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

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26 **Running title:** Indolamides and β -lactams synergism in *M. abscessus*
27

28 **Keywords:** *Mycobacterium abscessus*, indole-2-carboxamide, β -lactam, MmpL3, drug
29 synergism, macrophage, therapeutic activity.

30 **ABSTRACT**

31

32 New drugs or therapeutic combinations are urgently needed against *Mycobacterium abscessus*.
33 Previously, we demonstrated the potent activity of indole-2-carboxamides **6** and **12** against *M.*
34 *abscessus*. We show here that these compounds act synergistically with imipenem and ceftazidime
35 *in vitro* and increase the bactericidal activity of the β -lactams against *M. abscessus*. In addition,
36 compound **12** also displays synergism with imipenem and ceftazidime within infected
37 macrophages. The clinical potential of these new drug combinations requires further
38 evaluation.

39 *Mycobacterium abscessus*, is a fast-growing mycobacterial species particularly frequent in
40 patients with cystic fibrosis (CF), bronchiectasis and chronic obstructive pulmonary diseases
41 (COPD) (1, 2). In the context of CF and COPD, *M. abscessus* has emerged as an important
42 opportunistic pathogen responsible for significant mortality (3). However, treatment of *M.*
43 *abscessus* lung disease remains particularly challenging, largely due to intrinsic resistance of *M.*
44 *abscessus* to most antibiotic classes (1, 2). The typical treatment regimen includes a
45 combination of macrolides, aminoglycosides and intravenous β -lactams (cefoxitin or imipenem)
46 for at least 12 months (2). There is no reliable therapeutic strategy for the treatment of *M.*
47 *abscessus* pulmonary infections and the lengthy treatment duration and drug toxicity effects
48 are often accompanied with severe undesirable outcomes. Thus, there is an unmet clinical need
49 for new drug regimens with improved efficacy to treat these infections. Along with the
50 development of repurposed drugs, the drug pipeline has recently been fueled with chemical
51 entities acting on new targets in *M. abscessus*, such as the mycolic acid transporter MmpL3
52 inhibited by a wide range of structurally-unrelated small molecules (4). Chemical inhibition of
53 MmpL3 abolishes the export of trehalose monomycolate to the outer membrane, leading to
54 significant bacterial growth inhibition. In *M. abscessus*, these chemotypes include a piperidinol-
55 based compound (PIPD1) (5), benzimidazoles (6) as well as indole-2-carboxamide derivatives (7,
56 8). They exhibit high activity against clinical isolates *in vitro*, in macrophages, zebrafish or in an
57 acute murine model of *M. abscessus* infection (5–7, 9). Due to their pronounced role in
58 modulating the cell wall architecture and composition, it may be speculated that chemical
59 inhibition of MmpL3 would increase the efficacy of other drugs. Although this has been
60 reported in *M. tuberculosis* whereby the indolecarboxamides and adamantyl-ureas act
61 synergistically with rifampin, bedaquiline, clofazimine and β -lactams (10), to date this has not
62 been investigated in *M. abscessus*.

63 Indolecarboxamides **6** and **12** (**Figure 1**) present favorable absorption, distribution,
64 metabolism, and excretion (ADME) properties (7, 11) and the ease to obtain them in high yields
65 prompted us to investigate their interaction profiles with different classes of antibiotics active
66 against *M. abscessus* and/or used as part of clinical treatment regimens. These include
67 sutezolid; an oxazolidinone inhibiting bacterial translation (12), clofazimine; affecting the
68 energy metabolism (13), and particularly β -lactams (the cephalosporin cefoxitin (FOX) and the
69 carbapenem imipenem (IPM)); inhibiting peptidoglycan biosynthesis and reported to act in
70 synergy with different drugs against *M. abscessus* (14, 15) (**Figure 1**). The minimal inhibitory

71 concentrations (MIC) were determined according to the CLSI guidelines (16) in Cation-Adjusted
72 Mueller-Hinton Broth (CaMHB; Sigma-Aldrich). Pair combinations between Cpd6 and Cpd12
73 with other drugs were tested in CaMHB in a typical checkerboard assay (17) with resazurin
74 reduction as a metabolic readout. This allowed us to establish the fractional inhibitory
75 concentration index (FICI) of each drug combination, where FICI was determined using formula:
76 MIC_A with B/ MIC_A alone + MIC_B with A/ MIC_B alone and values ≤ 0.5 were considered synergistic,
77 between 0.5 and 4 as indifferent and ≥ 4 as antagonist (10). While Cpd12 showed a FICI value \leq
78 0.5 with IPM or FOX, indicative of synergistic interactions, no interaction (indifference) was
79 recorded with clofazimine or sutezolid. A similar interaction profile was also observed when
80 combining these drugs with Cpd6 (**Table 1**).

