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Dissecting the antibacterial activity of oxadiazolone-core derivatives against Mycobacterium abscessus

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Abstract

Mycobacterium abscessus (M. abscessus), a rapidly growing mycobacterium, is an emergent opportunistic pathogen responsible for chronic bronchopulmonary infections in individuals with respiratory diseases such as cystic fibrosis. Most treatments of M. abscessus pulmonary infections are poorly effective due to the intrinsic resistance of this bacteria against a broad range of antibiotics including anti-tuberculosis agents. Consequently, the number of drugs that are efficient against M. abscessus remains limited. In this context, 19 oxadiazolone (OX) derivatives have been investigated for their antibacterial activity against both the rough (R) and smooth (S) variants of M. abscessus. Several OXs impair extracellular M. abscessus growth with moderated minimal inhibitory concentrations (MIC), or act intracellularly by inhibiting M. abscessus growth inside infected macrophages with MIC values similar to those of imipenem. Such promising results prompted us to identify the potential target enzymes of the sole extra and intracellular inhibitor of M. abscessus growth, i.e., compound iBpPOX, via activity-based protein profiling combined with mass spectrometry. This approach led to the identification of 21 potential protein candidates being mostly involved in M. abscessus lipid metabolism and/or in cell wall biosynthesis. Among them, the Ag85C protein has been confirmed as a vulnerable target of iBpPOX. This study clearly emphasizes the potential of the OX derivatives to inhibit the extracellular and/or intracellular growth of M. abscessus by targeting various enzymes potentially involved in many physiological processes of this most drug-resistant mycobacterial species.

Introduction

Non-tuberculous mycobacteria (NTM) are naturally-occurring bacterial species mostly found in soil and water that do not cause tuberculosis or leprosy [1]. NTM are opportunistic
pathogens able to infect humans with predisposing conditions like cystic fibrosis (CF) or immunosuppression and responsible for wide range of infections like skin infections, pulmonary infections or disseminated diseases [2–4]. In the last decades, NTM infections are increasing worldwide, the most frequently reported species being Mycobacterium avium complex (MAC) and M. abscessus complex [3, 5].

M. abscessus can be isolated from solid medium with either a smooth (S) or a rough (R) colony morphotype [6]. The difference between both morphotypes is related to the presence of glycopeptidolipids (GPLs) in the cell wall of the S variant, while absent in the R one [7]. This latter R strain is also associated with severe and persistent infections [8]. In CF patients, treatment of M. abscessus complex infections requires a multidrug therapy including a daily oral macrolide (clarithromycin or azithromycin) in conjunction with intravenous amikacin and a β-lactam (imipenem or cefoxitin) [9]. However, almost 60% of M. abscessus strains could develop both intrinsic and acquired resistance to currently available antibiotics, including macrolides [4, 10]. As a direct consequence, treatment of such infections has become very complicated with very limited alternative options [5, 11].

Due to the worldwide increasing incidence and prevalence of M. abscessus and the inherent difficulties to manage such resistant pulmonary infections, new active molecules are urgently needed. In this context, we recently investigated the antibacterial activities of 19 oxadiazolone-core (OX) derivatives (Fig 1) against three pathogenic slow-growing mycobacteria: M. marinum, M. bovis BCG as well as M. tuberculosis H37Rv the etiologic agent of tuberculosis [12].

These OX compounds exhibited not only encouraging minimal inhibitory concentrations (MIC), but above all, they were also found to display a diversity of actions by acting either only on extracellular M. tuberculosis growth, or both intracellularly on infected macrophages as well as extracellularly on bacterial growth. Remarkably, all OX derivatives exhibited very low

Fig 1. Chemical structure of the OX derivatives. Rm (or p)PPOX nomenclature is as follows: m (or p)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., M; methyl, E, ethyl; B, butyl; iB, isobutyl; H, hexyl; O, octyl; Eh, 2-ethylhexyl; D, decyl; Do, dodecyl; Be, benzylxyethyI; Me, methoxyethyl). Adapted from [12].
toxicity towards host cell macrophages [12]. Of interest, only the \textit{iBpPPOX} derivative exhibited moderate (MIC$_{50} = 32.0$ μM) to quite good (MIC$_{50} = 8.5$ μM) antibacterial activity against both extracellular and intramacrophagic \textit{M. tuberculosis} H37Rv, respectively [12]. Following an activity-based protein profiling (ABPP) approach combined with mass spectrometry, 18 putative target(s) of \textit{HPOX}, a selective inhibitor of \textit{M. tuberculosis} extracellular growth, were identified. All these proteins were (Ser/Cys)-enzymes possessing a catalytic serine or cysteine residue, and involved in \textit{M. tuberculosis} lipid metabolism and/or in cell wall biosynthesis. Above all, the results of this study imply that such \textit{OX} derivatives represent a novel class of multi-target mycobacterial inhibitors via the formation of a covalent bond with the catalytic residue of various mycobacterial (Ser/Cys)-containing enzymes involved in various physiological processes.

Given all these previous findings, in the present study we have further assessed the antibacterial activity of these 19 \textit{OXs} against \textit{M. abscessus} growth. The determined MIC revealed that some \textit{OXs} were able to inhibit \textit{M. abscessus} growth \textit{in vitro} in culture broth medium and/or intracellularly inside macrophages. In addition, using a similar ABPP assay as previously reported for \textit{M. tuberculosis} [12], the potential target enzymes of \textit{iBpPPOX}, the most active inhibitor of extra- and intracellular bacterial growth, were further identified.

**Materials and methods**

**Bacterial strains and growth conditions**

\textit{M. abscessus} CIP104536 with either a smooth (S) or rough (R) morphotype was grown in Middlebrook 7H9 broth (BD Difco, Le Pont de Claix, France) supplemented with 0.2% glycerol, 0.05% Tween 80 and 0.2% glucose (Sigma-Aldrich, St. Quentin Fallavier, France) (7H9-S).

**Chemicals**

Clarythromycine and Imipenem mixture w/Cilastatin were purchased from Euromedex (Souffelweyersheim, France). The Oxadiazolone derivatives were synthesized as previously reported and were at least 98% pure as determined by HPLC analysis [12]. Stock solutions of each inhibitor (4 mg/mL) were prepared in DMSO and stored at -20 °C before use.

