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Oxadiazolone derivatives, new promising multi-target inhibitors against M. tuberculosis

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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) and Totally drug-resistant (TDR) TB strains has led to the emergence of multidrug-resistant TB with poor outcomes and high mortality, especially in people co-infected with HIV/AIDS [7].

Abbreviations: ABPP, activity-based protein profiling; CC50, compound concentration leading to 50% of cell cytotoxicity; CyC, Cyclipostins & Cyclophostin analogs; ETO, ethionamide; FM, foamy macrophages; HSL, hormone-sensitive lipase; ILI, 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 microtitre assay; RIF, rifampicin; TB, tuberculosis; TAG, triacylglycerols; TDR, totally drug-resistant strains; XDR, extensively drug-resistant strains; xI, inhibitor molar excess related to 1 mol of enzyme; xI50, inhibitor molar excess leading to 50% enzyme inhibition

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Fig. 1. Chemical structure of (A) 5-(pyridin-4-yl)-1,3,4-oxadiazol-2(3H)-one SS7, and (B) 5-methoxy-3-(3-phenoxophenyl)-1,3,4-oxadiazol-2(3H)-one MmPPOX and its mode of action. (C) General procedure for the one step preparation of 5-alloxy-3-phenyl substituted-1,3,4-oxadiazol-2(3H)-one compounds from either (3-phenoxophenyl)hydrazine hydrochloride (1), (4-phenoxophenyl)hydrazine hydrochloride (2) or phenylhydrazine hydrochloride (3), giving OX derivatives 5a-k (i.e., RmPPOX), 6a,d,e,k (i.e., RpPPOX) or 7a,d,e,k (i.e., RPOX), respectively. Reagents and conditions: (i) Allyl chloroformate 4a-k, Pyridine, 0 °C to RT; (ii) ClCO2CCl3, CHCl3, Pyridine, 0 °C to RT, 40–85%. Adapted from [27].

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 phenox group (in meta or para position) when present (Fig. 1C), and synthesized as previously reported [27]. The (phenoxophenyl) 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) represents the meta (or para) Phenoxy group when present; P the phenyl group; OX the Oxadiazolone core; and R the alkyl chain (i.e., Me, benzyloxyethyl; M, methyl, E, ethyl; B, butyl; iB, isobutyl; H, hexyl; O, octyl; Eh, 2-ethylhexyl; D, decyl; Do, dodecyl; Me, methoxyethyl).

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 mc<sup>2</sup>6300, a H37Rv strain with its RD1 region and panCD genes deleted, resulting in an avirulent pan(−) phenotype [28]. The corresponding MIC<sub>50</sub> 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 (MIC<sub>50</sub>)}
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 32.1 ± 1.0 µM). In all other cases, MIC50 values were indicative either of a weak (mean MIC50 = 84.1 ± 9.1 µM for MmpPOX, MPOX and MemPPOX), or a moderate (mean MIC50 = 46.9 ± 5.1 µM for BmpPOX, iBmpPOX, HmpPOX, HpPOX, HPOX, OmPPOX, EhmPPOX, BepPOX 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, iBPOX, 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 oxadiazolone 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. MpPOX; iBmpPOX vs. iBpPOX; HmpPOX vs. HpPOX; BmpPPOX vs. BepPPOX). Remarkably, iBPOX 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 HmpPOX & iBmpPOX on the one hand, and BmpPOX & BepPPOX on the other hand. Finally, in absence of the bulky phenoxy group, the best MIC50s against M. tb mc²6230 were also obtained with HPOX and BePOX vs. MPOX and iBPOX. 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, methoxyethyl, 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.

Table 1

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MIC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M. marinum</td>
</tr>
<tr>
<td>INH</td>
<td>20.5</td>
</tr>
<tr>
<td>RIF</td>
<td>1.4</td>
</tr>
<tr>
<td>MmPPOX</td>
<td>7.0</td>
</tr>
<tr>
<td>MpPPOX</td>
<td>11.5</td>
</tr>
<tr>
<td>MPOX</td>
<td>52.9</td>
</tr>
<tr>
<td>EmPPOX</td>
<td>3.4</td>
</tr>
<tr>
<td>MemPPOX</td>
<td>2.7</td>
</tr>
<tr>
<td>iBmPPOX</td>
<td>4.1</td>
</tr>
<tr>
<td>iBmpPPOX</td>
<td>13.6</td>
</tr>
<tr>
<td>iBpPPOX</td>
<td>6.9</td>
</tr>
<tr>
<td>iBPOX</td>
<td>2.5</td>
</tr>
<tr>
<td>HmpPOX</td>
<td>11.1</td>
</tr>
<tr>
<td>HpPPOX</td>
<td>3.4</td>
</tr>
<tr>
<td>HPOX</td>
<td>2.6</td>
</tr>
<tr>
<td>BmpPPOX</td>
<td>5.6</td>
</tr>
<tr>
<td>BepPPOX</td>
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</tr>
<tr>
<td>BePOX</td>
<td>1.9</td>
</tr>
<tr>
<td>OmPPOX</td>
<td>8.0</td>
</tr>
<tr>
<td>EhmpPPOX</td>
<td>8.3</td>
</tr>
<tr>
<td>DmPPOX</td>
<td>10.5</td>
</tr>
<tr>
<td>DomPPOX</td>
<td>19.5</td>
</tr>
</tbody>
</table>

