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ABSTRACT

A set of 19 oxadiazolone (OX) derivatives have been investigated for their antimycobacterial activity against two pathogenic slow-growing mycobacteria, Mycobacterium marinum and Mycobacterium bovis BCG, and the avirulent Mycobacterium tuberculosis (M. tb) mc²6230. The encouraging minimal inhibitory concentrations (MIC) values obtained prompted us to test them against virulent M. tb H37Rv growth either in broth medium or inside macrophages. The OX compounds displayed a diversity of action and were found to act either on extracellular M. tb growth only with moderated MIC₅₀, or both intracellularly on infected macrophages as well as extracellularly on bacterial growth. Of interest, all OX derivatives exhibited very low toxicity towards host macrophages. Among the six potential OXs identified, HPOX, a selective inhibitor of extracellular M. tb growth, was selected and further used in a competitive labelling/enrichment assay against the activity-based probe Desthiobiotin-FP, in order to identify its putative target(s). This approach, combined with mass spectrometry, identified 18 potential candidates, all being serine or cysteine enzymes involved in M. tb lipid metabolism and/or cell wall biosynthesis. Among them, Ag85A, CaeA, TesA, KasA and MetA have been reported as essential for in vitro growth of M. tb and/or its survival and persistence inside macrophages. Overall, our findings support the assumption that OX derivatives may represent a novel class of multi-target inhibitors leading to the arrest of M. tb growth through a cumulative inhibition of a large number of Ser- and Cys-containing enzymes involved in various important physiological processes.

1. Introduction

With 10.4 million new cases and 1.7 million deaths in 2016, tuberculosis (TB) caused by the pathogenic species Mycobacterium tuberculosis (M. tb) remains the leading cause of death worldwide from a single infectious agent [1]. Despite the quadritherapy treatment involving isoniazid (INH), pyrazinamide, rifampicin (RIF) and ethambutol, the introduction of new molecules on the market to strengthen or replace this first-line antibiotics regimen is a slow and tedious process [2,3]. Only a few drugs were able to pass the selection stages (e.g., bedaquiline [4], delamanid [5] and PA-824 [6]). However, the appearance and spread of multidrug-resistant (MDR), extensively drug-resistant (XDR) related to 1 mol of enzyme; x₅₀, inhibitor molar excess leading to 50% enzyme inhibition

Abbreviations: ABPP, activity-based protein profiling; CC₅₀, compound concentration leading to 50% of cell cytotoxicity; CyC, Cyclosporins & Cyclophostin analogs; ETO, ethionamide; FM, foamy macrophages; HSL, hormone-sensitive lipase; ILL, intracytoplasmic lipid inclusions; INH, isoniazid; MDR, multidrug-resistant strains; MIC₅₀, minimal inhibitory concentration leading to 50% of growth inhibition; M. tb, Mycobacterium tuberculosis; pNP, para-nitrophenyl; REMA, resazurin microtiter assay; RIF, rifampicin; TB, tuberculosis; TAG, triacylglycerols; TDR, totally drug-resistant strains; XDR, extensively drug-resistant strains; xI₅₀, inhibitor molar excess related to 1 mol of enzyme

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resistant (XDR) and totally drug-resistant (TDR) strains [7–10] highlights the urgent need for finding new therapeutic options to fight against M. tb.

In this context, oxadiazolone-core (OX) compounds represent attractive tools. Compound S57 (Fig. 1A), initially described in 1954 [11,12] as being active against TB [13–15], was used as template for the synthesis of 1,3,4-oxadiazole-2-one derivatives [16,17]. These molecules were found to exhibit interesting anti-mycobacterial activity against M. tb H37Rv, with minimal inhibitory concentrations (MIC) of 1.25–8 µg/mL, comparable to those of INH (0.5 µg/mL) and Rif (1.0 µg/mL) [16,17]. Molecular modeling indicated that these compounds possessed all necessary features to block the enzymatic activity of the mycobacterial cytochrome P450-dependent 14α-sterol demethylase (P45014DMs) [18], also a target for antifungal drug design [19]. These findings thus suggest the potential use of such OX derivatives as alternative therapeutic agents for TB [20].

Few years ago, we reported that the OX compound MmPPOX (Fig. 1B), a reversible inhibitor of the hormone-sensitive lipase (HSL) family of proteins [21,22], was able to inhibit the growth of M. tb with MIC values of around 15–25 µg/mL as determined on solid medium [23]. Keeping in mind its strong affinity towards the HSL family member proteins (Lip-HSL), we further investigated the in vitro inhibition of recombinant M. tb Lip-HSL. As expected, the nine purified Lip-HSL enzymes tested were all strongly inhibited by MmPPOX, which reacted with the catalytic serine residue by forming a covalent but reversible bond (i.e., carbamate or thio carbamatic bond, Fig. 1B). Such an inhibitor could then be considered as a long-life substrate rather than a true inhibitor as already observed with Orlistat (also named Tetrahydrolipstatine or THL), a representative member of α-sterol demethylases (P45014DMs) [18], also a target for antifungal drug design [19]. These findings thus suggest the potential use of such OX derivatives as alternative therapeutic agents for TB [20].

