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Targeting Mycolic Acid Transport by Indole-2-carboxamides for the Treatment of *Mycobacterium abscessus* Infections

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KEYWORDS: *Mycobacterium abscessus*, pathogen, indole-2-carboxamides, mycolic acid transporter, antimicrobial agent, cystic fibrosis.

ABSTRACT

Mycobacterium abscessus is a fast-growing, multidrug-resistant organism that has emerged as a clinically significant pathogen in cystic fibrosis (CF) patients. The intrinsic resistance of *M. abscessus* to most commonly available antibiotics seriously restricts chemotherapeutic options. Herein, we report the potent activity of a series of indolecarboxamides against *M. abscessus.* The lead compounds, **6** and **12**, exhibited strong activity *in vitro* against a wide panel of *M. abscessus* isolates and in infected macrophages. High resistance levels to the indolecarboxamides appear to be associated with an A309P mutation in the mycolic acid transporter MmpL3. Biochemical analyses demonstrated that while *de novo* mycolic acid synthesis remained unaffected, the indolecarboxamides strongly inhibited the transport of trehalose monomycolate, resulting in the loss of trehalose dimycolate production and abrogating mycolylation of arabinogalactan. Our data introduce a hereto unexploited chemical structure class active against *M. abscessus* infections with promising translational development possibilities for the treatment of CF patients.

INTRODUCTION

Fighting multidrug-resistant bacteria requires repurposing of existing drugs or the development of new more rapidly acting and cost effective antibiotics. Chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) are associated with unresolved therapeutic needs, resulting from severe and even fatal infections by multidrug-resistant bacteria. Lung infections in CF patients represent the most frequent and most serious manifestations, since they are responsible for more than 90% of CF patient deaths.[1] In the context of CF and COPD, *Mycobacterium abscessus*, a fast-growing mycobacterial species, has emerged in recent years as an important opportunistic pathogen increasingly responsible for mortality.[2] The incidence of *M. abscessus* infection in the CF population rises, suggesting a potential link between a genetic defect in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) and *M. abscessus* lung infection. In this population, *M. abscessus* accelerates inflammatory lung damage, leading to increased morbidity and mortality.[3] A recent genomic study of a global collection of clinical isolates indicated that the majority of *M. abscessus* infections are acquired through aerosol transmission of recently emerged dominant circulating clones.[4]

Although a rapid grower, *M. abscessus* shares important physiopathologic features with pathogenic slow-growing mycobacteria such as the ability to persist silently for years and even decades[5] in the human host and to induce lung disease associated with caseous lesions and granuloma formation in lung parenchyma.[6, 7] Infections caused by *M. abscessus* are difficult to treat and to eradicate because *M. abscessus* is naturally resistant to most antibiotics, including nearly all current antitubercular drugs.[8] The recommended treatment for pulmonary infections includes a combination of a macrolide (clarithromycin or azithromycin), an aminoglycoside (amikacin), and an intravenous β-lactam (cefoxitin or imipenem) to be taken at least for 12 months[9, 10] (**Figure 1**).

The cure rate is estimated to only 25-40% in the case of resistance to macrolides, which appears in 40-60% of the isolates.[11] In addition, unsuccessful eradication of *M. abscessus* represents a contraindication for lung transplantation by several CF centers, leaving patients without therapeutic options. Therefore, more specific, active, and at the same time less toxic antimicrobial agents are urgently needed.

Despite being a formidable respiratory mycobacterial pathogen, little is known with respect to the molecular and cellular mechanisms leading to immunopathogenesis and infection. However, recent genomic data highlighted the considerable sequence similarity between *Mycobacterium tuberculosis* and *M. abscessus*[12, 13], suggesting that these species may share common biochemical pathways. From this, it can be inferred that compounds inhibiting biosynthetic pathways in *M. tuberculosis* may also be active against *M. abscessus*, and that already existing data generated during previous tuberculosis (TB) drug discovery programs would be useful to identify new chemotypes with strong activity against *M. abscessus,* an approach that would obviate the need to initiate chemical screens *de novo*. Indeed, we have recently validated this approach and undertaken an *M. abscessus* "cross-screen" with a confirmed chemical series arising from a known set of potent non-toxic antitubercular hits.[14] This led to the discovery of a new piperidinol-based compound **1** (PIPD1) (**Figure 1**), showing selective activity against *M. abscessus* both *in vitro* and *in vivo*.[15] This success prompted us to further explore this strategy by testing a small library of indole-2-carboxamide derivatives, previously shown to exhibit strong activity against *M. tuberculosis*.[16-18]

