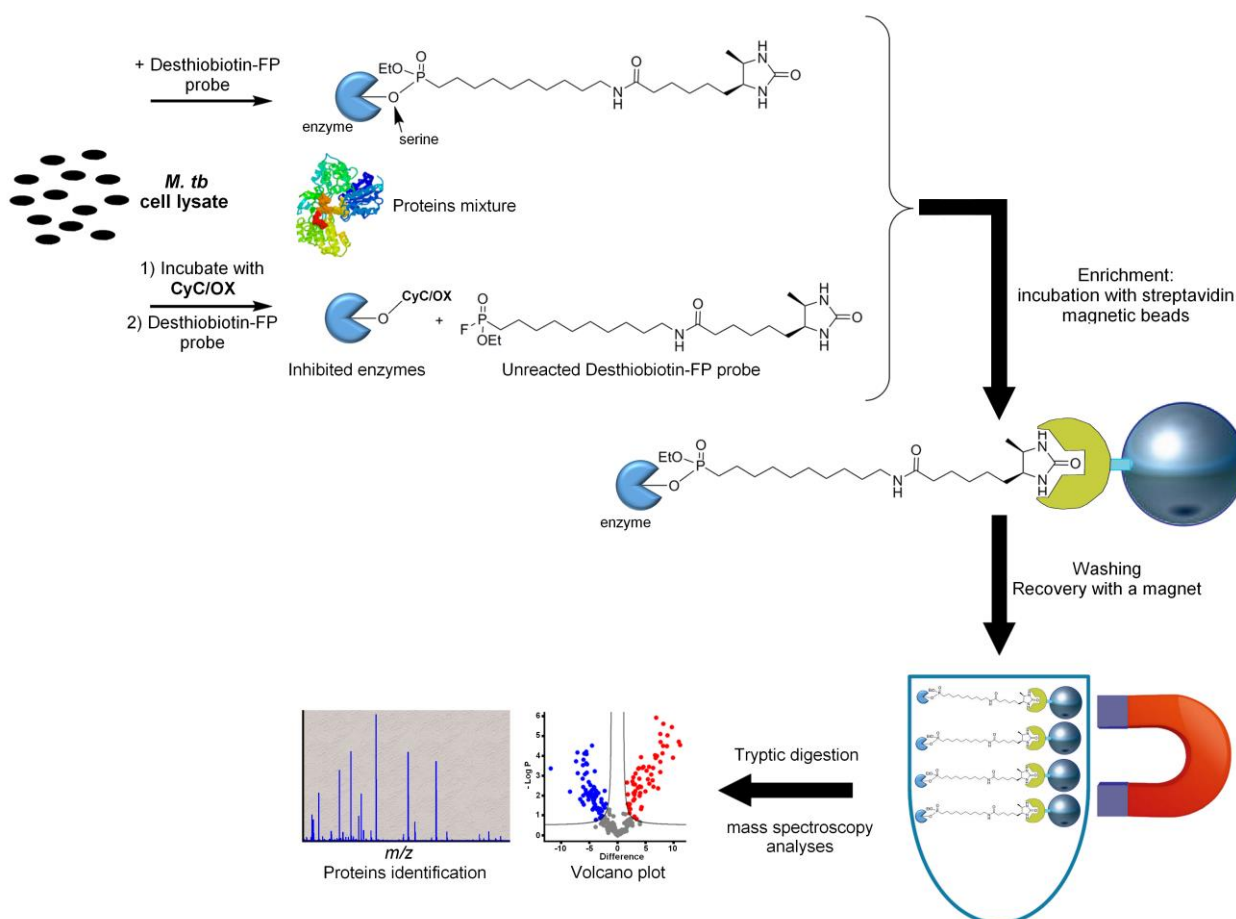
301 and to excellent (500 nM for **CyC17**) MIC₅₀ values. Of great importance, beside their inhibitory
 302 activity against bacterial growth, both set of compounds exhibited very low toxicity towards
 303 host macrophages (CC₅₀ > 100 μM). Data show that both **OXs** and **CyCs** can be divided into
 304 two different classes based on their antibacterial activity (**Figure 4** and **Table 3**).
 305