From these first results, a new series of 18 lipophilic OX derivatives based on MmPPOX core-structure have been synthesized (Fig. 1C) and tested for their anti-mycobacterial activities. More precisely, each oxadiazolone molecule has been tested against M. tb H37Rv for (i) its capacity to inhibit in vitro growth; (ii) its antitubercular activity on M. tb-infected macrophages, and (iii) its cytotoxicity towards macrophages. Interestingly, some analogs were found to inhibit M. tb growth in vitro and/or inside macrophages without any significant toxicity to host cells. In addition, using an activity-based protein profiling (ABPP) assay, the potential target enzymes of HPOX, acting only on M. tb extracellular growth, were further identified.

2. Results and discussion

2.1. Synthesis of oxadiazolone-core (OX) derivatives

The set of new 18 lipophilic OX derivatives based on MmPPOX core-structure was designed by varying the nature of the R chain and/or the positioning of the phenoxo group (meta or para position) when present (Fig. 1C), and synthesized as previously reported [27]. The (phenoxo)phenyl group, proposed to be responsible for strong hydrophobic interactions and structural stiffening [27], was conserved in most of the new candidate inhibitors. In addition, modifying the R chain born by the oxadiazolone ring allow an investigation of the influence of the lipophilicity on the antibacterial activity exerted by these molecules. To remain consistent with previous studies involving such compounds [23,27], we have decided to use the specific nomenclature already developed for these derivatives noted Rm (or p)PPOX; where m (or p)P represents the meta (or para)-Phenoxo group when present; P the phenyl group; OX the Oxadiazolone core; and R the alkyl chain (i.e., n-Bee, benzoxylethyl; M; methyl, E, ethyl; B, butyl; iB, isobutyl; H, hexyl; X, xyl); O; octyl; Eth, 2-ethylhexyl; D, decyl; Do, docetyl; Me, methoxylethyl).

2.2. Susceptibility testing on selected mycobacteria

The antibacterial properties of the OX compounds were first evaluated towards three slow-growing mycobacteria: M. marinum, M. bovis BCG and M. tb mc²6230, a H37Rv strain with its RD1 region and panCD genes deleted, resulting in an avirulent pan(−) phenotype [28]. The corresponding MIC₅₀ values, as determined by the REMA assay [29–32], are reported in Table 1. First, it is noteworthy that the concentrations needed to inhibit 50% of the bacterial growth (MIC₅₀)
obtained for rifampicin (RIF) and isoniazid (INH), used here as reference antibiotics, were in agreement with literature data [33–35]. Nearly all 19 OXs were active against *M. bovis* BCG and *M. marinum* growth (Table 1). However, with MIC₅₀ values in the range 1.9–53 µM, *M. marinum* was nearly 2-times more sensitive to OX compounds than *M. bovis* BCG (MIC₅₀ from 3.5 to > 120 µM). BePOX, HPOX and iBPOX, for which the phenoxy substituent is absent, exhibited the most potent antibacterial activity towards *M. marinum*, with mean MIC₅₀ of 2.3 ± 0.33 µM, comparable to that of RIF (1.4 µM). Interestingly, these three OX compounds were also among the best inhibitors of *M. bovis* BCG growth; HPOX being the best one (MIC₅₀ = 3.5 µM).

From these encouraging data obtained using two slow-growing mycobacteria, it was tempting to extrapolate that these OXs would behave in a similar way against *M. tb* growth and conclude that the same three compounds, i.e., BePOX, HPOX and iBPOX, would then be promising anti-TB molecules.

Drug susceptibility testing of the 19 OXs was thus further assessed using the non-virulent *M. tb* mc²6230 strain. Among all tested compounds, 14 OXs were active against *M. tb* mc²6230. The two best growth inhibitors obtained were *iBpPOX* and *BePOX*, which displayed similar MIC₅₀ value (mean 2.1 ± 1.0 µM). In all other cases, MIC₅₀ values were indicative either of a weak (mean MIC₅₀ = 8.1 ± 9.1 µM for *MmPPOX*, MPOX and *MemPPOX*), or a moderate (mean MIC₅₀ = 46.9 ± 5.1 µM for *BmPPOX*, *iBmPPOX*, *HmPPOX*, *HpPPOX*, *HPOX*, *OmPPOX*, *EhmPPOX*, *BemPPOX* and *BepPPOX*) antibacterial activity (Table 1). From these data, *M. tb* mc²6230 was found nearly 13- and 5-times less sensitive to the OX compounds than *M. marinum* and *M. bovis* BCG, respectively.

Surprisingly, *iBpPOX*, which differs from BePOX and HPOX by the length of its R substituent, was not active against this mycobacteria. Moreover, no clear trends or rules in terms of structure-activity relationships (SAR) have emerged regarding the potency of these oxadiazolone-core compounds against *M. marinum* and *M. bovis* BCG. Indeed, increasing the lipophilicity by varying the nature of the R chain on the oxadiazole ring and/or the positioning of the phenoxy group when present had no real impact on the anti-mycobacterial activity.

