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Synergistic interactions of indole-2-carboxamides and β-lactam antibiotics against *Mycobacterium abscessus*

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Running title: Indolamides and β-lactams synergism in *M. abscessus*

Keywords: *Mycobacterium abscessus*, indole-2-carboxamide, β-lactam, MmpL3, drug synergism, macrophage, therapeutic activity.
New drugs or therapeutic combinations are urgently needed against *Mycobacterium abscessus*. Previously, we demonstrated the potent activity of indole-2-carboxamides 6 and 12 against *M. abscessus*. We show here that these compounds act synergistically with imipenem and cefoxitin *in vitro* and increase the bactericidal activity of the β-lactams against *M. abscessus*. In addition, compound 12 also displays synergism with imipenem and cefoxitin within infected macrophages. The clinical potential of these new drug combinations requires further evaluation.
Mycobacterium abscessus, is a fast-growing mycobacterial species particularly frequent in patients with cystic fibrosis (CF), bronchiectasis and chronic obstructive pulmonary diseases (COPD) (1, 2). In the context of CF and COPD, M. abscessus has emerged as an important opportunistic pathogen responsible for significant mortality (3). However, treatment of M. abscessus lung disease remains particularly challenging, largely due to intrinsic resistance of M. abscessus to most antibiotic classes (1, 2). The typical treatment regimen includes a combination of macrolides, aminoglycosides and intravenous β-lactams (cefoxitin or imipenem) for at least 12 months (2). There is no reliable therapeutic strategy for the treatment of M. abscessus pulmonary infections and the lengthy treatment duration and drug toxicity effects are often accompanied with severe undesirable outcomes. Thus, there is an unmet clinical need for new drug regimens with improved efficacy to treat these infections. Along with the development of repurposed drugs, the drug pipeline has recently been fueled with chemical entities acting on new targets in M. abscessus, such as the mycolic acid transporter MmpL3 inhibited by a wide range of structurally-unrelated small molecules (4). Chemical inhibition of MmpL3 abolishes the export of trehalose monomycolate to the outer membrane, leading to significant bacterial growth inhibition. In M. abscessus, these chemotypes include a piperidino-based compound (PIPD1) (5), benzimidazoles (6) as well as indole-2-carboxamide derivatives (7, 8). They exhibit high activity against clinical isolates in vitro, in macrophages, zebrafish or in an acute murine model of M. abscessus infection (5–7, 9). Due to their pronounced role in modulating the cell wall architecture and composition, it may be speculated that chemical inhibition of MmpL3 would increase the efficacy of other drugs. Although this has been reported in M. tuberculosis whereby the indolecarboxamides and adamantyl-ureas act synergistically with rifampin, bedaquiline, clofazimine and β-lactams (10), to date this has not been investigated in M. abscessus.

Indolecarboxamides 6 and 12 (Figure 1) present favorable absorption, distribution, metabolism, and excretion (ADME) properties (7, 11) and the ease to obtain them in high yields prompted us to investigate their interaction profiles with different classes of antibiotics active against M. abscessus and/or used as part of clinical treatment regimens. These include sutezolid; an oxazolidinone inhibiting bacterial translation (12), clofazimine; affecting the energy metabolism (13), and particularly β-lactams (the cephalosporin cefoxitin (FOX) and the carbapenem imipenem (IPM)); inhibiting peptidoglycan biosynthesis and reported to act in synergy with different drugs against M. abscessus (14, 15) (Figure 1). The minimal inhibitory
concentrations (MIC) were determined according to the CLSI guidelines (16) in Cat
ion-Adjusted Mueller-Hinton Broth (CaMHB; Sigma-Aldrich). Pair combinations between Cpd6 and Cpd12 with other drugs were tested in CaMHB in a typical checkerboard assay (17) with resazurin reduction as a metabolic readout. This allowed us to establish the fractional inhibitory concentration index (FICI) of each drug combination, where FICI was determined using formula: MIC_A with B/MIC_A alone + MIC_B with A/MIC_A alone and values ≤ 0.5 were considered synergistic, between 0.5 and 4 as indifferent and ≥ 4 as antagonist (10). While Cpd12 showed a FICI value ≤ 0.5 with IPM or FOX, indicative of synergistic interactions, no interaction (indifference) was recorded with clofazimine or sutezolid. A similar interaction profile was also observed when combining these drugs with Cpd6 (Table 1).