306
 307 **Figure 4.** (A-C) Activity of **HPOX**, **CyC7β** & **CyC17** against GFP-labelled *M. tb* H37Rv
 308 replicating in culture medium expressed as normalized relative fluorescence units (RFU%). (**B-**
 309 **D**) Activity of **iBPOX** and **CyC7β** against *M. tb* H37Rv-GFP replicating inside Raw264.7
 310 macrophages. Adapted from [54, 61].
 311

312 First, 9 out of 14 compounds exhibited higher activity against intracellular bacteria than
 313 against extracellular ones: *i.e.*, **iBpPPOX**, **iBPOX**, **HpPPOX**, **BepPPOX**, **CyC6β**, **CyC7α,β** and
 314 **CyC8α,β**. Such feature supposes that the intracellular mode of action of these inhibitors may
 315 differ from that of those acting on extracellularly-replicating bacilli. It can therefore be
 316 hypothesized that the vulnerability of the corresponding bacterial target(s) of these 4 **OXs** and
 317 5 **CyCs** is higher and/or more critical during the intracellular life of *M. tb* than for

318 extracellularly-growing bacteria. Alternatively, a specific stringent response of the macrophage
 319 caused by the compounds and leading to bacterial death can, however, not be excluded. The
 320 second type of compounds are active against extracellular bacteria and display only poor or
 321 even no activity against intracellular *M. tb*: **HPOX**, **BePOX**, **CyC₁₇**, **CyC_{18 α,β}** ; a property
 322 already reported for 1,2,4-Oxadiazole EthR inhibitors [63]. The observed differences in the
 323 behavior of studied compounds, particularly the high ratio between the intracellular vs.
 324 extracellular antibacterial effects, may translate in the prevention of entry of actively replicating
 325 bacilli into the persistence phase and/or prevent reactivation of dormant bacilli within FM.



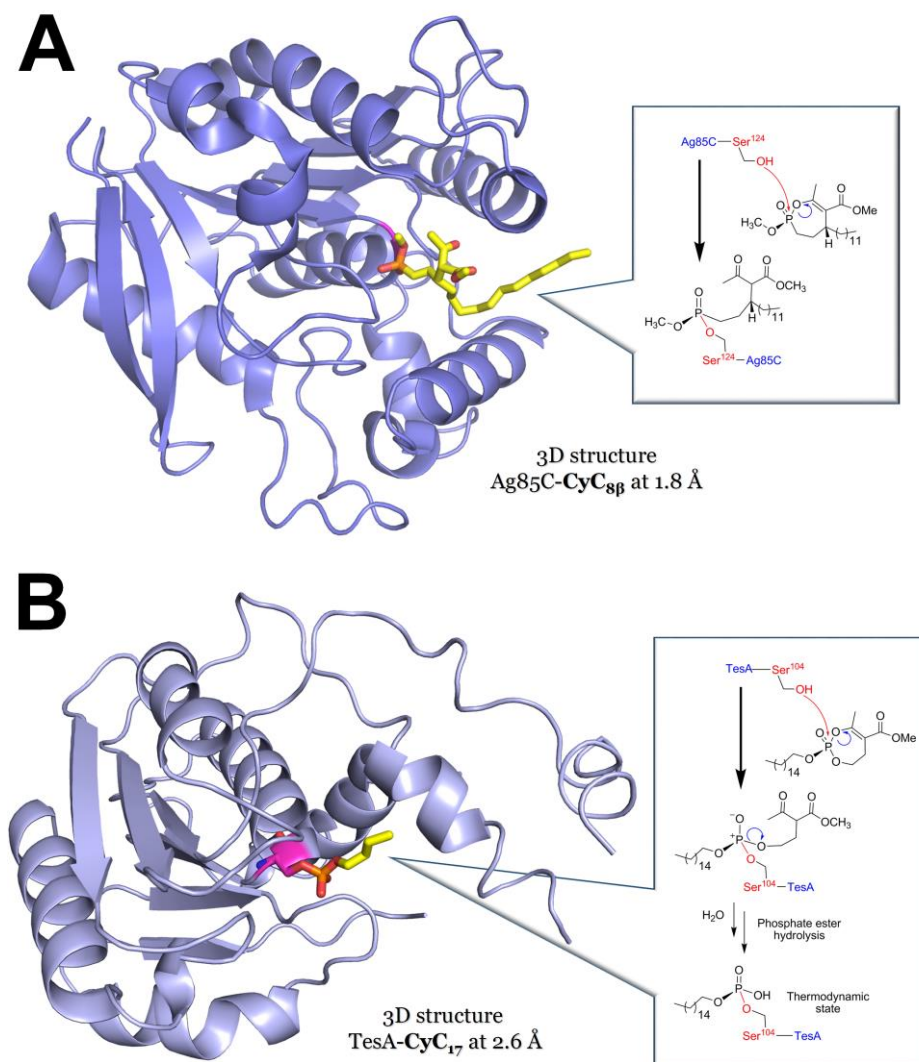
326
 327 **Figure 5.** General scheme for the identification of the target enzymes of our inhibitors using
 328 ABPP approach.
 329

