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

## 2 **Discovery**

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#### 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 nontuberculous mycobacteria (NTM) responsible for cutaneous and pulmonary infections, 24 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.

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

#### 45 Abbreviations:

- 46 ABP: activity-based probe
- 47 ABPP: Activity-based protein profiling
- 48 AChE: acetylcholinesterase
- 49 CC<sub>50</sub>: 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 IC<sub>50</sub>: compound concentration leading to 50% enzyme activity inhibition
- 56 ILI: intracytoplasmic lipid inclusions
- 57 ILI<sup>+3</sup>: 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 MIC<sub>50</sub>: compound concentration leading to 50% mycobacterial growth inhibition
- 63 MIC<sub>50Raw</sub>: 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  $x_{I}$ : 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 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].

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 drugresistant and clinically relevant mycobacterial species.

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

119 2.1. Orlistat,  $\beta$ -lactones and related compounds. Among the potent lipolytic enzyme 120 inhibitors,  $\beta$ -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  $\beta$ -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<sub>50</sub> values of 0.2-0.8  $\mu$ M toward Cut6, compared with Orlistat (IC<sub>50</sub> = 3.8  $\mu$ M) [26]. More 148 recently, the  $\beta$ -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].



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Scheme 1. Chemical structures of Orlistat & Orlistat probe [27], and related β-lactones Cpd12, Cpd-17-20 [26], EZ120 & EZ120P probe [34]; as well as the human lysosomal acid lipase
inhibitor Lalistat and its corresponding alkyne-modified probe [33].

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

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].



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

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171

We further showed that **MmPPOX** efficiently inhibited pure recombinant *M. tb* enzymes belonging to the hormone-sensitive lipase (HSL) family member proteins (*i.e.*, Lip-HSL) [28], including LipY (Rv3097c) the major *M. tb* Lip-HSL lipase involved in TAG acquisition from the host and in ILI breakdown [2, 10, 17, 41]. The mechanism of action of **MmPPOX** involving the formation of a covalent bond with the catalytic serine residue of the enzymes (**Figure 1**  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 µM 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, nearly 90% of the ILI<sup>+3</sup> profiles were retained in the presence vs. absence of **MmPPOX** (Figure 195 196 **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 exposed for 24h to VLDL in the absence or presence of MmPPOX. When exposed to VLDL 206 only (C) many mycobacterial profiles are  $ILI^{+3}$ ; whereas exposure to VLDL+MmPPOX (D) 207 results in small ILI with no ILI<sup>+3</sup> profiles. (E-G) Effect of MmPPOX during TAG consumption 208 209 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  $ILI^{+3}$ profiles. Conversely, in the presence of **MmPPOX** (G) cells still contain  $ILI^{+3}$  profiles. (B, E) 212 213 Both bar graphs represent the quantitative evaluation of the percentage of each category of ILI profiles ±SD (\*, p<0.05; \*\*,<sup>##</sup>, p<0.01; \*\*\*, p<0.001). Bars in panels A, 1 µm; C-D-E-F, 0.5 214 215 µm. Adapted from [10, 42].

216

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 219 *p*)**PPOX**; where m(or p)**PP** (= R<sup>1</sup>) 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*<sub>150</sub>), 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*<sub>150</sub> = 2.4), but 225 also than Orlistat (*x*<sub>150</sub> = 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,  $R^3=C_{15}-C_{18}$ ) possess a core structure 233 similar to that of Cyclophostin (**Figure 3** – X=O,  $R^3=CH_3$ ) 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].



240

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].

244

From these findings, we first reported the total synthesis of natural Cyclophostin CyC<sub>1</sub>, its phosphonate analogues CyC<sub>2 $\alpha,\beta$ </sub> [48, 49], and related monocyclic enol-phosphonates CyC<sub>3-10,19-22</sub> [51-53] and phosphates CyC<sub>23-30</sub> [53]; as well as Cyclipostin P CyC<sub>18 $\beta$ </sub>, its *trans*-( $\alpha$ ) diastereoisomer CyC<sub>18 $\alpha$ </sub> [49], and the corresponding monocyclic enol-phosphonates CyC<sub>11-13</sub> [52, 54], difluorophosphonates CyC<sub>14-15</sub> and phosphates CyC<sub>16-17</sub> [55, 56] (Figure 3). Of particular importance, diastereomeric *cis*- and *trans*-monocyclic enolphosphonates CyC<sub>6-10</sub> 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.*,  $R^2$ ) group – Figure 3) or at the phosphorous center (*i.e.*,  $R^3$  group – Figure 3), strongly impacted 258 259 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,  $CyC_7$ 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  $cis(\beta)$ -isomer [52]. The four stereoisomers of CyC<sub>7</sub> 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].