**Resazurin microtiter assay (REMA) for MIC determination—Extracellular assay**

Susceptibility testing was performed using the Middlebrook 7H9 broth microdilution method. MICs of the \textit{OXs} were determined in 96-well flat-bottom Nunclon Delta Surface microplates with lid (Thermo-Fisher Scientific, ref. 167008) using the resazurin microtiter assay (REMA) [12–15]. Briefly, log-phase bacteria were diluted to a cell density of $5 \times 10^6$ cells/mL and 100 μL of this inoculum was grown in a 96-well plate in the presence of serial dilutions of each \textit{OX} compound. After 3–5 days incubation at 37 °C, 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 (\textit{i.e.}, bacteria without antibiotics). Fluorescence of the resazurin metabolite resorufin ($\lambda_{\text{excitation}}$, 530 nm; $\lambda_{\text{emission}}$, 590 nm) was then measured [13, 16] and the concentration leading to 50% and 90% growth inhibition was defined as the MIC$_{50}$ and MIC$_{90}$, respectively. See S1 Appendix for detailed protocol.
Intramacrophage killing assay—Intracellular assay

The intracellular growth of *M. abscessus* S was assessed following a 24 h exposure of infected Raw264.7 murine macrophages cell line (American Type Culture Collection TIB-71) to each of the 19 OX compounds at a final concentration of 30 μM [17]. To avoid growth of extracellular mycobacteria, cells were extensively washed and treated with amikacin (200 μg/mL = 340 μM; 87 × MIC50) prior to treatment with the OX analogs. Imipenem (IMP; 80 μg/mL = 267 μM; 64 × MIC50) was used as positive control for this intracellular killing assay. In each case, the viability of infected macrophages was checked by addition of trypan blue [18] before cell lysis and plating for CFU count. See S1 Appendix for detailed protocol.

iBpPPOX target enzymes identification

Activity-Based Protein Profiling (ABPP) for the identification of iBpPPOX target enzymes. Bacterial suspension of *M. abscessus* R in 7H9-S was adjusted at an OD600 corresponding to 6 × 10⁹ cells/mL and then incubated with iBpPPOX 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 iBpPPOX-treated *M. abscessus* and DMSO-control lysate samples (750 μL – 0.75 mg total proteins) were labeled with 2 μM Desthiobiotin-FP probe for 90 min at room temperature. Samples were enriched for biotinylated proteins using 0.8 μm Nanolink streptavidin magnetic beads (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 tryptic digestion, peptide extraction, and LC-MS/MS analysis as described below.

Alternatively, *M. abscessus* R total lysates (500 μL – 1 mg total proteins) were further preincubated with iBpPPOX (400 μM final concentration) or DMSO as control for 60 min at 37 °C, and then treated with 2 μM ActivX Desthiobiotin-FP probe (ThermoFisher Scientific) and processed as described above for *M. abscessus* R living cells. Detailed protocol regarding ABPP experiments is given in S1 Appendix.

Mass spectrometry analysis for enzyme 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 [19]. Peptides extracts were reconstituted with 0.1% trifluoroacetic acid in 4% acetonitrile and analyzed by liquid chromatography (LC)-tandem mass spectrometry (MS/MS) using Orbitrap Mass Spectrometers (Thermo Electron, Bremen, Germany) online with a nanoLC Ultimate 3000 chromatography system (Dionex, Sunnyvale, CA). Protein identification and quantification were processed using the MaxQuant computational proteomics platform, version 1.5.3.8 [20] using a UniProt *M. abscessus* ATCC 19977 (Taxon 561007) database (date 2017.02; 4940 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. Detailed Materials and Methods are given in S1 Appendix.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (www.proteomeexchange.org) [21] via the PRIDE partner repository with the dataset identifier PXD015680.
Validation of Ag85C<sub>Mabs</sub> by iBpPPOX

**Plasmids and DNA manipulations.** All specific oligonucleotides and plasmids used in this study are listed in S1 Appendix (see S3 and S4 Tables—page S8). All cloned fragments were amplified using purified <i>M. abscessus</i> genomic DNA. The <i>mab<sub>0175</sub></i> gene encoding Ag85C was amplified by PCR using the specific forward (<i>pMyc::ag85C-F</i>) and reverse (<i>pMyc::ag85C-R</i>) primers. For the inactivated Ser124Ala mutant <i>ag85C<sup>S124A</sup>-R</i> were used, the second fragment containing the mutation was generated using the primer sets <i>pMyc::ag85C-F</i> and <i>pMyc::ag85C<sup>S124A</sup>-R</i>. The two fragments were further purified, mixed in 1:1 (v/v) ratio and used as template to amplify the complete insert containing the mutation, using the primer pairs <i>pMyc::ag85C-F</i> and <i>pMyc::ag85C-R</i>. The respective PCR products were cloned into pMyC vector, following digestion with NcoI and HindIII, enabling the incorporation of a α-His-tag in the C-terminus of the Ag85C or Ag85C<sup>S124A</sup> protein. Deletion mutant Δ<i>mab<sub>0175</sub></i> (= Δ<i>ag85C</i>) was obtained by a simple and rapid gene disruption strategy in <i>M. abscessus</i> developed by Viljoen et al. [22].<i>Ag85C </i>gene was amplified using primer pairs <i>pUX1::Δag85C-F</i> and <i>pUX1::Δag85C-R</i>, then cloned into pUX1 vector using NheI and BamHI restriction sites by classical cloning. Finally, for complementation strain, the <i>mab<sub>0175</sub></i> gene was amplified using the primer pairs <i>pVV16::ag85C-F</i> and <i>pVV16::ag85C-R</i>, and cloned into pVV16 plasmid in frame with a α-His-tag located in C-terminal and downstream of the hsp60 promoter also containing a kanamycin resistance cassette using restriction free cloning (SLIC) [23] to generate <i>pVV16::ag85C</i>. Sequence integrity of each construct was confirmed by DNA sequencing (Eurofins Genomics). All the constructs were further transformed in electrocompetent <i>M. abscessus</i> S and R types and selected on respective antibiotic agar plates as described previously [22]. Positive transformants were further grown in 7H9<sup>OADC</sup> medium (i.e., 7H9 broth + 0.2% glycerol + 0.05% Tween 80 + 10% oleic acid, albumin, dextrose, catalase) supplemented with either hygromycin (1000 µg/mL; i.e., overexpression and inactivated strains), kanamycin (250 µg/mL; i.e., deletion strain) or both antibiotics (1000 µg/mL hygromycin + 250 µg/mL kanamycin; i.e., complementation strain), up to OD<sub>600</sub> of 1. The overproduction of the recombinant proteins in the overexpression and inactivated strains as well as in the complementation strain was checked by Western blot using the HisProbe™ HRP conjugate (ThermoFisher Scientific). Regarding the deletion strain, the selection was made based on red fluorescent colonies followed by PCR amplification and sequencing strategy as described in [22].