330 Based on these aforementioned results, and given the strong affinity of our **OXs** and **CyCs**
 331 for *M. tb* lipolytic enzymes, these inhibitors might target and impair the activity of various

332 (Ser/Cys)-based enzymes involved in several processes of *M. tb* life cycle, thus resulting in
333 bacterial death without any (or only very low) cytotoxicity towards host cells. Accordingly,
334 target(s) identification experiments were next conducted by applying ABPP approach [27, 33,
335 34, 64-67] (**Figure 5**). Here, **HPOX** and **CyC₁₇**, which selectively inhibit *M. tb* growth in
336 culture broth medium only, were selected for such experiments [54, 61]. *M. tb* total cell lysate
337 was incubated with each inhibitor and then subjected to competitive probe labelling/enrichment
338 assays using an activity-based probe (ABP), *i.e.*, the ActivX Desthiobiotin-FP widely exploited
339 to screen for reversible and irreversible inhibitors of drug targets [54, 61, 64, 68]. This resulted
340 in the identification of a panel of 18 and 23 distinct proteins for the **HPOX**- and
341 **CyC₁₇**-pretreated lysate, respectively [54, 61]. Remarkably, these 41 identified proteins were
342 **all** (Ser/Cys)-based enzymes, most of them participating in *M. tb* lipid metabolism and in cell
343 wall biosynthesis. Among them, several are annotated as essential enzymes for the *in vitro*
344 growth of *M. tb* and/or its survival of inside macrophages [69-71]. These included the antigen
345 85 complex, Ag85A (Rv3804c), Ag85B (Rv1886c) and Ag85C (Rv0129c) [35]; the
346 thioesterase TesA (Rv2928) [72]; the carboxylesterase CaeA (Rv2224c) [73]; the β -ketoacyl
347 synthase KasA (Rv2245) [74]; a bifunctional thioesterase-phospholipase enzyme LipG
348 (Rv0646c) [75], and the hydrolase HsaD (Rv3569c) [76, 77].

349 Through biochemical and structural studies, the antigen 85 complex (*i.e.*, Ag85A/B/C) was
350 validated as an effective target of **CyC₁₇**, but also of **CyC_{7 β}** and **CyC_{8 β}** [78]. These three **CyCs**
351 are indeed able to block the synthesis of trehalose dimycolate (TDM) as well as the
352 mycolylation of arabinogalactan in *M. tb* which results in the disruption of the cell envelope
353 integrity [78]. Among the targets of both the **CyCs** and **OxS**, TesA, a putative thioesterase
354 involved in the synthesis of phthiocerol dimycocerosates (PDIM) and phenolic glycolipids
355 (PGL), has been identified. These two lipids (PDIM and PGL), non-covalently linked to the
356 outer cell wall of pathogenic mycobacteria such as *M. tb*, are important virulence factors. In

357 this context, TesA was validated as an effective pharmacological target of **CyC₁₇** ($x_{150} = 12.4$)
358 [79] and **HPOX** ($x_{150} = 0.59$) [61].
359



360
361 **Figure 6.** Crystal structures of **(A)** Ag85C in complex with **CyC_{8β}** (PDB ID: 5OCJ; 1.8Å
362 resolution) [78] and **(B)** TesA in complex with **CyC₁₇** (PDB ID: 6FVJ; 2.6Å resolution) [79].
363 The mechanism of action of the phosphonate (**CyC_{8β}**) and the phosphate (**CyC₁₇**) analog based
364 on mass spectrometry analyses are illustrated in inset. Each inhibitor is in stick representation,
365 and the catalytic Serine residue is colored in magenta. Structures were redrawn as ribbon
366 representations from their respective modified PDB IDs, with PyMOL Molecular Graphics
367 System (Schrödinger, LLC).
368