Interestingly, with *M. tb* mc²6230, some SAR tendencies can however be set up. First, and as mentioned above, the positioning of the phenoxy group in *meta* or *para* position has no real impact on the antibacterial activity of the corresponding compounds (i.e., *MmPPOX* vs *MppPOX*; *iBmPPOX* vs *iBpPOX*; *HmPPOX* vs *HpPPOX*; *BemPPOX* vs *BepPPOX*). Remarkably, *iBpPOX* bearing the short chain isobutyl has no activity as compared to the phenoxyphenyl derivatives *iBpPOX* and *iBmPPOX*. This is however not the case with the medium chains hexyl and benzyloxyethyl OXs, for which the respective activity of HPOX and BePOX is retained and even slightly better than HPOX & iPPOX on the one hand, and BemPOX & BepPOX on the other hand. Finally, in absence of the bulky phenoxy group, the best MIC₅₀ against *M. tb* mc²6230 were also obtained with HPOX and BePOX vs MPOX and iPPOX. In brief, the R chain length thus seems to affect the potency of the tested compounds. More globally, the best growth inhibitors were found to carry a middle chain length of around 6–9 carbon atoms (i.e., hexyl, 2-ethylhexyl, octyl or benzyloxyethyl chains). Longer or shorter chain’s OXs (i.e., methyl, methoxymethyl, ethyl, decyl or dodecyl) exhibited no or only very poor activity.

In summary, *iBpPOX* displayed the best, while still moderate, antibacterial activity against *M. tb* mc²6230. The reason why such differences exist between the activity of the OX compounds against *M. marinum* and *M. bovis* BCG, compared to *M. tb*, is not clear and will need further studies to be elucidated; but differences in membrane composition and permeability are likely playing a role in this phenotype.
The presence of increasing concentrations of candidate inhibitors determine the MIC50 of the compound [36,39,40]. In subsequent experiments, infected cells, as well as the number of living host cells allowed us to infected cells, the total number of bacteria, the number of bacteria per infected cells, as well as the number of living host cells allowed us to determine the MIC50 of the compound [36,39,40]. In subsequent experiment, the concentration leading to 50% of host cell cytotoxicity, i.e. CC50, were also determined in absence of infection.

Among all tested molecules, 6 potential OX candidates exhibited interesting antitubercular properties (Table 2 and Fig. 2). BePOX and HPOX impaired exclusively \( M. \) \( tb \) growth in culture broth medium with the same moderate MIC50 (30.8 and 44.6 µM, respectively) than obtained previously on \( M. \) \( tb \) mc\(^2\)6320. In contrast, iBPOX, HppPOX and BepPOX showed a clear preference against intracellularly-replicating mycobacteria with similar MIC50 values (3.5–17.1 µM) than the first line antibiotics. Of interest, only iPPOX exhibited moderate (32.0 µM) to quite good (8.5 µM) activity against both extracellular and intramacrophagic \( M. \) \( tb \), respectively (Fig. 2).

Beside antibacterial activity, significantly, all these 6 OX inhibitors exhibited very low toxicity towards host macrophages with CC50 > 100 µM, similarly to INH (CC50 > 150 µM) and ethionamide (CC50 ≤ 120 µM), two potent anti-TB drugs (Table 2). Their respective selectivity index (SI = CC50/MIC50) on intramacrophagic \( M. \) \( tb \) vs Raw264.7 cells was thus found to be in a range from 5.8 and up to 28. While these are preliminary results that would need to be confirmed in other cell types including hepatocytes, they are encouraging for further improvement of the OX compounds. This absence of cytotoxicity was actually not obvious, given the number of (Ser/Cys)-enzymes present in host cells and the predicted potency of the corresponding OX from extracellular- (i.e., HPOX & BePOX) to intracellular-replicating bacilli (i.e., HppPOX & BepPOX). On the other hand, with the short isobutyl chain, both iPPOX and iBPOX are found most

### Table 2

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Extracellular growth</th>
<th>Intracellular macrophage growtha</th>
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<tbody>
<tr>
<td></td>
<td>MIC50 (µM)</td>
<td>CC50 (µM)</td>
</tr>
<tr>
<td>INHb</td>
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</tr>
<tr>
<td>BePOX</td>
<td>30.8</td>
<td>No</td>
</tr>
</tbody>
</table>

* Experiments were performed as described in Section 4. MIC50, compound minimal concentration leading to 50% of growth inhibition. CC50, compound concentration leading to 50% of cell cytotoxicity. Each value is the mean ± SD for a triplicated dose-response. Experiments were conducted at least twice with consistent results.
* Raw264.7 macrophages were infected by \( M. \) \( tb \) H37Rv-GFP at a MOI of 2:1.
* Data from [36]. INH, isoniazid; RIF, rifampicin; ETO, ethionamide.