**Functional validation of Ag85C<sub>Mabs</sub> target enzyme**

The abovementioned transformed bacteria, i.e., the <i>M. abscessus</i> _pMyc::ag85C_ overexpressing strains, the inactivated <i>M. abscessus</i> _pMyc::ag85C<sup>S124A</sup>_ overexpressing strains, the <i>M. abscessus</i> _Δag85C_ deletion strains and their complemented counterparts <i>M. abscessus</i> _Δag85C::C_ were grown in 7H9<sup>OADC</sup> medium supplemented with either hygromycin (1000 µg/mL; i.e., overexpression and inactivated strains), kanamycin (250 µg/mL; i.e., deletion strain), or both antibiotics (1000 µg/mL hygromycin + 250 µg/mL kanamycin; i.e., complementation strain) until the OD<sub>600</sub> reached 2. In the case of the overexpression and inactivated strains, induction was further done with 0.2% acetamide and the culture was incubated at 37°C for additional 24 h. Susceptibility testing of each of the <i>M. abscessus</i> mutant strains against various concentrations of iBpPPOX was further performed as described above.
**Expression and purification of *M. abscessus* antigen Ag85C**

The plasmid harboring the *mab_0175* gene was used to transform the *M. smegmatis* ΔGroEl expression strain. Transformed bacteria were grown in 7H9 medium containing hygromycin (200 μg/mL) until the OD_{600} reached 2.0. Induction was done with 0.2% acetamide and the culture was further incubated at 37 °C for 24 h. One L of bacterial pellets were collected by centrifugation (8,000 \times g, 4 °C, 1 h), re-suspended in 30 mL ice-cold buffer (50 mM Tris pH 8.0 containing 200 mM NaCl), and were broken using a French Pressure cell at 1,100 psi. The lysate was clarified by centrifugation (12,000 \times g, 4 °C, 30 min) prior to purification by nickel affinity chromatography with Ni-NTA sepharose beads and elution with the previous Tris (pH 8.0) buffer containing 500 mM imidazole. Purified protein was concentrated at 1 mg/mL and stored at –80 °C [24, 25].

**In vitro inhibition of pure recombinant *M. abscessus* Ag85C by iBpPPOX**

A 14 μM (*i.e.*, 25 μg) concentration of Ag85C_{Mabs} was incubated for 1 h in its native form with increasing molar excess of iBpPPOX (*i.e.* enzyme/inhibitor molar ratio, E/I = 1:1; 1:5, 1:10, 1:25, 1:50, and 1:75) in a reaction mixture containing 10 mM Tris buffer (pH 8), 150 mM NaCl and 0.1% (w/v) Triton X-100. Each sample was further treated with 10 μM ActiveX TAMRA-FP fluorescent probe (ThermoFisher Scientific) for 1 h at room temperature in the darkness. The reaction was stopped by adding 5X Laemmli reducing buffer followed by boiling, and equal amounts of proteins (12 μg) were separated by 12% SDS-PAGE. Subsequently, TAMRA FP-labeled proteins were detected by fluorescent gel scanning (TAMRA: λ_{ex} 557 nm, λ_{em} 583 nm) using the Cy3 filter of a ChemiDoc MP Imager (Bio-Rad) before staining the gel with Coomassie Brilliant Blue dye. Finally, relative fluorescence quantification of each band was performed using the ImageLab™ software version 5.0 (Bio-Rad) by taking the labeled Ag85C_{Mabs}-TAMRA adduct as 100% absolute fluorescence level.

**Mass spectrometry analysis of Ag85C_{Mabs}-iBpPPOX complex**

Purified Ag85C_{Mabs} Recombinant protein (14 μM– 100 μg) was further incubated for 1 h in its native form with iBpPPOX, using an enzyme/inhibitor molar ratio E/I = 1:100 to ensure total inhibition. Samples of the resulting Ag85C_{Mabs}-iBpPPOX complex were analysed on a MALDI-TOF-TOF Bruker Ultraflex III spectrometer (Bruker Daltonics, Wissembourg, France) controlled by the Flexcontrol 3.0 package (Build 51), as described previously [24] (see S1 Appendix for full details). The total mass of the untreated protein (theoretical Mw = 32,057.83 Da; experimental Mw = 32,048.7 Da) is corresponding to the native enzyme lacking the 36 first N-terminal amino acids (*i.e.*, M^{SVRVKARRVLSALLAFAVMPSMAATAH}_{36}) consisting of a Sec signal peptide cleaved at the Ala-X-Ala (*i.e.*, A^{35}-H_{36}-A^{37}) site, as confirmed by N-terminal Edman sequencing [26].

**Statistical analysis**

Graphpad Prism 5 was used to perform the statistical analyses of the intracellular activity of the OX compounds, and of all susceptibility testing on *M. abscessus* mutant strains. The statistical analysis related to MIC_{50Raw} was completed using a Student’s *t*-test. The statistical significance of differences in the MIC_{50} or MIC_{90} values between each mutant strain was analyzed by one-way ANOVA followed by a post hoc Fisher’s test.
Results and discussion

In vitro activity of oxadiazolone derivatives against *M. abscessus*

Drug susceptibility testing of the OX derivatives was assessed against both S and R variants of *M. abscessus*, with amikacin (AMK) as standard drug. The corresponding MIC$_{50}$/MIC$_{90}$ values for each OX compound, as determined by the REMA assay [12–16], are reported in Table 1. Among all tested compounds, 14 OXs were able to block the growth of *M. abscessus* S variant. The best growth inhibitors were iBpPPOX (33.0 ± 2.0 μM), HpPPOX (32.5 ± 2.2 μM), MemPPOX (41.8 ± 1.6 μM) and BePOX (45.1 ± 3.4 μM) which displayed interesting MIC$_{50}$ values (Table 1). In all other cases, MIC$_{50}$ values were indicative either of a moderate (MIC$_{50}$ around

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MIC$<em>{50}$/MIC$</em>{90}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>M. abscessus</em> CIP104536$^T$</td>
</tr>
<tr>
<td>AMK</td>
<td>3.9 ±0.19 / 5.8 ±0.20</td>
</tr>
<tr>
<td>IMP</td>
<td>4.2 ±0.19 / 6.3 ±0.26</td>
</tr>
<tr>
<td>MnnPPOX</td>
<td>60.7 ±5.0 / 119.3 ±4.2</td>
</tr>
<tr>
<td>MpPPOX</td>
<td>88.2 ±7.3 / 157.5 ±6.2</td>
</tr>
<tr>
<td>MPOX</td>
<td>&gt;200</td>
</tr>
<tr>
<td>EmPPOX</td>
<td>82.8 ±6.5 / 101.8 ±4.6</td>
</tr>
<tr>
<td>MemPPOX</td>
<td>41.8 ±1.6 / 44.4 ±2.0</td>
</tr>
<tr>
<td>BmPPOX</td>
<td>78.1 ±5.3 / &gt;200</td>
</tr>
<tr>
<td>iBmPPOX</td>
<td>122.1 ±7.8 / &gt;200</td>
</tr>
<tr>
<td>iBpPPOX</td>
<td>33.0 ±2.0 / 85.9 ±5.5</td>
</tr>
<tr>
<td>iBPOX</td>
<td>61.3 ±5.1 / 68.8 ±2.4</td>
</tr>
<tr>
<td>HmPPOX</td>
<td>&gt;200</td>
</tr>
<tr>
<td>HpPPOX</td>
<td>32.5 ±2.2 / 79.4 ±3.3</td>
</tr>
<tr>
<td>HPOX</td>
<td>92.9 ±4.2 / 99.9 ±5.5</td>
</tr>
<tr>
<td>BemPPOX</td>
<td>126.7 ±7.3 / 145.7 ±6.9</td>
</tr>
<tr>
<td>BepPPOX</td>
<td>53.7 ±3.1 / 73.5 ±3.2</td>
</tr>
<tr>
<td>BePOX</td>
<td>45.1 ±3.4 / 46.5 ±2.0</td>
</tr>
<tr>
<td>OmPPOX</td>
<td>&gt;200</td>
</tr>
<tr>
<td>EhmPPOX</td>
<td>145.1 ±7.7 / &gt;200</td>
</tr>
<tr>
<td>DmPPOX</td>
<td>&gt;200</td>
</tr>
<tr>
<td>DomPPOX</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