To determine the optimal concentration of Cpd12 showing no or little activity against M. abscessus CIP104536T (S variant), cultures were exposed to concentrations ranging from 0.03 to 0.125 µg/ml Cpd12 prior to colony-forming units (CFU) determination at 5 days post-exposure. While at the MIC (0.125 µg/ml) there was a ~6 to 7 Log drop in the CFU counts, no decrease was observed at 0.03 µg/ml (Figure 2A). This concentration was thus chosen to investigate the potential synergistic activity of Cpd12 with β-lactams. IPM was used at 4, 8 and 16 µg/ml and FOX was used at 16 and 32 µg/ml, corresponding to concentrations 4- and 2-fold lower than their MIC, respectively (Table 1). At these sub-MIC, Cpd12 plus IPM decreased CFU counts by ~4 to 6 Log, as compared to Cpd12 or IPM alone. Similarly, FOX alone at 16 and 32 µg/ml was accompanied by a reduction in the CFU counts, while the addition of 0.03 µg/ml Cpd12 further reduced the CFU by ~2 to 3 Log (Figure 2A). Comparable results were obtained when assessing the synergistic activity of Cpd6 with IPM or FOX (Figure 2B). At 0.06 µg/ml and 0.125 µg/ml of Cpd6, the CFU were reduced by ~1 and 5 Log, respectively, and no further decrease in the CFU was observed at 0.25 µg/ml. The addition of 4 µg/ml or 8 µg/ml IPM to 0.06 µg/ml Cpd6 resulted in a ~3 Log decrease in the CFU as compared to IPM alone. Similarly, the simultaneous addition of 0.06 µg/ml Cpd6 to FOX (at 16 or 32 µg/ml) exacerbated the effect of FOX, leading to a ~4 Log decrease in the CFU as compared to FOX alone (Figure 2B). To assess whether these interactions are due to the chemical inhibition of MmpL3, the CFU killing assay was repeated using a strain highly resistant to both Cpd12 and Cpd6 due to the presence of an A309P missense mutation in MmpL3 (MIC_Cpd12/Cpd6 of 32 µg/ml, (7)). Figure 2C shows the Cpd12 plus IPM or Cpd12 plus FOX synergistic interactions were abolished, indicating that inhibition of MmpL3 is necessary to establish drug synergism with the β-lactams. This confirms a previous...
study demonstrating that synergistic interactions between the indolecarboxamides NITD-304 and NITD-349 with other clinically-relevant drugs is diminished in an MmpL3 mutant of *M. tuberculosis* resistant to indolecarboxamides (10).

The *M. abscessus* complex comprises three subspecies, *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense* (18), displaying different drug susceptible profiles. We therefore tested the activity of the Cpd12/β-lactam combinations against a panel of *M. abscessus* complex clinical isolates (19, 20) by determining the CFU counts of two *M. abscessus* subsp. *abscessus* strains (1298 and 2587), two *M. abscessus* subsp. *bolletii* strains (97 and 116) and two *M. abscessus* subsp. *massiliense* strains (120 and 122). In general, the combination of Cpd12 plus IPM or Cpd12 plus FOX resulted in significantly reduced CFU counts as compared to the cultures exposed to Cpd12, IPM or FOX alone. However, the 6 strains responded differently to each of these drug combinations (Figure 3). Overall, CFU determination *in vitro* was in direct agreement with the checkerboard results and indicate that low concentrations of Cpd6 and Cpd12 improve the activity of IPM or FOX against the *M. abscessus* complex *in vitro*.

The activity of IPM and FOX alone or in combination with Cpd12 was next evaluated using THP-1 macrophages infected with *M. abscessus* CIP104536^T^ (S variant) carrying pTEC27, as previously described (6). Infected cells were either left untreated or exposed for 2 days to Cpd12, IPM or FOX alone or in combination, lysed and plated for subsequent intracellular bacterial load determination. While IPM and FOX displayed only minor effects at the concentrations tested, the addition of 0.06 µg/ml Cpd12 significantly reduced the bacterial burden by ~0.5 Log (Figure 4A). This effect was further exacerbated (1 Log reduction) when 0.25 µg/ml of Cpd12 was used. A microscopy-based infectivity assay reported earlier (6, 21) was subsequently used to quantify the impact of drug treatment on the percentage of infected THP-1 cells. The results confirm the pronounced reduction in the number of infected macrophages treated with Cpd12 plus IPM or Cpd12 plus FOX (~50% decrease with 0.06 µg/ml Cpd12), as compared to cells treated with the drugs alone at day 2 post-infection (Figure 4B and 4C). Collectively, these findings suggest that the Cpd12/IPM and Cpd12/FOX combinations are effective on intracellular *M. abscessus*.