Fig. 2. In vitro and ex vivo dose-response activity of the OX derivatives against \( M. \) \( tb \) H37Rv. (A) Activity of BePOX, iBPOX and HPOX against GFP-expressing \( M. \) \( tb \) replicating in broth medium, expressed as normalized relative fluorescence units (RFU%). The dashed line represents the level of inhibition (~70%) reached with 1 µg/mL (~7.3 µM) INH as control. The MIC50 of BePOX, iBPOX and HPOX replicating in culture broth medium were 30.8 µM, 32.0 µM and 44.6 µM, respectively. (B) Activity of iPPOX, BePOX, HppPOX and BePOX against \( M. \) \( tb \) replicating inside Raw264.7 macrophages. Results are expressed as the percentage of infected macrophages after 5 days post-infection. The dashed line represents the level of inhibition (~87%) reached with 10 µg/mL (~73 µM) INH as control. The MIC50 of BePOX, iPPOX, HppPOX and iPPOX replicating inside macrophages were 3.5 µM, 8.5 µM, 9.5 µM and 17.1 µM, respectively. Each value is the mean ± SD for a triplicated dose-response. Experiments were conducted at least twice with consistent results.
active against intramacrophagic-replicating *M. tb*.

From these findings, it is tempting to assume that these OX compounds thus lead to the inhibition of specific but most likely distinct mycobacterial target enzymes between intramacrophagic- vs. extra-cellularly-replicating bacilli.

### 2.4. Targets identification by Activity-based protein profiling

Based on the aforementioned results, and taking into account their strong affinity for Serine and/or Cysteine (Ser/Cys)-based enzymes (Fig. 1B), one can hypothesize that OX inhibitors might target and impair the activity of various enzymes involved in several processes of *M. tb* pathogenic life cycle, thus resulting in bacterial death without any cytotoxicity towards host cells. Accordingly, target(s) identification experiments were conducted by applying an activity-based protein profiling (ABPP) approach [41–44]. In order to take into account the penetration/diffusion of the inhibitor through the mycobacterial cell wall, all experiments have been performed on living bacterial cells and not with a lysate, as previously described [31].

Here, HPOX, that selectively inhibits *M. tb* growth only in culture broth medium, was selected for such experiments. *M. tb* mc*c*6230 cells were grown to log phase and then incubated with HPOX compound or DMSO as a control. After cell lysis, part of the lysate was used for competitive probe labelling/enrichment assay using the Desthiobiotin-FP probe, targeting (Ser/Cys)-based enzymes [43] (Fig. 3). In parallel, the remaining lysate was incubated with TAMRA-FP, also targeting (Ser/Cys)-based enzymes [43], to reveal the candidates presumably reacting with HPOX on SDS-PAGE gel, using fluorescence scanning [31]. Around 9 distinct bands labelled by TAMRA-FP were visible in the fluorescence readout (Fig. 3B – lane E) and could also be detected by silver staining after release of the enzymes captured by Desthiobiotin-FP (Fig. 3B – lane B). In contrast, pre-treatment with HPOX (Fig. 3A) resulted in a decrease in fluorescence intensity of all visible bands, as exemplified by the black arrows in Fig. 3B – lane D. Indeed, the enzymes previously inactivated by HPOX inhibitor will thus be unable to further react with the probes. The respective enriched mixtures (Fig. 3B – lanes A-B) were digested with trypsin and the resulting peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) followed by subsequent label free quantification analysis. The proteins that were also found in the control experiment (i.e., Fig. 3B – lane A: DMSO alone for unspecific binding to streptavidin-magnetic beads) were not taken into account.

The resulting mycobacterial targets of HPOX were displayed as volcano plot (Fig. 4). Only proteins identified with a permutation false discovery rate (pFDR) of 5% and a score threshold value ≥60 were selected, therefore leading to a panel of 18 distinct proteins (Table 3). These identified enzyme candidates ranged in their functional category from intermediary metabolism/respiration (6 proteins), lipid metabolism (5 proteins), cell wall/cell processes (6 proteins), and virulence/ detoxification/adaptation (1 protein) (Table 3).

As expected, the identified proteins targeted by HPOX were all (Ser/Cys)-based enzymes. Among them, a variety of Ser/Cys hydrolases were detected. These included the putative β-lactamase *Rv1367c* possibly involved in cell wall biosynthesis; two amidases AmidC (*Rv2888c*) and AmidD (*Rv3375*); BpeOc (*Rv0554*) a putative serine hydrolase [43]; two members of the lipase family Lip (LipH [45] and LipV [46]); three Cutinase-like proteins (Cfp21, Cut2 and Cut3) [47]; and the monoacylglycerol lipase *Rv0183* [48].