* Experiments were performed as described in Materials and Methods. MIC$_{50}$/MIC$_{90}$: compound minimal concentration leading to 50% or 90% of growth inhibition, respectively, as determined by the REMA assay. Values are mean of at least two independent assays performed in triplicate. AMK, amikacin, IMP, imipenem.

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53–61 μM for MmPPOX, iBPOX, and BepPPOX), a weak (MIC_{50} around 78–93 μM for MmPPOX, EmPPOX, BmPPOX, and HPOX), or a poor (MIC_{50} > 120 μM for iBmPPOX, EhmPPOX, and BemPPOX) antibacterial activity (Table 1).

Considering the MIC_{50} values reached on M. abscessus S, they are up to 2.5-fold greater than the corresponding MIC_{50}; except for HPOX (MIC_{50} = 92.9 ±4.2 μM / MIC_{90} = 99.9 ±5.5 μM), MmPPOX (MIC_{50} = 41.8 ±1.6 μM / MIC_{90} = 44.4 ±2.0 μM) and BePPOX (MIC_{50} = 45.1 ±3.4 μM / MIC_{90} = 46.5 ±2.0 μM) for which both MICs are in the same order of magnitude (Table 1).

Compared to the S morphotype, M. abscessus R variant was nearly 1.3- to 3.6-times less sensitive to the OX compounds (Table 1); a property already observed for many drugs including AMK [27]. The best inhibitors of M. abscessus R growth were iBpPPOX (MIC_{50} = 53.2 ±1.8 μM / MIC_{90} = 104.3 ±5.1 μM), HpPPOX (MIC_{50} = 45.8 ±1.9 μM / MIC_{90} = 103.8 ±4.0 μM), and BepPPOX (MIC_{50} = 52.6 ±2.5 μM / MIC_{90} = 111.1 ±4.1 μM) which exhibited similar MIC_{50} and MIC_{90} values, respectively (Table 1). Interestingly, MmPPOX bearing a short methyl chain has no antibacterial effect as compared to the three abovementioned para-phenoxyphenyl derivatives. In summary, iBpPPOX, HpPPOX, and BepPPOX all possessing the phenoxy group in a para position as well as bulky ester chains, displayed the best antibacterial activity against M. abscessus R. No other clear trends or rules in terms of structure-activity relationships (SAR) have emerged regarding the potency of these oxadiazolone-core compounds against M. abscessus.

It is noteworthy that with MIC_{50} values ranging from 31 to >120 μM [12], M. tuberculosis susceptibility to the OX compounds is similar to that of the S variant of M. abscessus; iBpPPOX being the best growth inhibitor of both species. The increased tolerance of the most-virulent M. abscessus R variant towards the OX compounds is in line with its high resistance to classical antibiotics [4] compared to M. tuberculosis; a result that supports M. abscessus R’s nickname of “antibiotics nightmare” [28].

### Intramacrophagic susceptibility of Mycobacterium abscessus to OX derivatives

Macrophages, as the primary target, represent the host’s first line of defense but also an important reservoir of mycobacteria in lungs. From our previous work, the OXs were able to inhibit the growth of M. tuberculosis inside infected macrophages, and found to be non-toxic for Raw264.7 murine macrophages cell line with a CC_{50} > 100 μM (i.e., compound concentration leading to 50% cell toxicity) [12]. Considering such properties, we further investigated the ability of OXs to inhibit the intra-macrophagic growth of M. abscessus.

The intrinsic nature of the R variant is to form bacterial clumps and cords in culture medium with time. As reported by Bernut et al., M. abscessus R cording prevents its phagocytosis by macrophages. Consequently, the strain continues to grow extracellularly, and rapidly induces cell toxicity leading to cell death [29, 30]. Such cording characteristic makes macrophage infection experiments using M. abscessus R very difficult to handle. Indeed, nearly all macrophages were lysed at 24 h post-infection with M. abscessus R variant, making it impossible to quantify the intracellular effect of the OXs. This is, however, not the case with M. abscessus S for which more homogenous bacterial suspensions can be obtained for macrophages infection studies [25, 31, 32].

Therefore, Raw264.7 cells were infected with M. abscessus S at a multiplicity of infection (MOI) of 10, and then incubated for 24 h with all the OX compounds at a final concentration of 90, 60 and 30 μM, or with imipenem (IMP) used as positive drug control. Among the 19 compounds tested, only 3 OXs (i.e., MPOX, MmPPOX, and iBpPPOX) exhibited an antibacterial activity against intracellular M. abscessus growth. Interestingly, MmPPOX and MPOX,
which are weakly and not active against extracellular bacilli, respectively, were however able to significantly decrease the intramacrophagic _M. abscessus_ present 24 h after infection (Fig 2). _MpPPOX_ displayed a moderate activity against intracellular _M. abscessus_ S (Fig 2) with an approximated MIC₅₀ of around 75 μM which is 2.6 times higher that of IMP (MIC₅₀ = 28.3 μM). In contrast, 24 h-treatment with 30–60 μM _MPOX_ led to a 53% reduction in mycobacteria which increased up to 73.5% at 90 μM, a percentage value comparable to the one elicited by IMP, i.e., 74.0% reduction following treatment with 60 μM (Fig 2). Remarkably, and as observed previously for _M. tuberculosis_[12], _iBpPPOX_ was the sole identified inhibitor able to impair extracellular as well as intracellular growth of _M. abscessus_. A plateau value corresponding to 58.5 ± 0.8% bacterial killing was indeed reached, whatever the _iBpPPOX_ concentration used (30–90 μM) to treat the infected cells.

Such a difference between the intra and extracellular activities has already been reported in our previous works with the _OX_ derivatives [12], as well as with another family of growth inhibitors, the Cyclipostins & Cyclophostin analogs [13, 14] acting against _M. tuberculosis_ and _M. abscessus_ [25]. Similar to _M. tuberculosis_[12], the intracellular and extracellular inhibition of _M. abscessus_ growth may probably result from several different mechanisms of action or penetration of the _OX_ derivatives. The short methyl chain _MpPPOX_ and _MPOX_ display a better antimycobacterial activity against intramacrophagic _M. abscessus_ than in broth medium.