369 From a molecular point of view, the obtained crystal structures of Ag85C inhibited by **CyC_{8β}**
370 (**Figure 6A**) [78] and TesA in complex with **CyC₁₇** (**Figure 6B**) [79], together with biochemical
371 and mass spectrometry experiments, have clearly stated that the inhibition of these enzymes
372 results from the phosphorylation of their respective catalytic Serine residue (**Figure 6**).
373 Interestingly, following **CyC₁₇** phosphorylation of the TesA-Ser104 or Ag85C-Ser124, further
374 rearrangement of the inner structure of the covalently bound inhibitor occurs resulting in the
375 loss of the β-ketoester moiety (**Figure 6B**). Such chemical modification can therefore be
376 considered as a signature of the **CyC₁₇** reactivity with Ser- and Cys-based enzymes [78, 79].
377 Finally, the bifunctional thioesterase-phospholipase enzyme LipG (Rv0646c), involved in the
378 modification and remodeling of the mycobacterial envelope and described as essential for the
379 survival and intracellular persistence of *M. tb* [75], has been biochemically characterized [80].
380 Inhibition kinetics indeed demonstrated that LipG was able to react with **CyC_{7α}** ($x_{150} = 5.0$) and
381 in near stoichiometry with **CyC₁₇** ($x_{150} = 0.98$), but not with **CyC_{7β}** and **CyC_{8α,β}** therefore
382 exhibiting a certain selectivity of action.

383

384 **3. The CyC And Ox Analogs are New Compounds for the Treatment of** 385 **Mycobacterial-Related Infections**

386 In view of the results obtained on *M. tb*, the specificity of our **CyC** compounds against various
387 bacteria was further investigated. Susceptibility testing conducted on 7 bacterial strains, *P.*
388 *aeruginosa*, *E. coli*, *B. subtilis*, *M. abscessus*, *M. marinum*, and *M. bovis* BCG against the 26
389 available **CyC₁₋₁₈** surprisingly showed that these compounds block specifically the growth of
390 the mycobacterial species without affecting the growth of Gram-positive and Gram-negative
391 bacteria [81]. More particularly, **CyC₁₇** exhibited MIC₅₀ values of 12.7 μM and 0.37 μM
392 towards the smooth and rough morphotype of *M. abscessus*, respectively; the latter value being

393 20- to 37-fold lower than that of reference antibiotics, amikacin (7.4 μM), imipenem (12 μM)
394 or cefoxitin (27 μM) [81].

395 *M. abscessus*, a rapidly growing mycobacterium, is an opportunistic pathogen responsible
396 for chronic lung infection in patients with respiratory diseases such as cystic fibrosis (CF) [82-
397 84]. *M. abscessus* exists in two variants, a smooth (S) and a rough (R) one, the latter being
398 considered the most virulent form in humans [85, 86]. Nicknamed the "antibiotics nightmare",
399 this nontuberculous mycobacterium (NTM) is one of the most drug-resistant mycobacteria for
400 which most standardized chemotherapeutic regimens are poorly effective, often leading to high
401 treatment failures and death [86-88].

402 When tested on 37 bacterial strains isolated from CF patients, including 26 clinical isolates
403 belonging to the *M. chelonae-abscessus* complex, the best candidate (**CyC₁₇**) showed high
404 selectivity for mycobacteria only, and MICs (<2-40 $\mu\text{g/mL}$) comparable with those of clinically
405 used antibiotics [81]. To refine the structure-activity relationships of this family of compounds,
406 12 new **CyC** analogs (**CyC₁₉₋₃₀** – **Figure 3**) were synthesized by varying the R⁴ chain on the
407 enolphosphorus ring [53]. Such structural modification was also guided by the isolation of the
408 antimalarial Salinipostin A, a natural product produced by a *Salinispora sp.* bacterium, which
409 is a Cyclipostin-like compound with variations in the alkyl enol substituent [89, 90]. Among
410 these 12 new **CyCs**, **CyC₂₅** and **CyC₂₆** exhibited very promising MIC₅₀ values against *M.*
411 *abscessus* R (**Table 4**) [53]. Remarkably, these latter three best inhibitors of *M. abscessus*
412 growth were all phosphate esters bearing a long lipophilic C10/C16 alkyl chain either at the R³
413 or R⁴ position (**Figure 3**). We also demonstrated that the absence of surface exposed
414 glycopeptidolipids (GPL) in the R variant was responsible for the higher susceptibility (up to
415 34 \times) to the **CyCs** as compared to the S strain. More recently, by combining fast quantitative
416 imaging (QI) atomic force microscopy (AFM) with hydrophobic tips, Viljoen *et al.*
417 demonstrated that GPL modulated the nanoscale surface hydrophobicity of *M. abscessus* [91].