More interestingly, 5 out of 18 identified proteins have been annotated as essential enzymes [49] (Table 3). These include the antigen 85 complex, *Ag85A* (*Rv3804c*), *Ag85B* (*Rv1886c*) and *Ag85C* (*Rv0129c*) [50]; the thioesterase *Tesa* (*Rv2928*) [51]; the carboxylesterase CaeA (*Rv2224c*) [52]; the beta-ketoacyl synthase KasA (*Rv2245*) [53]; and the sole putative αβ-hydrolase MetA (*Rv3341*) belonging to the homoserine O-acetyltransferase family proteins in *M. tb* [54].

### 2.5. *TesA, Rv0183 and Cfp21 are inhibited by HPOX*

In order to validate some targets of HPOX inhibitor, we further investigated its ability to efficiently inhibit the activity of three identified proteins; i.e., *TesA, Cfp21 and Rv0183*. Coding sequences were amplified from *M. tb* genome, cloned in E. coli and the enzymes produced in recombinant form and purified as previously reported [48,55]. Purified proteins were then individually incubated for 30 min at room temperature with HPOX at various inhibitor molar excess (x). The residual enzyme activity was then measured using *para*-nitrophenyl caprylate (*pNPC-8*) as substrate for *TesA* and *Cfp21* [55], and mono- olein as substrate in the case of *Rv0183* [48]. The variation in the residual enzyme activity allowed determination of the inhibitor molar excess leading to 50% enzyme inhibition, i.e., *x*50 value [27,56]. Thereby, a *x*50 value of 0.5 is synonymous with a 1:1 stoichiometric ratio between the inhibitor and the lipolytic enzyme, and is therefore the highest level of inhibitory activity that can be achieved.

As depicted in Fig. 5, a clear dose-dependent inhibition was observed with the three enzymes. *TesA, Rv0183* and *Cfp21* were indeed strongly inactivated, with 97.6% and 88.5% inhibition at *x* = 20, respectively (Fig. 5, inset). Interestingly, HPOX was found to react almost stoichiometrically with pure *TesA* and *Rv0183* as confirmed by their respective *x*50 values of around 0.60. These results clearly demonstrate that these three lipolytic enzymes are effective targets of HPOX.

The fact that these OXs derivatives behave against *M. tb* extracellular growth similarly to two other well-known non-specific (Ser/Cys)-enzyme inhibitors, namely Orlitstat (MIC ~ 25 μM) [41,57] and the human lysosomal acid lipase inhibitor Laliset (MIC ~ 25–50 μM) [42], support the assumption that HPOX, and certainly all the other active OX compounds, may act as multi-target inhibitors by impairing the activities of multiple non-essential lipolytic enzymes as well as essential proteins involved in various important physiological pathways of *M. tb* life cycle.

Overall, it is now acknowledged that the lipolytic enzymes containing a catalytic Ser or Cys residue in their active site are not only involved in the host-pathogen cross-talk [58], but also play several roles in the physiopathology of the disease, in particular by recycling fatty acids from host lipids, a key element favoring *M. tb* reactivation [48,59] and survival in dormancy [60]. *M. tb* indeed induces the formation of lipid bodies (LB) inside infected macrophages, giving the cells a foamy appearance. In foamy macrophages (FM), bacilli accumulate lipids within intracytoplasmic lipid inclusions (ILI) [61–64], which allow the bacteria to persist in a non-replicating state. In FM, several mycobacterial lipolytic enzymes of *M. tb* hydrolyze triacylglycerols (TAG) from LB and the resulting fatty acids are stored within ILI as newly synthesized TAGs. Consequently, although they exhibited moderate MIC50 values against *M. tb* H37Rv as compared to classical antibiotics or the more recent Cgyc analogs [31]; OX compounds may however represent attractive chemical tools for identifying such (Ser/Cys)-containing enzymes in living mycobacteria, studying the regulation of ILI formation in infected FM [65], and thus provide a better understanding of how bacilli can persist inside lipid-rich FM.
elimination of either actively replicating or latent bacilli from infected individuals. Accordingly, given the importance of such (Ser/Cys)-enzymes for \textit{M. tb} viability during infection, they should represent new attractive drug targets. Such experiments are currently underway, and will be reported in due course.

4. Experimental Section

4.1. Chemistry

The first 13 oxadiazolone derivatives 5a–k, 6k and 7k were synthesized as described previously [27,66]. The new six derivatives 6a,d,e and 7a,d,e were prepared from commercial (4-phenoxypyphenyl) hydrazine hydrochloride (2) and phenylhydrazine hydrochloride (3), respectively, by performing both the coupling reaction with alkyl chloroformate 2a–k (step i) and the cyclization reaction with diphenogene (step ii) in a one-pot two-steps reaction [27]. All compound were at least 98% pure as determined by HPLC analysis [27]. Stock solutions (4 mg/mL) in which the oxadiazolone compounds were found to be completely soluble in dimethyl sulfoxide (DMSO), were prepared prior to drug susceptibility testing. See Supplementary Material for NMR, HPLC analysis and HRMS spectra of the new six OX derivatives.