* Experiments were performed as described in Section 4. MIC50: compound minimal concentration leading to 50% of growth inhibition, as determined by the REMA assay. The best MIC50 obtained for each strain are highlighted in bold. Values are mean of at least two independent assays performed in triplicate. INH, isoniazid; RIF, rifampicin.
Table 2
Antitubercular activities of the most active OX derivatives against M. tb H37Rv.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Extracellular growth ( \text{MIC}_{50} ) (µM)</th>
<th>Intracellular macrophage growth ( \text{MIC}_{50} ) (µM)</th>
<th>CC\textsubscript{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH\textsuperscript{c}</td>
<td>1.2</td>
<td>1.2</td>
<td>&gt; 150</td>
</tr>
<tr>
<td>RIF\textsuperscript{c}</td>
<td>0.01</td>
<td>2.9</td>
<td>24</td>
</tr>
<tr>
<td>ETO\textsuperscript{c}</td>
<td>6.0</td>
<td>6.0</td>
<td>120</td>
</tr>
<tr>
<td>iBPOX</td>
<td>32.0</td>
<td>8.5</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>iBPOX\textsuperscript{b}</td>
<td>&gt; 50</td>
<td>17.1</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>HpPPOX</td>
<td>&gt; 50</td>
<td>9.5</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>HPOX</td>
<td>44.6</td>
<td>No effect</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>BePPOX</td>
<td>&gt; 50</td>
<td>3.5</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>BePOX</td>
<td>30.8</td>
<td>No effect</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Experiments were performed as described in Section 4. MIC\textsubscript{50} compound minimal concentration leading to 50% of growth inhibition. CC\textsubscript{50} 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.

\textsuperscript{b} Raw264.7 macrophages were infected by M. tb H37Rv-GFP at a MOI of 2:1.

\textsuperscript{c} Data from [36]. INH, isoniazid; RIF, rifampicin; ETO, ethionamide.

Consequently, each of the 19 OX derivatives have been further evaluated for their specific antitubercular activity against extracellularly- and intracellularly-growing virulent M. tb H37Rv-GFP strain, using a high-content screening assay based on the fluorescence measurement of GFP-expressing bacteria. In vitro growth (i.e., extracellular assay) of M. tb H37Rv-GFP was first monitored after 5 days at 37 °C in presence of increasing concentrations of candidate inhibitors [31,36–38]. Intracellular growth of M. tb H37Rv-GFP was also assessed following a 5-day exposure of infected Raw264.7 murine macrophage cell line to the various OX compounds. In this case, the percentage of 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 MIC\textsubscript{50} of the compound [36,39,40]. In subsequent experiment, the concentration leading to 50% of host cell cytotoxicity, i.e. CC\textsubscript{50}, 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 MIC\textsubscript{50} (30.8 and 44.6 µM, respectively) than obtained previously on M. tb mc\textsuperscript{2}H37Rv. In contrast, iBPOX, HpPPOX and BePPOX showed a clear preference against intracellularly-replicating mycobacteria with similar MIC\textsubscript{50} values (3.5–17.1 µM) than the first line antibiotics. Of interest, only iBPPPOX 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 CC\textsubscript{50} > 100 µM, similarly to INH (CC\textsubscript{50} > 150 µM) and ethionamide (CC\textsubscript{50} ~ 120 µM), two potent anti-TB drugs (Table 2). Their respective selectivity index (SI = CC\textsubscript{50}/MIC\textsubscript{50}) 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 OX compounds to target these classes of enzymes.

Interestingly, the observed differences in the behavior of OX compounds; in particular the higher activity against intracellular bacteria than against extracellular ones, have also been reported in the case of the CyC compounds family [31]. As previously discussed above, inhibition of M. tb H37Rv may result from several (and probably different) mechanisms of action or penetration of the OX derivatives. With the hexyl and benzoxoyethyl chain, the absence/presence of the phe-noxy group in para position clearly shifted the activity 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 iBPPPOX and iBPOX are found most
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. extracellularly-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* mc²6230 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 Desthioibiotin-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 Desthioibiotin-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 AmiC (Rv2888c) and AmiD (Rv3375); BpoC (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 α/β-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 monoolein 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., x50 value [27,56]. Thereby, a x50 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 x50 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 Orlistat (MIC ~ 25 µM) [41,57] and the human lysosomal acid lipase inhibitor Lalistat (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 CyaC 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.