Cutinase displayed a high diastereoselectivity for the  $(S_p)$  configuration with a Stereoselectivity Index (SI) derived from  $x_{150}$  values of 94.9% (**Table 2**) when using **CyC**<sub>7</sub>-( $S_c$ ) inhibitors, whereas no obvious stereopreference at the phosphorus center was observed with the **CyC**<sub>7</sub>-( $R_c$ ) 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<sub>7</sub><sub>B</sub>-( $R_c$ , $R_p$ ) 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 nonracemic 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].

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 anti-mycobacterial activity on a M. tb H37Rv-GFP strain using a high-content screening assay 290 291 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 MIC<sub>50</sub> 297 (compound concentration leading to 50% growth inhibition) and CC<sub>50</sub> (compound 298 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  $\mu$ M) to good (3-4  $\mu$ M) and to excellent (500 nM for  $CyC_{17}$ ) MIC<sub>50</sub> values. Of great importance, beside their inhibitory activity against bacterial growth, both set of compounds exhibited very low toxicity towards host macrophages ( $CC_{50} > 100 \mu$ M). Data show that both **OXs** and **CyCs** can be divided into two different classes based on their antibacterial activity (**Figure 4** and **Table 3**).

305



306

307 Figure 4. (A-C) Activity of HPOX, CyC<sub>7β</sub> & CyC<sub>17</sub> against GFP-labelled *M. tb* H37Rv
308 replicating in culture medium expressed as normalized relative fluorescence units (RFU%). (B309 D) Activity of iBPOX and CyC<sub>7β</sub> against *M. tb* H37Rv-GFP replicating inside Raw264.7
310 macrophages. Adapted from [54, 61].

311

First, 9 out of 14 compounds exhibited higher activity against intracellular bacteria than against extracellular ones: *i.e.*, **iBpPPOX**, **iBPOX**, **HpPPOX**, **BepPPOX**, **CyC**<sub>6</sub>, **CyC**<sub>7a,β</sub> and **CyC**<sub>8a,β</sub>. 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 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 even no activity against intracellular M. tb: HPOX, BePOX, CyC17, CyC180, B; a property 321 322 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

Figure 5. General scheme for the identification of the target enzymes of our inhibitors usingABPP 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

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 CyC<sub>17</sub>, 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 CyC<sub>17</sub>, but also of CyC<sub>7β</sub> and CyC<sub>8β</sub> [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 CyC<sub>17</sub> ( $x_{150} = 12.4$ )

358 [79] and **HPOX** (
$$x_{150} = 0.59$$
) [61].

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**Figure 6.** Crystal structures of (**A**) Ag85C in complex with **CyC**<sub>8β</sub> (PDB ID: 5OCJ; 1.8Å resolution) [78] and (**B**) TesA in complex with **CyC**<sub>17</sub> (PDB ID: 6FVJ; 2.6Å resolution) [79]. The mechanism of action of the phosphonate (**CyC**<sub>8β</sub>) and the phosphate (**CyC**<sub>17</sub>) 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<sub>8B</sub> 369 370 (Figure 6A) [78] and TesA in complex with CvC<sub>17</sub> (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 CyC<sub>17</sub> 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  $\beta$ -ketoester moiety (Figure 6B). Such chemical modification can therefore be 376 considered as a signature of the  $CvC_{17}$  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<sub>7a</sub> ( $x_{150} = 5.0$ ) and 381 in near stoichiometry with  $CyC_{17}$  ( $x_{150} = 0.98$ ), but not with  $CyC_{7\beta}$  and  $CyC_{8\alpha,\beta}$  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

In view of the results obtained on *M. tb*, the specificity of our **CyC** compounds against various bacteria was further investigated. Susceptibility testing conducted on 7 bacterial strains, *P. aeruginosa*, *E coli*, *B. subtilis*, *M. abscessus*, *M. marinum*, and *M. bovis* BCG against the 26 available **CyC1**-18 surprisingly showed that these compounds block specifically the growth of the mycobacterial species without affecting the growth of Gram-positive and Gram-negative bacteria [81]. More particularly, **CyC17** exhibited MIC<sub>50</sub> values of 12.7  $\mu$ M and 0.37  $\mu$ M 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  $\mu$ M), imipenem (12  $\mu$ M) 394 or cefoxitin (27  $\mu$ M) [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].