81 To determine the optimal concentration of Cpd12 showing no or little activity against *M.*
82 *abscessus* CIP104536^T (S variant), cultures were exposed to concentrations ranging from 0.03 to
83 0.125 $\mu\text{g/ml}$ Cpd12 prior to colony-forming units (CFU) determination at 5 days post-exposure.
84 While at the MIC (0.125 $\mu\text{g/ml}$) there was a ~ 6 to 7 Log drop in the CFU counts, no decrease
85 was observed at 0.03 $\mu\text{g/ml}$ (**Figure 2A**). This concentration was thus chosen to investigate the
86 potential synergistic activity of Cpd12 with β -lactams. IPM was used at 4, 8 and 16 $\mu\text{g/ml}$ and
87 FOX was used at 16 and 32 $\mu\text{g/ml}$, corresponding to concentrations 4- and 2-fold lower than
88 their MIC, respectively (**Table 1**). At these sub-MIC, Cpd12 plus IPM decreased CFU counts by ~ 4
89 to 6 Log, as compared to Cpd12 or IPM alone. Similarly, FOX alone at 16 and 32 $\mu\text{g/ml}$ was
90 accompanied by a reduction in the CFU counts, while the addition of 0.03 $\mu\text{g/ml}$ Cpd12 further
91 reduced the CFU by ~ 2 to 3 Log (**Figure 2A**). Comparable results were obtained when assessing
92 the synergistic activity of Cpd6 with IPM or FOX (**Figure 2B**). At 0.06 $\mu\text{g/ml}$ and 0.125 $\mu\text{g/ml}$ of
93 Cpd6, the CFU were reduced by ~ 1 and 5 Log, respectively, and no further decrease in the CFU
94 was observed at 0.25 $\mu\text{g/ml}$. The addition of 4 $\mu\text{g/ml}$ or 8 $\mu\text{g/ml}$ IPM to 0.06 $\mu\text{g/ml}$ Cpd6
95 resulted in a ~ 3 Log decrease in the CFU as compared to IPM alone. Similarly, the simultaneous
96 addition of 0.06 $\mu\text{g/ml}$ Cpd6 to FOX (at 16 or 32 $\mu\text{g/ml}$) exacerbated the effect of FOX, leading
97 to a ~ 4 Log decrease in the CFU as compared to FOX alone (**Figure 2B**). To assess whether these
98 interactions are due to the chemical inhibition of MmpL3, the CFU killing assay was repeated
99 using a strain highly resistant to both Cpd12 and Cpd6 due to the presence of an A309P
100 missense mutation in MmpL3 ($MIC_{Cpd12/Cpd6}$ of 32 $\mu\text{g/ml}$, (7)). **Figure 2C** shows the Cpd12 plus
101 IPM or Cpd12 plus FOX synergistic interactions were abolished, indicating that inhibition of
102 MmpL3 is necessary to establish drug synergism with the β -lactams. This confirms a previous

103 study demonstrating that synergistic interactions between the indolecarboxamides NITD-304
104 and NITD-349 with other clinically-relevant drugs is diminished in an MmpL3 mutant of *M.*
105 *tuberculosis* resistant to indolecarboxamides (10).

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

118 The activity of IPM and FOX alone or in combination with Cpd12 was next evaluated using
119 THP-1 macrophages infected with *M. abscessus* CIP104536^T (S variant) carrying pTEC27, as
120 previously described (6). Infected cells were either left untreated or exposed for 2 days to
121 Cpd12, IPM or FOX alone or in combination, lysed and plated for subsequent intracellular
122 bacterial load determination. While IPM and FOX displayed only minor effects at the
123 concentrations tested, the addition of 0.06 $\mu\text{g/ml}$ Cpd12 significantly reduced the bacterial
124 burden by ~ 0.5 Log (**Figure 4A**). This effect was further exacerbated (1 Log reduction) when
125 0.25 $\mu\text{g/ml}$ of Cpd12 was used. A microscopy-based infectivity assay reported earlier (6, 21) was
126 subsequently used to quantify the impact of drug treatment on the percentage of infected THP-
127 1 cells. The results confirm the pronounced reduction in the number of infected macrophages
128 treated with Cpd12 plus IPM or Cpd12 plus FOX ($\sim 50\%$ decrease with 0.06 $\mu\text{g/ml}$ Cpd12), as
129 compared to cells treated with the drugs alone at day 2 post-infection (**Figure 4B and 4C**).
130 Collectively, these findings suggest that the Cpd12/IPM and Cpd12/FOX combinations are
131 effective on intracellular *M. abscessus*.