In the recent past, we have designed a series of indolecarboxamides with potent activity against both drug-susceptible and drug-resistant strains of *M. tuberculosis* by targeting the putative mycolic acid transporter MmpL3.[18] We identified a single point mutation in MmpL3, which confers high resistance to the indolecarboxamide class, while remaining susceptible to currently used first- and

second-line TB drugs, signifying a lack of cross-resistance. The lead compound **2** (**Figure 1**) was found to be active against *M. abscessus*, thus prompting further investigation of other indolecarboxamide analogs. The bioavailability of the indolecarboxamides, combined with their ability to kill tubercle bacilli as well as non-tubercular mycobacteria (NTM), indicates great potential for the translational development of this structural class for the treatment of *M. abscessus* infections in CF patients. Herein, we describe SAR studies of a series of indolecarboxamide analogs against *M. abscessus* and provide biological evidence that they are working through inhibition of mycolic acid transport.

(Figure 1)

RESULTS

The synthesis of compounds **2-29** was carried out by following procedures reported previously.[16-18] Briefly, starting from the appropriate indole-2-carboxylic acid, a standard amide coupling protocol (**Scheme 1**) was performed to furnish the amides **2-23** and **25-28**. Compound **24** was obtained by $LiAlH_4$ reduction of the amide **2**, whereas compound **29** was prepared by coupling intermediate **45** with cycloheptanecarbonyl chloride.

(Scheme 1)

Structure-activity relationship of new indole-2-carboxamides against *M. abscessus*.

The indoles **2-29** were initially screened *in vitro* against the *M. tuberculosis*[16, 17] and *M. abscessus* strains to obtain their respective MIC values (**Table 1**). The lead compound **2** with a cyclooctyl group displayed excellent activity against both *M. tuberculosis* and *M. abscessus*. Presence of only a single methyl at positions 4, 5, or 6 of the indole ring (compounds **3-5**) resulted in a two-fold

decrease in activity against *M. abscessus* in comparison to **2**. A similar trend in activity was observed when these compounds were screened against *M. tuberculosis*[16], thus indicating that the presence of two non-polar substituents in the indole ring helps to improve activity. Likewise, compound **6** that possesses a lipophilic bromine atom at position 6 displayed a two-fold increase in activity when compared to its 6-methyl counterpart, compound **5**. Compound **7** bearing an *N*-adamantyl group was nearly 130-fold less active than compounds **6** and **8**. These findings further support the notion that the activity of these compounds is driven by their lipophilicity, and that the tolerance for steric bulk at the amide N is fairly high but limited. Additionally, the effect of a single methyl group at position 7, as in compound **9,** was evaluated. Compound **9** showed a large loss in activity against *M. tuberculosis* and *M. abscessus* as compared to other mono-substituted compounds with methyl groups at positions 4, 5, or 6 (**3**-**5**), indicating that substitution at this position is unfavorable. Next, we investigated the effect of di-substitution at other positions of the indole ring. The 5,7-dimethylindole **10** showed 16- and 2-fold loss of activity against the *M. tuberculosis* and *M. abscessus* strains, respectively, as compared to its 4,6-dimethyl counterpart **2**. In this case, both molecules have equal CLogP values, and the differential activities observed are thus a consequence of the substitution pattern in the indole ring.

(**Table 1**)

After finding that substituents at the 4 and 6-positions of the indole ring were optimal for activity, other 4,6-disubstituted derivatives, such as the 4,6-dimethoxy, 4,6-dichloro, 4,6-difluoro, and 4,6-bis-(trifluoro)methyl analogs, were investigated for their activity against *M. abscessus*. Compound **11** bearing the 4,6-dimethoxyindole moiety displayed an 8-fold loss of activity compared to compound **2** (4,6-dimethyl substitution), suggesting that the more polar methoxy substituents are less favorable compared to methyl due to their reduced lipophilicity. Next, we replaced the two

methyl groups of **2** with more lipophilic and metabolically stable halogen atoms to afford compounds **12** (4,6-dichloroindole analog; CLogP = 6.16) and **13** (4,6-difluoroindole analog; CLogP = 5.02). Both of these compounds showed activities identical (0.12 μg/mL) to that of the lead compound **2** against *M. abscessus*. In the case of *M. tuberculosis*, compound **12** displayed the same activity as did the lead **2**, while compound **13** was approximately 8-fold less active than compound **2**. Of interest is the fact that while compound **12** is more lipophilic than **13**, this property did not enhance its activity in the *M. abscessus* screening assay in contrast to the result that was observed in the case of the *M. tuberculosis* assay. Both compounds **14** and **15** were inactive against *M. abscessus* while retaining activity against *M. tuberculosis*.