418 While S morphotype displayed unusual variations of nanoscale hydrophobic properties, the R
419 variant showed homogeneous surface hydrophobicity conferred by surface exposed mycolic
420 acids when GPL were lacking [91]. Given together, the antibacterial activity of the **CyCs**
421 against the R and S variants, respectively, can thus be directly correlated with the GPL-
422 dependent variation in the nanoscale distribution of *M. abscessus* hydrophobicity.

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

429 *M. abscessus*, like *M. tb*, is also able to survive and replicate inside macrophages [93, 94].
430 Altogether, high intrinsic resistance and intracellular forms of *M. abscessus* are thus mostly
431 contributing to treatment failures since only few antimicrobials can penetrate the cell membrane
432 of phagocytes [95]. In this context, the 38 **CyCs** and 19 **OXs** were tested for their capability to
433 inhibit the intramacrophagic growth of *M. abscessus* inside Raw264.7 macrophages. Only
434 **CyC_{7α,β}** and **CyC_{8α,β}** were found active against intracellularly replicating *M. abscessus* (**Table**
435 **4**). With MIC_{50Raw} values of 7.9 and 2.0 μM, respectively, **CyC_{8α}** and **CyC_{8β}** were 3.6- and 14-
436 fold more potent against intracellular *M. abscessus* than imipenem (28.3 μM), used as reference.
437 It is noteworthy that such MIC_{50Raw} are of the same order of magnitude than those previously
438 obtained with the same **CyC_{8α,β}** against intracellular *M. tb* (4-12 μM – **Table 3**). Regarding the
439 **OXs**, only **MPOX** and **MpPPOX** (inactive against extracellular bacterial growth) and
440 **iBpPPOX** were able to block intracellularly-growing *M. abscessus*. As previously observed for
441 *M. tb* [61], **iBpPPOX** impairs both extracellular (MIC₅₀ = 33.0 μM) and intracellular (~59%

442 bacterial clearance at 30 μ M) replicating bacteria with similar potency/efficiency to that of
443 imipenem (**Table 4**).

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

452 The fact that Ag85A/B/C proteins have been identified and validated as effective targets of
453 both the **CyC** and **OX** compounds in *M. tb* [61, 78] and *M. abscessus* [53, 92], but also of
454 Orlistat and related analogs [25, 30, 34], suggest that these mycolyltransferases represent a
455 common primary target of such inhibitors, regardless of the mycobacterial strain tested. Indeed,
456 as Ag85A/B/C proteins are secreted enzymes, they may be easily accessible to the **CyCs** and
457 **OXs**. Due to their importance in mycolic acid metabolism, the Ag85 enzymes have often been
458 proposed as attractive targets for future chemotherapeutic developments against mycobacteria
459 [35, 78]. Overall, our studies provide compelling evidence that both the **CyC** analogs and the
460 **OX** compounds inhibit the activity of the Ag85 complex *in vitro* and in mycobacteria [78, 92],
461 therefore opening the way to a new strategy to fight against pathogenic mycobacteria.