This clear preference against intracellularly-replicating mycobacteria may imply that the intracellular activity and/or the targets of these two compounds might differ from that of _OXs_ acting on extracellularly-replicating bacilli. Several factors may indeed account for these discrepancies, such as the metabolic status/fitness which varies between extra- and intracellular replicating bacteria. Another hypothesis could be that their corresponding target(s) would be more accessible and/or vulnerable during the intracellular lifestyle of _M. abscessus_. A specific response of the macrophage stimulated by the action of these compounds and leading to bacterial clearance cannot, however, be excluded. On the other hand, the _iBpPPOX_ retains a similar
activity against *M. abscessus* both extracellularly (MIC\textsubscript{50} = 33.0 μM) and inside macrophages (~59% bacterial clearance at 30 μM). Regarding its intracellular antibacterial activity, the presence of a plateau value, whatever the concentration used, might underline a different effect of iBP\textsubscript{PPOX} towards infected macrophages compared to M\textsubscript{m}PPOX and MPOX for which a more classical dose-response has been reached. As mentioned above, one can speculate that the cellular stress caused by the action of iBP\textsubscript{PPOX} on the infected macrophages might induce a specific stringent response of these host cells, such as possible cell metabolism, therefore leading to bacterial death.

Given the previously determined very low toxicity of the three selected compounds toward Raw264.7 cells with CC\textsubscript{50} > 100 μM [12] similar to AMK (CC\textsubscript{50} ≥ 150 μM) [33], the selectivity index (SI = CC\textsubscript{50}/MIC\textsubscript{50}\textsubscript{Raw}) of these best intracellular inhibitors on *M. abscessus* vs. Raw264.7 cells was thus valued to be in a range from around 1.3 for M\textsubscript{p}PPOX and up to >3 for iBP\textsubscript{PPOX}.

From these findings, it can be assumed that the observed inhibitory potency of the OX compounds i) might result from the inhibition of specific but most likely distinct mycobacterial target enzymes between intramacrophagic- vs. extracellularly-replicating bacilli; or ii) may reflect differences in the uptake and accumulation of the different compound inside the macrophage. Overall, these results suggest that both M\textsubscript{p}PPOX, MPOX and iBP\textsubscript{PPOX} would be able to enter the macrophages and arrest bacterial replication without exhibiting significant toxicity for the host cell.

**iBP\textsubscript{PPOX} inhibit *M. abscessus* by targeting various serine/cysteine enzymes**

Given the previous results obtained with the HPOX on target enzymes identification during *M. tuberculosis in vitro* growth in broth medium [12], we thus performed a similar ABPP approach [12, 13, 34–37] to identify the potential target enzymes impacted by iBP\textsubscript{PPOX}, the sole extra and intracellular inhibitor of *M. abscessus* growth.

The R variant being associated to the most virulent form of *M. abscessus* and thus to severe pulmonary infections [6, 28, 38]; a crude lysate of *M. abscessus* R was, in the first approach, incubated with the iBP\textsubscript{PPOX} inhibitor (or DMSO as a control) and then subjected to competitive probe labelling/enrichment assay with the ActivX™ Desthiobiotin-FP probe (Thermo-Fisher Scientific), as reported previously in the case of *M. tuberculosis* [12, 13]. The obtained enriched mixtures were further 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 also found in the control experiment (i.e., DMSO alone for unspecific binding to streptavidin-magnetic beads) were not considered. A panel of 58 distinct protein candidates were then identified with a permutation false discovery rate (pFDR) of 10%, which was reduced to 21 and 11 when applying a pFDR of 5% and 1%, respectively (see S1 Table).

Since most of the identified proteins were putative in *M. abscessus*, the corresponding orthologs in *M. tuberculosis* H37Rv have been reported to bring more information about their essentiality, activity and predicted location [39]. Eleven out of 21 identified proteins (at a pFDR of 5%) were (Ser/Cys)-based enzymes, mainly involved in lipid metabolism and cell wall biosynthesis [40, 41]. These included the probable serine protease PepD (MAB\_1078); the D-amino acid aminohydrolase MAB\_2605c (i.e., Rv2913c); the probable carboxylesterase MAB\_1919 (i.e., Rv2223c); and the putative β-lactamase MAB\_2833 (i.e., Rv1367c) possibly involved in cell wall biosynthesis. Three members of the lipase family Lip [42], LipH (MAB\_2039), LipN (MAB\_3270c) and LipI (MAB\_2814); three Cutinase-like proteins [41], Cut2 (MAB\_3263), Cut3 (MAB\_3765) and Cut4 (MAB\_3766); and MAB\_175 (Ag85C), a
member of the antigen 85 (Ag85) complex [24, 43] which catalyzes the biosynthesis of trehalose dimycolate, triacylglycerol as well as the mycolylation of arabinogalactan, were also uncovered with iB\textsubscript{P}PPOX.

In a second approach, similar ABPP experiments were performed on living bacterial cells in order to take into account the ability of iB\textsubscript{P}PPOX to penetrate/diffuse through the mycobacterial cell wall. Accordingly, \textit{M. abscessus} R cells were grown to log phase and incubated with iB\textsubscript{P}PPOX or DMSO as a control. After cell lysis, the obtained total lysate was processed as described above with ActivX™ Desthiobiotin-FP probe and streptavidin magnetic beads. Tryptic digestion followed by tandem mass spectrometry analysis led to the identification of 21 protein candidates at a pFDR of 5%, and only 5 at a pFDR of 1% (Table 2 and S2 Table).

Although 4 of the identified proteins are only conserved hypotheticals, the remaining 17 ranged in their functional category from intermediary metabolism/respiration (8 proteins), lipid metabolism (4 proteins), regulatory pathways (3 proteins), cell wall/cell processes (1 protein), and information pathways (1 protein). Among them, MAB\_1675, the probable DNA repair protein RecO (\textit{i.e.}, Rv2362c), and MAB\_1053c (\textit{i.e.}, Rv0948c) a putative chorismate mutase possibly involved in phenylalanine, tyrosine and tryptophan biosynthesis, are annotated as essential enzymes for the \textit{in vitro} growth of \textit{M. tuberculosis} [44, 45]. In good agreement with our previous work on \textit{M. tuberculosis} target enzymes [12], several hydrolases were