132 IPM use is usually associated with improved outcome for the treatment of *M. abscessus*
133 pulmonary disease (22) and IPM combined with other antibiotics exerts a synergistic or
134 additive effect contributing to its success (14, 15). However, resistance to IPM is also

135 emerging, highlighting the limiting application of IPM in the treatment of *M. abscessus*
136 infections (23, 24). The present results highlight the therapeutic potential of the Cpd**12**/IPM
137 combination against a panel of distinct clinical *M. abscessus* complex isolates. This
138 combination may help lowering the effective dose of IPM, contributing to reductions in the
139 emergence of IPM-resistance strains. Similarly, the use of indolecarboxamides as companion
140 drugs further reduces the effective concentrations of FOX, restricting the eventual emergence
141 of *M. abscessus*-resistant mutants. A plausible hypothesis explaining this synergistic activity
142 may rely on the fact that the indolecarboxamides, through inhibition of mycolic acid transport
143 at the bacterial surface, disorganise and disrupt the mycomembrane, which accelerates the
144 penetration of the β -lactam drugs to reach their targets (the L,D-transpeptidase for IPM and
145 the D,D-transpeptidase for FOX), leading to inhibition of peptidoglycan synthesis. Conversely,
146 inhibition of the peptidoglycan transpeptide linkages by the β -lactams may also facilitate the
147 access of Cpd**6**/Cpd**12** to their inner membrane target. However, other underlying
148 mechanisms may be responsible to the observed synergistic effects and further research is
149 required.

150 In summary, indole-2-carboxamides represent a promising chemotype improving the activity
151 of FOX and IPM; two recommended drugs for the treatment of *M. abscessus* pulmonary
152 infections (2). Future studies should evaluate whether β -lactamase inhibitors (33,34) would
153 further improve the observed synergistic interactions. Our results indicate that the Cpd**12**/ β -
154 lactam combinations are highly effective within macrophages by reducing the intracellular
155 bacterial burden and the percentage of infected cells, emphasizing the need for further
156 evaluation in pre-clinical animal models.

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161

162 The authors have no conflict of interest to declare.

163

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

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248 vaborbactam in combination with β -lactams against *Mycobacterium abscessus* complex clinical
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250

251 **Table 1.** Interaction of Cpd6 and Cpd12 with other antibiotics against *M. abscessus* CIP104536^T
 252 (smooth strain) assessed by checkerboards REMA in CaMH. Results are the mean of the FICI \pm
 253 SD of 3 independent experiments. SUT, sutezolid; IPM, imipenem; FOX, ceftazidime; CFZ,
 254 clofazimine.

Compound	MIC	Interaction with Cpd12			Interaction with Cpd6		
		FICI (mean)	SD	Outcome	FICI (mean)	SD	Outcome
Cpd12	0.125						
Cpd6	0.25						
SUT	16	0.84	± 0.27	Indifferent	0.62	± 0	Indifferent
IPM	16	0.5	± 0.18	Synergistic	0.5	± 0	Synergistic
FOX	64	0.45	± 0.14	Synergistic	0.44	± 0.16	Synergistic
CFZ	0.5	0.88	± 0.18	Indifferent	0.88	± 0.18	Indifferent

260 **FIGURE LEGENDS**

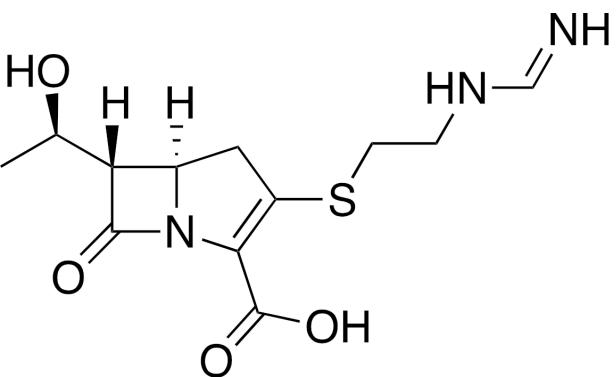
261
262 **Figure 1.** Structures of imipenem, ceftioxin and the lead indolecarboxamides **6** and **12** used in
263 this study.