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  $(CyC_{17})$  showed high 404 selectivity for mycobacteria only, and MICs (<2-40 µg/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 (CyC<sub>19-30</sub> – Figure 3) were synthesized by varying the  $R^4$  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, CyC<sub>25</sub> and CyC<sub>26</sub> exhibited very promising MIC<sub>50</sub> 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  $R^3$ 413 or  $R^4$  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 imaging (QI) atomic force microscopy (AFM) with hydrophobic tips, Viljoen et al. 416 417 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 GPLdependent variation in the nanoscale distribution of *M. abscessus* hydrophobicity.

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

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  $CyC_{7\alpha,\beta}$  and  $CyC_{8\alpha,\beta}$  were found active against intracellularly replicating *M. abscessus* (Table 435 4). With MIC<sub>50Raw</sub> values of 7.9 and 2.0  $\mu$ M, respectively, CyC<sub>8a</sub> and CyC<sub>8b</sub> 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<sub>50Raw</sub> are of the same order of magnitude than those previously 438 obtained with the same  $CyC_{8\alpha,\beta}$  against intracellular *M*. *tb* (4-12  $\mu$ M – 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 (MIC<sub>50</sub> = 33.0  $\mu$ M) and intracellular (~59%

bacterial clearance at 30  $\mu$ M) replicating bacteria with similar potency/efficiency to that of imipenem (**Table 4**).

444 By using similar strategy applied for *M. tb* (Figure 5), 39 and 21 potential target enzymes 445 of CyC<sub>17</sub> 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

pathogens. By impairing simultaneously, the activity of various (Ser/Cys)-base enzymes 467 468 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.

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.

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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		LipY			
Compounds		%inhil			
Compounds	n = 0  or  1	$x_{\rm I} = 4$	$x_{\rm I} = 20$	<i>X</i> I50 <sup><i>c</i></sup>	
Orlistat		26.8±1.5	87.6±4.8	7.1	
<b>M</b> <i>m</i> <b>PPOX</b>		63.6±4.6	85.9±4.9	2.4	
MpPPOX	er l	$60.8 \pm 2.1$	79.4±3.3	0.93	
MPOX		$34.4 \pm 2.4$	81.8±3.3	10.9	
<b>E</b> <i>m</i> <b>PPOX</b>	ese .	21.7±1.4	53.8±3.3	6.5	
MemPPOX	-2 <sup>-2</sup> -2-	92.4±0.30	93.7±1.3	0.69	
BmPPOX	22	15.6±0.50	36.7±1.8	>40	
iBmPPOX		37.4±2.4	49.4±1.5	3.9	
iBpPPOX	5	33.8±1.5	41.6±0.40	>40	
iBPOX		31.6±1.0	47.9±2.1	9.2	
H <b>mPPOX</b>		39.9±1.2	76.5±1.9	3.3	
HpPPOX	55 <u> </u>	38.7±1.7	77.2±2.7	2.9	
HPOX	· · ·	35.0±0.27	38.8±0.11	>40	
BemPPOX	S A	88.0±0.70	90.2±0.91	0.72	
BepPPOX	3 0	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	2 <sup>2</sup> 2	89.6±1.1	94.8±2.7	0.73	
<b>D</b> mPPOX	~~~~~ <sup>2</sup> č	91.5±2.8	94.2±3.1	0.67	
DomPPOX	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	88.7±4.2	90.4±2.2	0.77	

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

<sup>*a*</sup> Inhibition kinetics were performed using the microtiter plate assay with coated Pomegranate oil [44]. <sup>*b*</sup> Inhibition data (% of initial enzyme activity), at an inhibitor molar excess ( $x_I$ ) of 4 or 20 related to 1 mole of enzyme. Values are means of at least three independent assays. <sup>*c*</sup>  $x_{I50}$ : inhibitor molar excess leading to 50% lipase inhibition [28, 44].