<p>| Table 2. iB\textsubscript{P}PPOX target proteins identified at a pFDR of 1% and 5% in \textit{M. abscessus} R culture by LC-ESI-MS/MS analysis. |</p>
<table>
<thead>
<tr>
<th>Protein Ids</th>
<th>Mol. Weight [kDa]</th>
<th>M. tuberculosis orthologs</th>
<th>Location\textsuperscript{b}</th>
<th>Activity / Function</th>
<th>Functional category\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAB_0176</td>
<td>35.825</td>
<td>Rv3804c</td>
<td>CF/M</td>
<td>Secreted antigen 85-A FbpA (Ag85A)</td>
<td>LM</td>
</tr>
<tr>
<td>MAB_0177</td>
<td>34.909</td>
<td>Rv3804c</td>
<td>CF/M/WCL</td>
<td>Antigen 85-A/B/C precursor</td>
<td>LM</td>
</tr>
<tr>
<td>MAB_0274c</td>
<td>20.371</td>
<td>-</td>
<td>uncharacterized protein</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MAB_0401</td>
<td>46.209</td>
<td>Rv6517</td>
<td>-</td>
<td>Possible acyltransferase</td>
<td>IM/R</td>
</tr>
<tr>
<td>MAB_0520</td>
<td>38.811</td>
<td>Rv3626c</td>
<td>-</td>
<td>uncharacterized protein</td>
<td>-</td>
</tr>
<tr>
<td>MAB_0684c</td>
<td>26.813</td>
<td>Rv0774c</td>
<td>CF</td>
<td>Hypothetical extracellular esterase</td>
<td>CW/CP</td>
</tr>
<tr>
<td>MAB_1053c</td>
<td>10.305</td>
<td>Rv0948c</td>
<td>\textit{In vitro growth}</td>
<td>WCL</td>
<td>Chorismate mutase</td>
</tr>
<tr>
<td>MAB_1675</td>
<td>28.418</td>
<td>Rv2362c</td>
<td>\textit{In vitro growth}</td>
<td>CW</td>
<td>Possible DNA repair protein RecO</td>
</tr>
<tr>
<td>MAB_2366</td>
<td>33.804</td>
<td>Rv1701</td>
<td>-</td>
<td>Probable integrase</td>
<td>RP</td>
</tr>
<tr>
<td>MAB_2477c</td>
<td>55.217</td>
<td>Rv1393c</td>
<td>-</td>
<td>Probable monooxygenase</td>
<td>IM/R</td>
</tr>
<tr>
<td>MAB_2478c</td>
<td>15.382</td>
<td>-</td>
<td>uncharacterized protein</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MAB_2545c</td>
<td>35.436</td>
<td>Rv0480c</td>
<td>M/WCL</td>
<td>Possible amidohydrolase</td>
<td>IM/R</td>
</tr>
<tr>
<td>MAB_2943c</td>
<td>31.546</td>
<td>Rv1543</td>
<td>M/WCL</td>
<td>Possible fatty acyl-CoA reductase</td>
<td>LM</td>
</tr>
<tr>
<td>MAB_3336c</td>
<td>54.339</td>
<td>Rv2045c</td>
<td>-</td>
<td>Carboxylesterase LipT</td>
<td>IM/R</td>
</tr>
<tr>
<td>MAB_3398</td>
<td>17.635</td>
<td>Rv1718</td>
<td>-</td>
<td>uncharacterized protein</td>
<td>-</td>
</tr>
<tr>
<td>MAB_3661</td>
<td>57.093</td>
<td>Rv3308</td>
<td>M</td>
<td>Probable phosphomannomutase PmmB</td>
<td>IM/R</td>
</tr>
<tr>
<td>MAB_3689</td>
<td>26.374</td>
<td>Rv3342</td>
<td>WCL</td>
<td>Possible methyltransferase</td>
<td>IM/R</td>
</tr>
<tr>
<td>MAB_3705</td>
<td>19.995</td>
<td>Rv2506</td>
<td>CF/M</td>
<td>Putative TetR family regulatory protein</td>
<td>RP</td>
</tr>
<tr>
<td>MAB_4103c</td>
<td>30.192</td>
<td>Rv1523</td>
<td>-</td>
<td>Probable methyltransferase</td>
<td>IM/R</td>
</tr>
<tr>
<td>MAB_4201c</td>
<td>22.905</td>
<td>Rv3574</td>
<td>WCL</td>
<td>Transcriptional regulatory protein KsrR</td>
<td>RP</td>
</tr>
<tr>
<td>MAB_4750</td>
<td>27.932</td>
<td>Rv1544</td>
<td>M/WCL</td>
<td>Possible ketoacyl reductase</td>
<td>LM</td>
</tr>
</tbody>
</table>

In \textbf{bold}, the 5 proteins identified at a pFDR of 1%.

\textsuperscript{a} From [44, 45].

\textsuperscript{b} CF: Culture filtrate; CW: Cell wall; M: Membrane fraction; WCL: Whole cell lysate.

\textsuperscript{c} IM/R: intermediary metabolism/respiration; IP: information pathways; CW/CP: cell wall/cell processes; LM: lipid metabolism; RP: regulatory protein.

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detected, including one hypothetical extracellular esterase (MAB_2181c), three putative methyltransferases (MAB_3689, MAB_4103c, MAB_0401); the carboxylesterase LipT (MAB_3336c) belonging to the Lip-family members, and the mycolyltransferases MAB_176 (Ag85A) and MAB_177 (Ag85-A/B/C precursor) two members of the Ag85 complex (Table 2 and S2 Table).

It is noteworthy that among these 21 potential hits, only Ag85 proteins were previously detected in the iBpPPOX-treated total lysate (see S1 and S2 Tables); thus, implying that nearly 19 proteins had not been detected in the previous treated M. abscessus total lysate, or at least at a pFDR \( \leq 10\% \). On the other hand, such result suggests that Antigen 85 proteins may be the first target enzymes encountered and thus inhibited by the OX compounds.

**Validation of M. abscessus Ag85C as vulnerable target of iBpPPOX**

Knowing the importance of the Ag85 complex in mycobacterial membrane integrity due to its central role in cell envelope biogenesis, and given the fact that inhibiting the Ag85C was found to restrict *M. tuberculosis* growth [46], we decided to confirm the Ag85C \( \text{Abs} \), which shares nearly 58\% amino acid sequence identity with its *M. tuberculosis* ortholog and retains the same conserved catalytic triad (i.e., Ser\(^{124}\)-Glu\(^{228}\)-His\(^{266}\)), as a potential target of the OX compounds.

We thus followed two different strategies: the first one was based on the susceptibility testing of various *M. abscessus* mutant strains to the iBpPPOX; and the second one relied on the molecular interaction between the iBpPPOX and the purified recombinant Ag85C \( \text{Abs} \).

In the first step, genes encoding either Ag85C \( \text{Abs} \) or the inactivated Ag85C\(^{S124A}\) protein were cloned and overexpressed in *M. abscessus* S and R variants using the pMyc::ag85C \( / \) pMyc::ag85C\(^{S124A}\) inducible plasmids, where genes were cloned under the control of an acetamide promoter (Fig 3A). Moreover, a deletion mutant of Ag85C \( \text{Abs} \) named \( \Delta \text{ag85C} \) was generated by using a recent one-step single cross-over system with the pUX1 vector [22]; and its complemented counterpart \( \Delta \text{ag85C}:: \text{C} \) (Fig 3B) was obtained using the pVV16::ag85C complementation plasmid which allows the constitutive production of recombinant Ag85C \( \text{Abs} \), under the control of the *hsp60* promoter (see S1 Appendix for cloning details). In each case, the overexpression/complementation of antigen 85C protein was confirmed by Western blotting as compared to the parental strain (WT) (Fig 3).