3. Conclusion

In conclusion, we have developed a new series of promising oxadiazolone-core compounds, active against three pathogenic mycobacteria. By blocking extracellular and/or intracellular *M. tb* growth, we anticipate that these OX probes could thus provide interesting insights in the mechanisms operating during mycobacterial replication, persistence and/or reactivation, which are major issues for deciphering the susceptibility and the general development of TB. Moreover, as these compounds target bacterial pathway yet unexploited by anti-TB compounds, the identification of the mycobacterial enzymes inhibited by these OX compounds in vivo may contribute to background information for the development of new therapeutic strategies for
elimination of either actively replicating or latent bacilli from infected individuals. Accordingly, given the importance of such (Ser/Cys)-enzymes for 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-phenoxyphenyl) 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 diphosgene (step ii) in a one-pot two-steps reaction [27]. All compounds 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-(benzyloxy)ethoxy)-3-(3-phenoxyphenyl)-1,3,4-oxadiazol-2(3H)-one (6a = BepPPOX). (4-phenoxyphenyl)hydrazine hydrochloride [66] 2 (8.2 g, 34.6 mmol, 1 equiv.) and 1-methyl pyrrolidine (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-benzyloxyethyl...
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 (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 brine (3 × 100 mL), dried over MgSO4, and filtered. Purification by column chromatography using cyclohexane/ethyl acetate (98/2 to 95/5) was added using a syringe pump over a period of 1 h at room temperature. The reaction mixture was diluted with water (1 L) and brine (3 × 250 mL). The combined organic layers were washed with water (2 × 250 mL) and brine (3 × 100 mL), extracted with diethyl ether (3 × 250 mL). The combined organic layers were washed with water (2 × 250 mL) and brine (3 × 100 mL).

Table 3

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p<0.05 were selected, and are highlighted in black (non-essential) or in red (essential). Data results from two different experiments processed three times. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Volcano plot of proteomic analysis of HPOX in M. tb mc26230 culture. Volcano plot showing the significance two-sample t-test (Log p-value) versus fold-change (Log2 (FPK normalized intensity in HPOX versus Desthiobiotin-FP condition) on the y and x axes, respectively. Here the plot is zoomed on the positive difference between the two conditions. The full line is indicative of protein hits obtained at a permutation false discovery rate of 1% (pFDR). Only protein hits obtained at a pFDR of 5% (dashed line) and with a score threshold value ≥60 were selected, and are highlighted in black (non-essential) or in red (essential). Data results from two different experiments processed three times. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
4.1.1.3. 5-Hexyloxy-3-(4-phenoxophenyl)-1,3,4-oxadiazol-2(3H)-one (6e = HpPPOX). Prepared using Hexyl chloroformate 4e applying similar method as described above for 6a. Analytical data for HpPPOX: yellow oil (85%). Rf (AcOEt/Cyclohexane 1:3, v/v) 0.95. HRMS (ESI) m/z [M + H]+ calcd. for C14H21N2O4: 263.1390 Da; found: 263.1392 Da. 1H NMR δ 7.77 (dd, J = 9.0 Hz, J = 2.0 Hz, 2H), 7.30 (m, 2H), 6.97–7.12 (m, 5H), 4.37 (t, J = 6.6 Hz, 2H), 1.80 (m, 2H), 1.30–1.58 (m, 6H), 0.90 (t, J = 6.8 Hz, 3H), 13C NMR δ 157.16 (s), 155.33 (s), 154.67 (s), 148.38 (s), 131.65 (s), 129.83 (2 × d), 123.42 (d), 119.79 (2 × d), 119.49 (2 × d), 118.68 (2 × d), 71.79 (t), 31.36 (t), 28.35 (t), 25.17 (t), 22.50 (t), 13.99 (q).