4.1.1. General procedure for the one step preparation of 5-alkoxy-3-aryl-1,3,4-oxadiazol-2(3H)-one compounds

4.1.1.1. 5-(2-(benzyl)oxyethyl)-3-(3-phenoxypyphenyl)-1,3,4-oxadiazol-2(3H)-one (6a = BepPPOX). (4-phenoxypyphenyl)hydrazine hydrochloride [66] 2 (8.2 g, 34.6 mmol, 1 equiv.) and 1-methyl pyrrolidone (2.41 mL, 31.1 mmol, 0.9 equiv.) were dissolved in dry pyridine (700 mL). The solution was cooled in an ice bath to 0°C. Then, 2-benzylxyethyl...
chloroformate 4a (6.87 mL, 38.6 mmol, 1.1 equiv.) was added dropwise over a period of 30 min at 0-5 °C and allowed to stir for 1 h at 0 °C and 1 h at room temperature. The reaction mixture was diluted by addition of methylene chloride (300 mL) and dry pyridine (70 mL) and the mixture was cooled at -10 °C using an ice-salt bath. A solution of methylene chloride (300 mL) and dry pyridine (70 mL) and the mixture was cooled at -10 °C with an ice-salt bath. After the addition is complete the reaction mixture stirred 1 h at −10 °C and 2 h at room temperature. The reaction mixture was diluted with water (1 L) and extracted with diethyl ether (3 × 250 mL). The combined organic phase was filtered. Purification by column chromatography using cyclohexane/ethyl acetate (98/2 to 95/5, v/v) in methylene chloride (30 mL) was added dropwise using a syringe pump over a period of 1 h while maintaining −10 °C with an ice-salt bath. After the addition is complete the reaction mixture stirred 1 h at −10 °C and 2 h at room temperature. The reaction mixture was diluted with water (1 L) and extracted with diethyl ether (3 × 250 mL). The combined organic layers were washed with water (2 × 250 mL) and brine (3 × 100 mL), dried over MgSO4, and filtered. Purification by column chromatography using cyclohexane/ethyl acetate (98/2 to 95/5, v/v) as eluent gave the title compound 6a (BepPPOX) as a yellow oil (9.94 g, 71%). Analytical data for BepPPOX: Rf (AcOEt/Cyclohexane 1:3, v/v) 0.36. HRMS (ESI) m/z [M+H]+ calcd. for C23H21N2O5: 405.1445 Da; found: 405.1446 Da. 1H NMR δ 7.71 (dd, J = 9.1 Hz, J = 2.2 Hz, 2H), 7.31 (m, 7H), 6.98–7.13 (m, 5H), 4.60 (m, 2H), 4.54 (m, 2H), 3.83 (m, 2H). 13C NMR δ 157.14 (s), 155.23 (s), 154.77 (s), 148.29 (s), 137.43 (s), 131.56 (s), 129.85 (2 × d), 128.55 (2 × d), 127.99 (2 × d), 127.79 (2 × d), 123.47 (d), 119.83 (2 × d), 119.47 (2 × d), 118.74 (2 × d), 73.42 (t), 131.66 (s), 129.83 (2 × d), 123.42 (d), 119.79 (2 × d), 119.49 (2 × d), 118.74 (2 × d), 73.42 (t), 70.56 (t), 66.99 (t).

4.1.1.2. 5-isoButyloxy-3-(4-phenoxyphenyl)-1,3,4-oxadiazol-2(3H)-one (6d = iBpPPOX). Prepared using Isobutyl chloroformate 4d applying similar method as described above for 6a. Analytical data for iBpPPOX: pale yellow oil (87%). Rf (AcOEt/Cyclohexane 1:3, v/v) 0.56. HRMS (ESI) m/z [M+H]+ calcd. for C18H19N2O4: 327.1339 Da; found: 327.1342 Da. 1H NMR δ 7.72 (dd, J = 9.2 Hz, J = 2.3 Hz, 2H), 7.30 (m, 2H), 6.97–7.12 (m, 5H), 4.14 (t, J = 6.6 Hz, 2H), 2.15 (m, 1H), 1.0 (d, 6H). 13C NMR δ 157.17 (s), 155.42 (s), 154.67 (s), 148.36 (s), 131.66 (s), 129.83 (2 × d), 123.42 (d), 119.79 (2 × d), 119.49 (2 × d), 118.69 (2 × d), 71.39 (t), 22.75 (t), 18.70 (2 × q).

Table 3

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* Only positive hits with a pDFR of 5% and a score threshold value ≥60 were selected.
* CF: Culture filtrate; CW: Cell wall; M: Membrane fraction; WCL: Whole cell lysate.
4.1.1.3. 5-Hexyloxy-3-(4-phenoxyphenyl)-1,3,4-oxadiazol-2(3H)-one (6e = HPOX).
Prepared using Hexyl chlorofluorate 4e applying similar method as described above for 6a. Analytical data for HPOX: yellow oil (85%). R<sub>f</sub> (AcOEt/Cyclohexane 1:3, v/v) 0.95. HRMS (ESI) m/z [M + H]<sup>+</sup> calcd. for C<sub>20</sub>H<sub>23</sub>N<sub>2</sub>O<sub>4</sub>: 355.1652 Da; found: 355.1652 Da.