In order to examine whether the overexpression, inactivation or deletion/complementation of the Ag85C \( \text{Abs} \) protein affect the strain susceptibility to the iBpPPOX compound, their respective MICs were further determined.

As depicted in Table 3, the overexpression of Ag85C \( \text{Abs} \) protein (i.e., *M. abscessus* S_pMyc::ag85C and *M. abscessus* R_pMyc::ag85C) led to a significant increase in MIC\(_{50}\) values by 2.7-fold for both the S (87.3 \( \pm \)3.4 \( \mu \)M; \( p\)-value \( < 0.01 \)) and R variant (148.2 \( \pm \)2.1 \( \mu \)M; \( p\)-value \( < 0.01 \)), as well as in MIC\(_{90}\) values (\( > 200 \) \( \mu \)M), compared to the respective pMyc vector control and wild-type strains. These results clearly suggest that Ag85C \( \text{Abs} \) is responsible for the decreased susceptibility to the iBpPPOX, thus confirming this protein as one of the targets of our compound.

Regarding the inactivated Ag85C\(^{S124A}\) mutant *M. abscessus* S_pMyc::ag85C\(^{S124A}\), the gene deletion mutant *M. abscessus* S_\( \Delta \text{ag85C} \) and its complemented counterpart *M. abscessus* S_\( \Delta \text{ag85C}:: \text{C} \), as well as the wild-type *M. abscessus* S strain, they all responded similarly to iBpPPOX. In the case of *M. abscessus* R, although no significant variation was observed in MIC\(_{90}\) values (mean MIC\(_{90}\) = 111.1 \( \pm \)8.4 \( \mu \)M), a slight decrease in MIC\(_{90}\) of around 0.89- to 0.58-fold was reached for the inactivated Ag85C\(^{S124A}\) (47.5 \( \pm \)2.0 \( \mu \)M; \( p\)-value \( < 0.05 \)) and the \( \Delta \text{ag85C} \) (30.9 \( \pm \)2.1 \( \mu \)M; \( p\)-value \( < 0.01 \)) mutants, respectively, compared to the wild-type strain...
(53.2 ± 1.8 μM); while complementation of Ag85C_Mabs (i.e., M. abscessus R_Δag85C::C) restored the wild-type R phenotype (51.8 ± 3.1 μM — Table 3).

Based on these results, purified Ag85C_Mabs recombinant protein [25] was further incubated with iBpPPOX, using increasing enzyme/inhibitor molar ratio (E/I) ranging from 1:1 to 1:75, and then treated with ActivX TAMRA-FP fluorescent probe, as reported previously [24, 25]. Equal amounts of proteins were separated on SDS-PAGE and visualized by Coomassie staining or in-gel fluorescence for TAMRA detection (Fig 4A). Relative fluorescence quantification of each band was done using the ImageLab™ software version 5.0 (Bio-Rad) by taking as 100% absolute fluorescence level, the labeled Ag85C_Mabs-TAMRA adduct (Fig 4A). As expected, pre-treating Ag85C_Mabs with iBpPPOX, resulted in a significant loss in fluorescence intensity by around 32.8 ± 1.8% (E/I = 1:1 to 1:10), 58.5 ± 0.70% (E/I = 1:25), 64.0 ± 1.8% (E/I = 1:50) and up
583 nm) using the Cy

Fig 4. Inhibition of the Ag85C

quantification of each band was performed using the ImageLab

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1:100 to ensure total inhibition, as determined using an MALDI-TOF-TOF mass spectrometer in linear mode. (C) Mechanism of inhibition of Ag85C

PPOX to the catalytic serine as previously observed [24, 25]. TAMRA-labeled Ag85C

Table 3. Variation of MIC (µM) of iBpPPOX against M. abscessus-Ag85C-mutant strains

<table>
<thead>
<tr>
<th>M. abscessus strains</th>
<th>MIC_{50} / MIC_{90} (µM)</th>
<th>MIC_{50} / MIC_{90} ratio mutant vs. WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. abscessus S WT</td>
<td>33.0 ± 2.0ˆ / 85.9 ± 5.5^</td>
<td>1.0 / 1.0</td>
</tr>
<tr>
<td>M. abscessus S_pMyc empty vector</td>
<td>31.9 ± 1.7 / 82.4 ± 0.92</td>
<td>0.97 / 1.06</td>
</tr>
<tr>
<td>M. abscessus S_pMyc::ag85C^{226A}</td>
<td>34.4 ± 3.0 / 83.1 ± 6.8</td>
<td>1.04 / 0.97</td>
</tr>
<tr>
<td>M. abscessus S_Ag85C</td>
<td>33.7 ± 1.9 / 81.5 ± 7.4</td>
<td>1.02 / 1.05</td>
</tr>
<tr>
<td>M. abscessus S_Ag85C::C</td>
<td>32.6 ± 1.3 / 87.4 ± 1.5</td>
<td>0.99 / 1.02</td>
</tr>
<tr>
<td>M. abscessus S_pMyc::ag85C</td>
<td>87.3 ± 3.4^ / &gt;200^</td>
<td>2.65 / &gt;3.0</td>
</tr>
<tr>
<td>M. abscessus R WT</td>
<td>53.2 ± 1.8^ / 104.3 ± 5.1^</td>
<td>1.0 / 1.0</td>
</tr>
<tr>
<td>M. abscessus R_pMyc empty vector</td>
<td>49.9 ± 2.6 / 109.2 ± 10.4</td>
<td>0.94 / 1.05</td>
</tr>
<tr>
<td>M. abscessus R_pMyc::ag85C^{226A}</td>
<td>47.5 ± 2.0^ / 119.0 ± 9.6</td>
<td>0.89 / 1.14</td>
</tr>
<tr>
<td>M. abscessus R_Ag85C</td>
<td>30.9 ± 2.1^ / 114.9 ± 8.2</td>
<td>0.58 / 1.10</td>
</tr>
<tr>
<td>M. abscessus R_Ag85C::C</td>
<td>51.8 ± 3.1^ / 108.2 ± 4.6</td>
<td>0.97 / 1.04</td>
</tr>
<tr>
<td>M. abscessus R_pMyc::ag85C</td>
<td>148.2 ± 2.1^ / &gt;200^</td>
<td>2.78 / &gt;2</td>
</tr>
</tbody>
</table>

* Experiments were performed as described in Materials and Methods. MIC_{50} / MIC_{90} compound minimal concentration leading to 50% or 90% growth inhibition, respectively. Values are mean of two independent assays performed in triplicate. MIC values with a common symbol are significantly different (^; p-value<0.05; †; ^, †, ¶; p-value<0.01; ANOVA followed by Fisher’s test).