462

463 **4. Conclusion and future perspectives**

464 Collectively all our results strengthen the fact that the **OX** and the **CyC** inhibitors are not only
465 non-toxic for mammalian cells, but above all represent a novel class of selective and efficient
466 multi-target inhibitors [98] leading to the arrest of *M. tb* and *M. abscessus*, two major infectious

467 pathogens. By impairing simultaneously, the activity of various (Ser/Cys)-base enzymes
468 participating in important physiological processes related to the whole bacterial lipid
469 metabolism, including the Ag85 complex proteins, both the **CyCs** and the **OxS** will inhibit
470 extracellular and/or intracellular *M. tb* and *M. abscessus* growth, therefore leading to bacterial
471 death. Consequently, the probability that a strain would develop resistance to such inhibitors
472 would be very low, because resistant mutants would require the simultaneous acquisition of
473 several mutations in the same bacterial genome; thus, making it difficult or impossible for the
474 bacteria to adapt themselves and survive.

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

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

492

493 **Declaration of competing interest**

494 The authors report no declarations of interest.

495

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508

509

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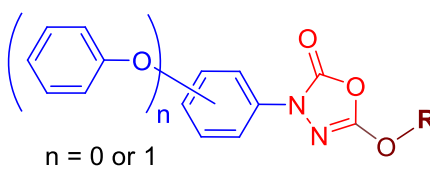


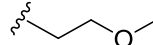
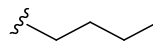
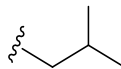
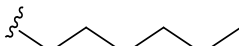
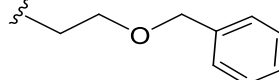
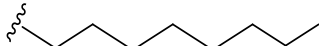
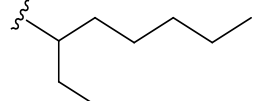
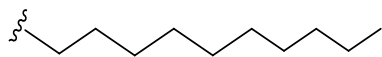
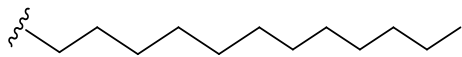
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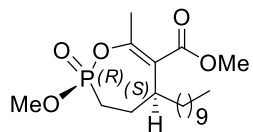
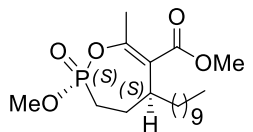
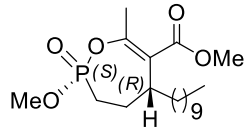
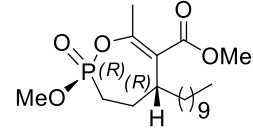
Table 1. Inhibition of the TAG-lipase LipY from *M. tb* after a 30-min incubation period with each **OX** compound ^a

Compounds	 n = 0 or 1	LipY		
		%inhibition ^b		x_{150} ^c
		$x_I = 4$	$x_I = 20$	
Orlistat		26.8±1.5	87.6±4.8	7.1
MmPPOX		63.6±4.6	85.9±4.9	2.4
MpPPOX		60.8±2.1	79.4±3.3	0.93
MPOX		34.4±2.4	81.8±3.3	10.9
EmPPOX		21.7±1.4	53.8±3.3	6.5
MemPPOX		92.4±0.30	93.7±1.3	0.69
BmPPOX		15.6±0.50	36.7±1.8	>40
iBmPPOX		37.4±2.4	49.4±1.5	3.9
iBpPPOX		33.8±1.5	41.6±0.40	>40
iBPOX		31.6±1.0	47.9±2.1	9.2
HmPPOX		39.9±1.2	76.5±1.9	3.3
HpPPOX		38.7±1.7	77.2±2.7	2.9
HPOX		35.0±0.27	38.8±0.11	>40
BemPPOX		88.0±0.70	90.2±0.91	0.72
BepPPOX		97.1±3.9	98.4±2.2	0.59
BePOX		89.0±1.9	92.2±0.20	0.61
OmPPOX		83.2±5.8	86.6±2.2	0.71
EhmPPOX		89.6±1.1	94.8±2.7	0.73
DmPPOX		91.5±2.8	94.2±3.1	0.67
DomPPOX		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].