1H NMR (DMSO-d<sub>6</sub>, 400 MHz, 298 K) δ 8.62 (m, 1H), 8.46 (d, J = 6.7 Hz, 1H), 7.80 (d, J = 8.3 Hz, 1H), 7.75 (dd, J = 1.7, 7.6 Hz, 2H), 7.44 (d, J = 7.6 Hz, 2H), 7.22 (t, J = 7.4 Hz, 1H), 4.40 (t, J = 6.6 Hz, 2H), 1.85 (m, 4H), 1.33–1.49 (m, 6H), 0.93 (t, 3H), 31C NMR δ 155.54 (s), 148.53 (s), 136.45 (s), 129.30 (2 × d), 125.67 (d), 118.13 (2 × d), 71.97 (t), 31.45 (t), 28.55 (t), 25.36 (t), 22.68 (t), 14.17 (q).

4.2. Biological evaluation

4.2.1. Bacterial strains and growth condition
M. marinum ATCC BAA-335/M, M. bovis BCG Pasteur 1173P2 and M. tb mc<sup>6</sup>230 (H37Rv JRD1 ΔpanCD [28]) strains were routinely grown in Middlebrook 7H9 broth (BD Difco) supplemented with 0.2% glycerol, 0.05% Tween 80 (Sigma-Aldrich), 10% oleic acid, albumin, dextrose, catalase (OADC enrichment; BD Difco) (7H9-S) and 24 µg/mL α-panthothenate (M. tb mc<sup>6</sup>230). For further intra and extracellular assays, M. tb H37Rv expressing GFP [36] was grown for 14 days in 7H9-S supplemented with 50 µg/mL hygromycin B (Euromex). All cultures were kept at 37 °C without shaking, except M. marinum which was grown at 32 °C.

4.2.2. Susceptibility testing on M. marinum, M. Bovis BCG and M. tb mc<sup>6</sup>230
The concentrations of compound leading to 50% of bacterial growth (MIC<sub>50</sub>) were first determined using the resazurin microtiter assay (REMA) [29,30]. Briefly, log-phase bacteria were diluted to a cell density of 5 × 10<sup>6</sup> cells/mL and 100 µL of this inoculum was grown in a 96-well plate in the presence of serial dilutions of compounds. After 7–14 days incubation, 20 µL of a 0.025% (w/v) resazurin solution was added to each well (200 µL) and incubation was continued until the appearance of a color change (from blue to pink) in the control well (bacteria without antibiotics). Fluorescence of the resazurin metabolite resorufin (λ<sub>excitation</sub> 530 nm; λ<sub>emission</sub> 590 nm) was then measured [30] and the concentration leading to 50% growth inhibition was defined as the MIC<sub>50</sub>. See Supplementary Material for detailed protocol.

4.2.3. High-content screening assay – extracellular assay
A 14 days old culture of M. tb H37Rv-GFP was washed twice with PBS and resuspended in 7H9 medium containing 10% OADC, 0.5% glycerol, 0.05% Tween 80 and 50 µg/mL hygromycin B. Bacteria were seeded in 384 well plates (7 × 10<sup>4</sup> bacteria/mL) containing 2-fold dilutions of the compounds in DMSO. The final volume of DMSO was kept under 0.3%. Plates were incubated at 37 °C, 5% CO<sub>2</sub> for 5 days. Bacterial fluorescence levels (RFU) were recorded using a fluorescent microplate reader (Victor X3, Perkin-Elmer). The MIC<sub>50</sub> of all tested compounds were determined using ten-point dose-response curves. In each plate, negative control with 1% DMSO; and positive controls containing 1 µg/mL INH and RIF were also included.

4.2.4. High-content screening assay in infected macrophages – intracellular assay
The growth of M. tb H37Rv-GFP strain in macrophages was monitored by automated fluorescence confocal microscope (Opera, Perkin-Elmer) as already described [36,67]. Briefly, bacteria were washed twice with PBS and resuspended in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen). Murine RAW264.7 macrophages (American Type Culture Collection TIB-71) were infected at a multiplicity of infection (MOI) of 2:1 and incubated 2 h at 37 °C in RPMI 1640 medium containing 10% PBS. Cells were then washed, treated with 50 µg/mL amikacin (Euromedex) for 1 h at 37 °C to kill all extra-cellular bacteria, washed again and finally seeded in 384-well plates (5 × 10<sup>5</sup> cells/mL) containing 2-fold dilutions of compounds in DMSO. The final volume of DMSO was kept under 0.3%. Plates were incubated for 5 days at 37 °C, 5% CO<sub>2</sub>. Infected cells were stained for 30 min using Syto60 dye (Invitrogen) at a final concentration of 5 µM before reading using fluorescence confocal microscope (20X water objective; GFP: λ<sub>ex</sub> 488 nm, λ<sub>em</sub> 520 nm;
Sytro60: $\lambda_{ex}$ 640 nm, $\lambda_{em}$ 690 nm). Dose-responses were fitted using Prism software (sigmoidal dose–response, variable slope model). The MIC$_{50}$ was determined using ten-point dose-response curves as an average of the MIC$_{50}$ of 4 parameters, the ratio of infected cells, the total area of bacteria, the cells number and the bacterial area per infected cell. In each plate, negative control with 1% DMSO (i.e., infected macrophages only); as well as positive controls containing 10 µg/mL INH, ETO and Rif were also included.