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to >90% (E/I = 1:75) as compared to the non-treated protein labeled by the TAMRA-FP probe (Fig 4A). This means that the TAMRA-FP probe cannot bind the catalytic serine when the Ag85C\_Mabs\_iBpPPOX complex has been formed, as revealed by the significant loss in fluorescence emission (Fig 4A).

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Fig 4. Inhibition of the Ag85C\_Mabs by iBpPPOX. (A) Ag85C\_Mabs was pre-treated with iBpPPOX (i.e. enzyme/inhibitor molar ratio of 1:1 to 1:75), incubated with ActiveX TAMRA-FP, separated by 12% SDS-PAGE, and visualized by Coomassie blue staining (upper panel) or in-gel fluorescence visualization (middle panel). The merged image is shown in the lower panel. Untreated protein (i.e., no TAMRA-FP and no iBpPPOX) was used as control. No TAMRA-FP labeling is detected in the presence of inactivated heat-treated Ag85C\_Mabs. TAMRA labeling of Ag85C\_Mabs is impaired in the Ag85C\_Mabs--iBpPPOX adducts, as evidenced by the loss of fluorescence in the iBpPPOX lanes, presumably resulting from the covalent binding of iBpPPOX to the catalytic serine as previously observed [24, 25]. TAMRA-labeled Ag85C\_Mabs was detected by fluorescent gel scanning (λ_{exc} 557 nm, λ_{em} 583 nm) using the Cy5 filter of a ChemiDoc MP Imager (Bio-Rad) before staining of the gel with Coomassie Brilliant Blue dye. Relative fluorescence quantification of each band was performed using the ImageLab™ software version 5.0 (Bio-Rad) by taking as 100% absolute fluorescence level the labeled Ag85C\_Mabs--TAMRA adduct. (B) Global mass modification of Ag85C\_Mabs pre-incubated with iBpPPOX, at an enzyme/inhibitor molar ratio of 1:100 to ensure total inhibition, as determined using an MALDI-TOF-TOF mass spectrometer in linear mode. (C) Mechanism of inhibition of Ag85C\_Mabs by the oxadiazolone iBpPPOX, based on mass spectrometry analysis. a.u., arbitrary units.
MALDI-TOF mass spectrometry was further used to confirm the (covalent) nature of the inhibition. Sample of the Ag85C\textsubscript{Mabs}\textsubscript{iBpPPOX} (E/I = 1:100) complex was subjected to MALDI-TOF mass spectrometry analyses. Mass increment of +305.3 Da was then observed within the global mass of the inhibited Ag85C\textsubscript{Mabs} as compared with the untreated protein (Fig 4B); whereas no changes in the global mass were observed with the inactivated heat-treated protein. Such result is thus consistent with the formation of a covalent enzyme-inhibitor adduct, as the reaction between the catalytic Ser124 and iBpPPOX is expected to yield a mass increase of +326 Da; and also, in agreement with the mechanism of action of such OX derivatives \[42\]. All these findings conclusively indicate that pure recombinant Ag85C\textsubscript{Mabs} protein is covalently modified by the iBpPPOX derivative (Fig 4C), in good agreement with the known classical mechanism of action of such OX compounds as previously demonstrated using pure lipolytic enzymes \[12, 42\].

Taken together, the in vitro inhibitory experiments conducted with iBpPPOX on pure recombinant Ag85C\textsubscript{Mabs} protein (Fig 4), as well as the statistically significant increased resistance levels when overexpressing the Ag85C\textsubscript{Mabs} protein in \textit{M. abscessus} S and R variants (Table 3), thus confirm the assertion that this enzyme is an effective target of iBpPPOX.

**Conclusion**

As already highlighted in the case of \textit{M. tuberculosis} \[12\], our series of oxadiazolone-core OX derivatives are able to impair different metabolic pathways during either extracellular and/or intracellular bacterial growth via the inhibition of various (Ser/Cys)-based enzymes, therefore resulting in \textit{M. abscessus} death. Although the efficiency of these OX molecules could not be considered as sufficient enough to obtain powerful anti-mycobacterial agents, they may however represent attractive tools for deciphering the lipid metabolism in \textit{M. abscessus} and/or in \textit{M. tuberculosis}. We have indeed reported that the MmPPOX compound was able to prevent intracytoplasmic lipid inclusion (ILI) catabolism \textit{in vivo} in \textit{M. bovis} BCG infected murine bone-marrow-derived macrophages (mBMDM) \[47–49\]; as well as \textit{in vitro} under carbon excess and nitrogen-deprived conditions allowing ILI biosynthesis and hydrolysis in \textit{M. abscessus} \[50\]. Taken together, all these findings support that the OX derivatives are able to abolish the activity of several (Ser/Cys)-containing enzymes involved in mycobacterial lipid metabolism and/or in cell wall biosynthesis. This is the case of the Ag85 complex proteins which are essential players in the biosynthesis of lipids from mycobacterial membrane as well as in intracellular lipid metabolism, but also of proteins belonging to the hormone-sensitive lipase (HSL) family member proteins (\textit{i.e.}, Lip-HSL) \[42\], including LipY the major Lip-HSL lipase involved in mycobacterial lipid catabolism \[49–52\]. Therefore, the respective effects of these OX compounds against lipid-poor vs. lipid-rich bacteria deserve to be investigated in more details. More especially, deciphering how the presence of intracytoplasmic lipid inclusions (ILI) in lipid-rich bacteria can actively contribute to substantially enhanced mycobacterial virulence and pathogenesis as compared to lipid-poor strains, as reported recently \[50\], will provide major insights for understanding the general development of mycobacterial-related diseases. Such experiments are currently underway, and will be reported in due course.

**Supporting information**

S1 Appendix. Detailed protocols regarding the MIC determination, targets identification and mass spectrometry analysis of Ag85C\textsubscript{Mabs} as well as the list of plasmids and primers used in this study.

(PDF)
S1 Fig. Uncropped and unadjusted image for Western Blotting of Fig 3. Each overexpressed protein was revealed using the HisProbe™ HRP conjugate (ThermoFisher Scientific) and compared to the M. abscessus wild type strain as well as the pure recombinant Ag85C-Mabs protein. (TIF)

S2 Fig. Uncropped and unadjusted images for SDS-PAGE gel of Fig 4A. SDS-PAGE gel visualized by Coomassie blue staining (upper panel) or by in-gel fluorescence visualization (middle panel). Superimposition of both images is reported in the lower panel. Molecular weights were derived from the Unstained Protein Molecular Weight Marker (Euromedex). (TIF)

S1 Table. iBpPPOX target proteins identified in M. abscessus R total lysate by LC-ESI-MS/MS analysis. Only positive hits with a pFDR of 1%, 5% and 10% are reported. (XLSX)

S2 Table. iBpPPOX target proteins identified in M. abscessus R culture cell by LC-ESI-MS/MS analysis. Only positive hits with a pFDR of 1% and 5% are reported. (XLSX)

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