IPM use is usually associated with improved outcome for the treatment of *M. abscessus* pulmonary disease (22) and IPM combined with other antibiotics exerts a synergistic or additive effect contributing to its success (14, 15). However, resistance to IPM is also
emerging, highlighting the limiting application of IPM in the treatment of *M. abscessus* infections (23, 24). The present results highlight the therapeutic potential of the Cpd12/IPM combination against a panel of distinct clinical *M. abscessus* complex isolates. This combination may help lowering the effective dose of IPM, contributing to reductions in the emergence of IPM-resistance strains. Similarly, the use of indolecarboxamides as companion drugs further reduces the effective concentrations of FOX, restricting the eventual emergence of *M. abscessus*-resistant mutants. A plausible hypothesis explaining this synergistic activity may rely on the fact that the indolecarboxamides, through inhibition of mycolic acid transport at the bacterial surface, disorganise and disrupt the mycomembrane, which accelerates the penetration of the β-lactam drugs to reach their targets (the L,D-transpeptidase for IPM and the D,D-transpeptidase for FOX), leading to inhibition of peptidoglycan synthesis. Conversely, inhibition of the peptidoglycan transpeptide linkages by the β-lactams may also facilitate the access of Cpd6/Cpd12 to their inner membrane target. However, other underlying mechanisms may be responsible to the observed synergistic effects and further research is required.

In summary, indole-2-carboxamides represent a promising chemotype improving the activity of FOX and IPM; two recommended drugs for the treatment of *M. abscessus* pulmonary infections (2). Future studies should evaluate whether β-lactamase inhibitors (33,34) would further improve the observed synergistic interactions. Our results indicate that the Cpd12/β-lactam combinations are highly effective within macrophages by reducing the intracellular bacterial burden and the percentage of infected cells, emphasizing the need for further evaluation in pre-clinical animal models.
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The authors have no conflict of interest to declare.

All data will be made publicly available upon publication and upon request for peer review.
REFERENCES


Table 1. Interaction of Cpd6 and Cpd12 with other antibiotics against *M. abscessus* CIP104536T (smooth strain) assessed by checkerboards REMA in CaMH. Results are the mean of the FICI ± SD of 3 independent experiments. SUT, sutezolid; IPM, imipenem; FOX, cefoxitin; CFZ, clofazimine.

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<th>Interaction with Cpd6</th>
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FIGURE LEGENDS

Figure 1. Structures of imipenem, cefoxitin and the lead indolecarboxamides 6 and 12 used in this study.

Figure 2. Synergistic activity of indole-2-carboxamide derivatives with IPM and FOX in vitro. CFU counts of Cpd12 (A) or Cpd6 (B) given alone or in combination with imipenem (IPM) or cefoxitin (FOX). M. abscessus cultures were incubated at 30 °C in CaMH for 5 days in the presence of the indicated compounds (µg/ml) and plated on LB agar prior to CFU enumeration. (C) CFU determination of M. abscessus mutant A309P (spontaneous resistant strain to Cpd12 carrying the A309P mutation in MmpL3) was exposed to the indicated antibiotics (µg/ml) at 30 °C in CaMH for 5 days. Graphs represent the mean of three independent experiments completed in triplicate. Data are expressed as the mean ± SD. The statistical test used is a non-parametric Mann Whitney t-test in which the combinations were compared to the drugs alone. **P ≤ 0.01, ***P ≤ 0.001.

Figure 3. CFU determination of clinical isolates exposed to Cpd12 given alone or in combination with imipenem (IPM) or cefoxitin (FOX). M. abscessus cultures were incubated at 30 °C in CaMH for 5 days in the presence of the indicated compounds (µg/ml) and plated on LB agar prior to CFU enumeration. Data are expressed as the mean ± SD from three independent experiments completed in triplicate. The statistical test used is a non-parametric Mann Whitney t-test in which the combinations were compared to the drugs alone. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

Figure 4. Impact of Cpd12 alone or in combination on intracellular-residing M. abscessus. THP-1 macrophages were infected with M. abscessus S expressing TdTomato (MOI of 2:1) and treated with the indicated compounds (µg/ml). (A) CFUs were determined at day 0 and day 2 post-infection. Data represents the mean ± SD of three independent experiments completed in triplicate. For statistical analysis, non-parametric Mann Whitney’s t-test was performed. **P ≤ 0.01, ***P ≤ 0.001. (B) Percentage of infected THP-1 macrophages at day 0 and day 2 post-infection. Data shown are mean values ± SD for one representative experiment completed in triplicate. One-way ANOVA kruskal-wallis was used as a statistical test. ***P ≤ 0.001. (C) Immunofluorescent fields were taken at day 2 post-infection at magnification x40 (using
confocal microscopy) showing the nuclei of macrophages (DAPI in blue), infected with red-fluorescent *M. abscessus* in absence or in presence of the drugs used alone or in combination. Yellow arrows emphasize red-fluorescent *M. abscessus* (tdTomato) within macrophages. Only intracellular bacteria that were individually observed under the microscope were recorded.