4.1.1.4. 5-Benzylxyloxy-3-phenyl-1,3,4-oxadiazol-2(3H)-one (7a = BePPOX). Prepared using 2-benzylxethyl chloroformate 4a and phenylhydrazine hydrochloride 3 applying similar method as described above for 6a. Analytical data for BePPOX: pale yellow oil (79%). Rf (AcOEt/Cyclohexane 1:3, v/v) 0.36. HRMS (ESI) m/z [M + H]+ calcd. for C14H19N2O3: 257.1255 Da; found: 257.1257 Da. 1H NMR δ 7.72 (dd, J = 9.0 Hz, J = 2.0 Hz, 2H), 7.30 (m, 2H), 7.18–7.73 (m, 8H), 4.60 (s, 2H), 4.54 (m, 2H), 3.83 (m, 2H), 13C NMR δ 155.23 (s), 148.23 (s), 137.44 (s), 136.17 (s), 129.12 (2 × d), 128.55 (2 × d), 127.98 (d), 127.79 (2 × d), 125.56 (d), 117.95 (2 × d), 73.41 (t), 70.55 (t), 66.98 (t).

4.1.1.5. 5-Isobutylxyloxy-3-phenyl-1,3,4-oxadiazol-2(3H)-one (7d = iBPOX). Prepared using Isobutyl chloroformate 4d and phenylhydrazine hydrochloride 3 applying similar method as described above for 6a. Analytical data for iBPOX: white crystals (77%). Mp: 76–77 °C. Rf (AcOEt/Cyclohexane 1:3, v/v) 0.55. HRMS (ESI) m/z [M + Na]+ calcd. for C14H19N2O4Na: 271.0897 Da; found: 271.0895 Da. 1H NMR δ 7.70 (m, 2H), 4.72 (t, J = 6.6 Hz, 2H), 2.75 (m, 1H), 4.18 (d, J = 6.6 Hz, 2H), 1.85 (m, 1H), 1.05 (d, 6H). 13C NMR δ 155.63 (s), 148.51 (s), 136.44 (s), 129.29 (2 × d), 125.67 (d), 118.14 (2 × d), 77.57 (t), 27.94 (t), 18.89 (2 × q).

4.1.1.6. 5-Hexyloxy-3-phenyl-1,3,4-oxadiazol-2(3H)-one (7e = HPOX). Prepared using Hexyl chloroformate 4e and phenylhydrazine hydrochloride 3 applying similar method as described above for 6a. Analytical data for HPOX: white crystals (71%). Mp: 34–35 °C. Rf (AcOEt/Cyclohexane 1:3, v/v) 0.59. HRMS (ESI) m/z [M + H]+ calcd. for C14H19N2O3: 263.1390 Da; found: 263.1390 Da. 1H NMR δ 7.80 (ddd, J = 8.4 Hz, J = 2.3 Hz, J = 0.75 Hz, 2H), 7.42 (dd, J = 7.6 Hz, J = 1.7 Hz, 2H), 7.22 (t, J = 7.4 Hz, 1H), 4.40 (t, J = 6.6 Hz, 2H), 1.85 (m, 2H), 1.33–1.49 (m, 6H), 0.93 (t, 3H). 13C 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-535/M, M. bovis BCG Pasteur 1173P2 and M. tb mc²6230 (H37Rv ΔαnCD [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 n-panthothenate (M. tb mc²6230). 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 (Euromedex). 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 mc²6230

The concentrations of compound leading to 50% of bacterial growth (MIC₅₀) were first determined using the resazurin microtiter assay (REMA) [29,30]. Briefly, log-phase bacteria were diluted to a cell density of 5 × 10⁶ 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% (v/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 (λex = 530 nm; λem = 590 nm) was then measured [30] and the concentration leading to 50% growth inhibition was defined as the MIC₅₀. 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 × 10⁶ 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% CO₂ for 5 days. Bacterial fluorescence levels (RFU) were recorded using a fluorescent microplate reader (Victor X3, Perkin-Elmer). The MIC₅₀ 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 × 10⁵ 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% CO₂. 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: λex 488 nm, λem 520 nm;
Syto60: $\lambda_{ex} = 640\text{ nm, } \lambda_{em} = 690\text{ nm}$. 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 $M. \text{tb}$ 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 3 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 $M. \text{tb}$ 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 (Suloflink), 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 trypsin digestion, peptide extraction, and LC-MS/MS analysis as described below.

Alternatively, the HPOX-treated $M. \text{tb}$ mc²6230 and DMSO-control lysate samples (100 µL–100 µg total proteins) were incubated with 2 µM 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) using 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 proteomes 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 $M. \text{tb}$, the thioesterase TesA, the monocyaglycerol 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 as previously described [27,56,71]. Briefly, an aliquot of each enzyme was pre-incubated at 25°C with HPOX at various inhibitor molar excess ($x_d$) 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-nitrophenyl (pNP) ester assay with pNP caprylate (pNP-C8) as substrate, as described previously [23]. Rv0183 residual activity was determined using monoolein as substrate, as reported in [48,56]. Dose-response curves were fitted in Kaleidagraph 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.

References