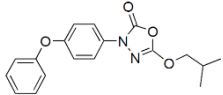
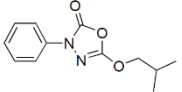
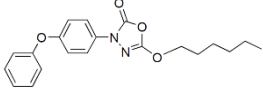
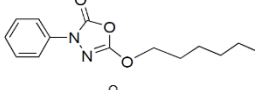
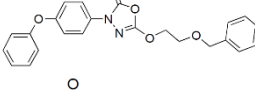
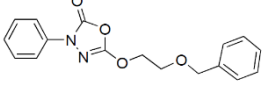
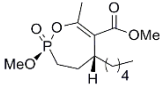
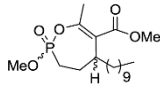
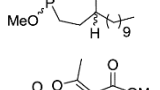
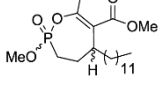
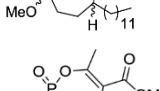
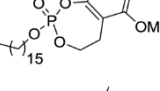
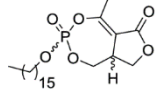
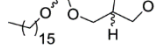
^b Inhibition data (% of initial enzyme activity), at an inhibitor molar excess (x_I) of 4 or 20 related to 1 mole of enzyme. Values are means of at least three independent assays. ^c x_{150} : inhibitor molar excess leading to 50% lipase inhibition [28, 44].

Table 2. Stereoselective inhibition of Cutinase, Rv0183, and LipY by enantiopure monocyclic enolphosphonates **CyC7**^a

x150								
	 CyC7_α-(S_c,R_p)		 CyC7_β-(S_c,S_p)		 CyC7_α-(R_c,S_p)		 CyC7_β-(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

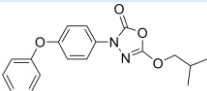
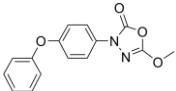
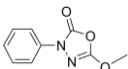
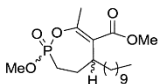
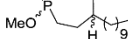
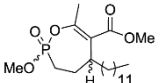
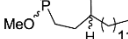
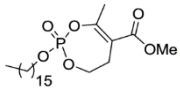
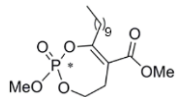
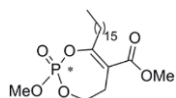
^a x_{150} , inhibitor molar excess leading to 50% lipase inhibition. SI, Stereoselectivity Index; $SI = 100 \times [(x_{150})_{\beta \text{ isomer}} - (x_{150})_{\alpha \text{ isomer}}] / [(x_{150})_{\beta \text{ isomer}} + (x_{150})_{\alpha \text{ isomer}}]$. Adapted from [59].

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

Compounds	<i>M. tb</i> 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				
iBpPPOX		32.0 ±0.89	8.5 ±0.30	>100
iBPOX		>50	17.1 ±1.10	>100
HpPPOX		>50	9.5 ±0.49	>100
HPOX		44.6 ±2.50	No effect	>100
BePPOX		>50	3.5 ±0.28	>100
BePOX		30.8 ±2.40	No effect	>100
CyC analogs				
CyC_{6β}		NE	12.6 ±0.90	>100
CyC_{7α}		92.6 ±6.5	4.5 ±0.39	>100
CyC_{7β}		16.6 ±0.78	3.1 ±0.11	>100
CyC_{8α}		40.4 ±2.5	4.0 ±0.30	>100
CyC_{8β}		>100	11.7 ±0.26	>20
CyC₁₇		0.50 ±0.02	No effect	>100
CyC_{18α}		24.4 ±2.14	No effect	>100
CyC_{18β}		1.7 ±0.03	No effect	>100

^a MIC₅₀, compound concentration leading to 50% bacterial growth inhibition in culture medium. ^b CC₅₀, 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	<i>M. abscessus</i> 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	<i>Not determined</i>	
Imipenem (IMP)	11.3 ±0.50	29.9 ±1.6	28.3 ±4.7	
OX derivatives				
iBpPPOX		33.0 ±2.0	53.2 ±1.8	< 30μM
MpPPOX		88.2 ±7.3	>200	75.2 ±5.1
MPOX		>200	>200	36.5 ±2.6
CyC analogs				
CyC_{7α}		>100	>100	29.3 ±2.9
CyC_{7β}		>100	>100	65.3 ±6.2
CyC_{8α}		>100	>100	7.9 ±0.23
CyC_{8β}		>100	>100	2.0 ±0.91
CyC₁₇		12.7 ±0.26	0.37 ±0.01	<i>No effect</i>
CyC₂₅		85.0 ±0.86	13.9 ±0.56	<i>No effect</i>
CyC₂₆		60.0 ±2.50	6.9 ±0.16	<i>No effect</i>

^a MIC₅₀, 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].