264
265 **Figure 2.** Synergistic activity of indole-2-carboxamide derivatives with IPM and FOX *in vitro*. CFU
266 counts of Cpd**12** (**A**) or Cpd**6** (**B**) given alone or in combination with imipenem (IPM) or ceftioxin
267 (FOX). *M. abscessus* cultures were incubated at 30 °C in CaMH for 5 days in the presence of the
268 indicated compounds (µg/ml) and plated on LB agar prior to CFU enumeration. (**C**) CFU
269 determination of *M. abscessus* mutant A309P (spontaneous resistant strain to Cpd**12** carrying
270 the A309P mutation in MmpL3) was exposed to the indicated antibiotics (µg/ml) at 30 °C in
271 CaMH for 5 days. Graphs represent the mean of three independent experiments completed in
272 triplicate. Data are expressed as the mean ± SD. The statistical test used is a non-parametric
273 Mann Whitney *t*-test in which the combinations were compared to the drugs alone. ns, non-
274 significant. ***P* ≤ 0.01, ****P* ≤ 0.001.

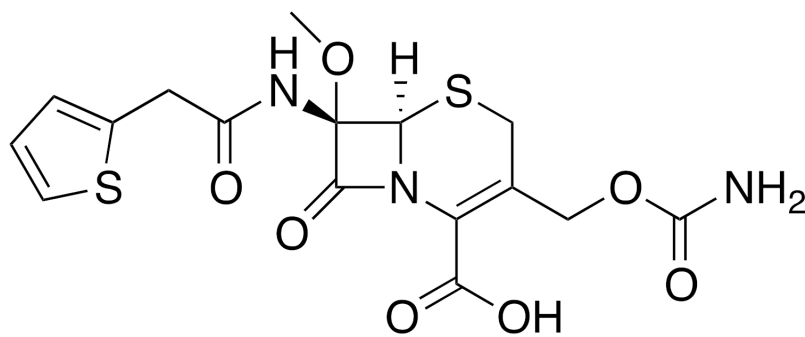
275
276 **Figure 3.** CFU determination of clinical isolates exposed to Cpd**12** given alone or in combination
277 with imipenem (IPM) or ceftioxin (FOX). *M. abscessus* cultures were incubated at 30 °C in CaMH
278 for 5 days in the presence of the indicated compounds (µg/ml) and plated on LB agar prior to
279 CFU enumeration. Data are expressed as the mean ± SD from three independent experiments
280 completed in triplicate. The statistical test used is a non-parametric Mann Whitney *t*-test in
281 which the combinations were compared to the drugs alone. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001.

282
283 **Figure 4.** Impact of Cpd12 alone or in combination on intracellular-residing *M. abscessus*. THP-1
284 macrophages were infected with *M. abscessus* S expressing TdTomato (MOI of 2:1) and treated
285 with the indicated compounds (µg/ml). (**A**) CFUs were determined at day 0 and day 2 post-
286 infection. Data represents the mean ± SD of three independent experiments completed in
287 triplicate. For statistical analysis, non-parametric Mann Whitney's *t*-test was performed. ***p* ≤
288 0.01, ****p* ≤ 0.001. (**B**) Percentage of infected THP-1 macrophages at day 0 and day 2 post-
289 infection. Data shown are mean values ± SD for one representative experiment completed in
290 triplicate. One-way ANOVA kruskal-wallis was used as a statistical test. ****p* ≤ 0.001. (**C**)
291 Immunofluorescent fields were taken at day 2 post-infection at magnification x40 (using

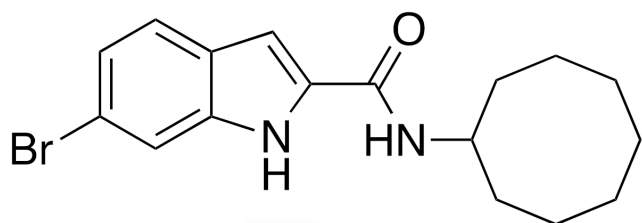
292 confocal microscopy) showing the nuclei of macrophages (DAPI in blue), infected with red-
293 fluorescent *M. abscessus* in absence or in presence of the drugs used alone or in combination.
294 Yellow arrows emphasize red-fluorescent *M. abscessus* (tdTomato) within macrophages. Only
295 intracellular bacteria that were individually observed under the microscope were recorded.



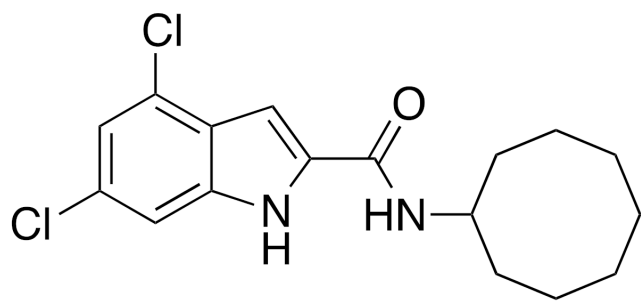
Imipenem



Cefoxitin



Compound 6



Compound 12