X150											
	MeO P(R)(S)	OMe Mg	9	MeC	O O O O O O O O O O O O O O O O O O O	O P MeO		OMe		C MeO	P(R)(R) H
	CyC <sub>7α</sub> -(S <sub>c</sub> ,	R <sub>p</sub> )			CyC <sub>7β</sub> -(S <sub>c</sub> ,S <sub>p</sub> )	Cyc	C <sub>7α</sub> -(R <sub>c</sub> ,	S <sub>p</sub> )		С	уС <sub>7β</sub> -( <i>R</i> <sub>c</sub> , <i>R</i> <sub>p</sub> )
Cutinase	97.3	←	<b>SI</b> = 94.9%	$\rightarrow$	2.52		4.07				3.38
Rv0183	11.3	←	<b>SI</b> = 81.4%	$\rightarrow$	1.16		1.24	←	<b>SI</b> = 72.0%	$\rightarrow$	7.61
LipY	25.9				20.0		15.4	←	$\mathbf{SI} = 80.7\%$	$\rightarrow$	1.64

Table 2. Stereoselective inhibition of Cutinase, Rv0183, and LipY by enantiopure monocyclic enolphosphonates CyC7<sup>*a*</sup>

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

		M. tb H37Rv-GFP				
Compounds		Extracellular	Intracel	Intracellular		
Compounds		growth <sup>a</sup>	macrophage growth <sup>b</sup>			
		MIC50 [µM]	MIC50Raw [µM]	CC <sub>50</sub> (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		
НрРРОХ		>50	9.5 ±0.49	>100		
НРОХ		44.6 ±2.50	No effect	>100		
BepPPOX		>50	3.5 ±0.28	>100		
BePOX		30.8 ±2.40	No effect	>100		
CyC analogs						
CyC <sub>6</sub> β	Meo <sup>P</sup> H 4	NE	12.6 ±0.90	>100		
CyC <sub>7α</sub>		$92.6 \pm 6.5$	4.5 ±0.39	>100		
CyC <sub>7β</sub>	MeO <sup>sf</sup> H	$16.6 \pm 0.78$	3.1 ±0.11	>100		
CyC <sub>8a</sub>		40.4 ±2.5	4.0 ±0.30	>100		
CyC <sub>8β</sub>	MeO <sup>s</sup> H 11	>100	11.7 ±0.26	>20		
CyC <sub>17</sub>	O, O O Me Me	0.50 ±0.02	No effect	>100		
CyC <sub>18a</sub>	0,0	24.4 ±2.14	No effect	>100		
CyC <sub>18β</sub>		1.7 ±0.03	No effect	>100		

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

<sup>*a*</sup> MIC<sub>50</sub>, compound concentration leading to 50% bacterial growth inhibition in culture medium. <sup>*b*</sup> CC<sub>50</sub>, compound concentration leading to 50% Raw264.7 macrophages toxicity. MIC<sub>50Raw</sub>, 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].

Compounds		M. abscessus CIP 104536 <sup>T</sup>				
		MIC <sub>5</sub>				
		S variant	R variant	MIC 50Raw [µM]		
Amikacin (AMK)		$3.9 \pm 0.19$	$7.4 \pm 0.26$	Not determined		
Imipenem (IMP)		11.3 ±0.50	$29.9 \pm 1.6$	28.3 ±4.7		
OX derivatives						
iBpPPOX		33.0 ±2.0	$53.2 \pm 1.8$	< 30µM		
MpPPOX		88.2 ±7.3	>200	75.2 ±5.1		
MPOX		>200	>200	36.5 ±2.6		
CyC analogs						
$CyC_{7\alpha}$	0.0	>100	>100	$29.3 \pm 2.9$		
$CyC_{7\beta}$	MeO <sup>3</sup> P H	>100	>100	$65.3 \pm 6.2$		
CyC <sub>8a</sub>	0.0	>100	>100	7.9 ±0.23		
CyC <sub>8β</sub>	MeO <sup>st</sup> H 11	>100	>100	$2.0\pm0.91$		
CyC <sub>17</sub>	CONCEPTION OME	12.7 ±0.26	0.37 ±0.01	No effect		
CyC <sub>25</sub>	MeO O OMe	$85.0 \pm 0.86$	13.9 ±0.56	No effect		
CyC <sub>26</sub>	O, O- P* MeO O OMe	60.0 ±2.50	$6.9\pm0.16$	No effect		

Table 4. Activities of the most active OX derivatives and CyC analogs against M. abscessus.

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