4.3. HPOX target enzymes identification

4.3.1. Activity-based protein profiling (ABPP)

Homogeneous bacterial suspension of M. tb mc²6230 in 7H9-S was adjusted at an OD$_{600}$ of 60 and then incubated with the selected HPOX inhibitor (400 µM final concentration) or DMSO (control) at 37 °C for 2–3 h. under gentle shaking at 75 rpm. Bacteria were then washed three times with PBS containing 0.05% Tween 80, resuspended in PBS buffer at a 1:1 (w/v) ratio and then lysed by mechanical disruption on a BioSpec Beadbeater. Both HPOX-treated M. tb mc²6230 and DMSO-control lysate samples (750 µL–0.75 mg total proteins) were labeled with 2 µM Dethiobiotin-FP probe for 90 min at room temperature. Samples were enriched for biotinylated proteins using Nanolink streptavidin magnetic beads 0.8 µm (Solulink), according to the manufacturer’s instructions. The resulting captured biotinylated proteins solution was mixed with 5X Laemmli reducing sample buffer, and heated at 95 °C for 5 min. The released denatured proteins were subjected to tropic digestion, peptide extraction, and LC-MS/MS analysis as described below.

Alternatively, the HPOX-treated M. tb mc²6230 and DMSO-control lysate samples (100 µL–100 µg total proteins) were incubated with 2µM ActivX TAMRA-FP probe (Thermo Fisher Scientific) for 90 min at room temperature and in absence of light. The reaction was stopped by adding 5x Laemmli reducing sample buffer and boiling at 95 °C for 5 min. The labeled proteins were further separated by SDS-PAGE electrophoresis. TAMRA fluorescence (TAMRA: $\lambda_{ex}$ 557 nm, $\lambda_{em}$ 583 nm) was detected using a ChemiDoc MP Imager (Bio-Rad). Detailed Materials and Methods is given in Supplementary Material.

4.3.2. Protein identification and quantification

Protein extract were loaded and stacked on a NuPAGE gel (Life Technologies). Stained bands were submitted to an in-gel trypsin digestion [68]. Peptides extracts were reconstituted with 0.1% trifluoroacetic acid in 4% acetonitrile and analyzed by liquid chromatography-(LC)-tandem mass spectrometry (MS/MS) via an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Electron, Bremen, Germany) online with an ultimate 3000QSLC nano chromatography system (Thermo Fisher Scientific, Sunnyvale, CA). Protein identification and quantification were processed using the MaxQuant computational proteomics platform, version 1.5.3.8 [69] using a UniProt M. tuberculosis ATCC 25,618 database (date 2018.01; 2164 entries). The statistical analysis was done with Perseus program (version 1.5.6.0). Differential proteins were detected using a two-sample t-test at 0.01 and 0.05 permutation based FDR. The mass spectrometry proteomics data, including search results, have been deposited to the ProteomeXchange Consortium (www.proteomexchange.org) [70] via the PRIDE partner repository with the dataset identifier PXD010255. Detailed Materials and Methods is given in Supplementary Material.

4.4. Inhibition assays on pure recombinant proteins

The three lipolytic enzymes from M. tb, the thiosterase TesA, the monoacylglycerol lipase Rv0183, and the Cutinase-like protein Cfp21 were produced and purified as previously reported [48,55].

The lipase-inhibitor pre-incubation method was used to test, in aqueous medium and in the absence of substrate, the possible direct reactions between lipases and inhibitors previously described [27,56,71]. Briefly, an aliquot of each enzyme was pre-incubated at 25 °C with HPOX at various inhibitor molar excess ($x$) ranging from 1 to 40 related to 1 mol of enzyme. A sample of the incubation medium was collected after 30 min incubation period and the residual enzyme activity was measured. The variation in the residual lipase activity allowed determination of the inhibitor molar excess which reduced the enzyme activity to 50% of its initial value ($x$)$_{50}$ [27,56,71]. In each case, control experiments were performed in the absence of inhibitor. The respective enzymatic activity of TesA and Cfp21 were assessed using para-nitrophenyle (pNP) ester assay with pNP caprylate (pNP-C8) as substrate, as described previously [23]. Rv0183 residual activity was determined using monooenole as substrate, as reported in [48,56]. Dose-response curves were fitted in Kaleidalog 4.2 Software (Synergy Software). Results are expressed as mean values ± SD of at least two independent assays.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bioorg.2018.08.025.

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