Next, the activities of the 4,6-dichloroindole analogs **16**–**22** bearing different substituents on the amide nitrogen were explored. Compounds **16** (containing a *N*-adamantyl group) and **17** (containing a *N*-(R)-(+)-bornyl group) showed poor activities (MIC > 32 μg/mL) against *M. abscessus*. These modifications thus resulted in an at least 256-fold decrease in activity of **16** and **17** as compared to lead compound **2**. Introduction of a simple *n*-octyl group or a *trans*-geranyl group on the amide nitrogen yielded the compounds **18** and **19**. Again, both of these compounds were found to be inactive (MIC > 32 μg/mL) against *M. abscessus*, suggesting that these lipophilic alkyl and alkenyl chains are likely unable to bind to the target site. The analog **20** bearing an α-branched cyclohexylmethyl group (MIC = 0.5 μg/mL) was moderately active whereas compound **21** bearing the indanyl group proved to be inactive against *M. abscessus*. The spiro compound **22** (CLogP = 7.04) proved to be the most active compound against *M. tuberculosis* (MIC = 0.00098 μg/mL) in the *in vitro* assay; disappointingly, this excellent activity was not duplicated in the *M. abscessus* screening (MIC > 32 μg/mL). Furthermore, the reverse amide derivative **23** was inactive against *M. abscessus*, while the amine **24** exhibited a 4-fold drop in activity compared to **2**.

Compound **25** containing a 4,6-bis(trifluoromethyl)indole, and the benzofuran derivatives **26-28**[17] showed MIC values between 0.5 and 1.0 μg/mL, while the benzothiazole derivative **29** was inactive against the susceptible strain of *M. abscessus*.

Overall, the SAR of these indole-2-carboxamides reveals some parallels in their activity against *M. tuberculosis* and *M. abscessus*, but this is not strictly true for all of the analogs that were studied. In most cases the indole analogs reported herein are more active against *M. tuberculosis* than against *M. abscessus*. Also, it is apparent that, based on the limited number of compounds studied so far, the cyclooctyl group is preferred for activity against *M. abscessus* and that in a few cases these cyclooctyl analogs are more active against *M. abscessus* than *M. tuberculosis*. We believe that the present data provide fertile ground to inspire the generation of a host of improved analogs that may show even better activity against *M. abscessus*.

Activity of compounds 12 and 6 against *M. abscessus* clinical isolates.

The two lead compounds, **12** and **6**, were further evaluated in *in vitro* and *in vivo* assays. Both exhibited an MIC of 0.125 μg/mL against the smooth (S) as well as the rough (R) variants of the reference *M. abscessus* strain CIP104536 (**Table 2**). Notably, both compounds were similarly active against 9 different clinical strains isolated from CF and non-CF patients. The *M. abscessus* complex is classified into three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense*[19, 20], and the distinction between these subspecies is clinically relevant because they can respond differently to antibiotics.[20] We found that 30 clinical isolates comprising 11 isolates of *M. abscessus* subsp. *abscessus*, 11 of *M. abscessus* subsp. *massiliense*, and 8 of *M. abscessus* subsp. *bolletii* with different susceptibility profiles to amikacin, were equally sensitive to **12** and **6**, generally exhibiting MICs of 0.125 μg/mL (**Table 2**). The lack

of cross-resistance between the indolecarboxamides and amikacin suggests that these compounds inhibit a different biological function, which is not targeted by amikacin.

To further understand the properties of the indolecarboxamides, exposure of *M. abscessus* 104536 (S) in exponential growth phase to 0.125 µg/mL of compound **12**, corresponding to 1 x MIC, resulted in a notable reduction in viable colony-forming units (CFU) of about 2 orders of magnitude in 3 days as compared to the initial inoculum. This *in vitro* killing effect of compound **12** was even more pronounced than the one obtained when exposing the cultures to 8 µg/mL imipenem (2 x MIC) **(Figure 2A)**. A comparable killing effect was obtained when cultures were treated with the same dose of compound **6 (Figure 2A)**.

(Figure 2 and Table 2)

Activity of compound 12 against intracellular *M. abscessus*.

The intracellular killing activity of **12** was next assessed after infection of PMA-activated THP-1 macrophages. To avoid growth of extracellular mycobacteria, cells were extensively washed and treated with amikacin. When left untreated for 4 days, the number of intracellular *M. abscessus* was increased by more than 2 orders of magnitude, while exposure to compound **12** (3 µg/mL, 24 x MIC) prevented any multiplication within the macrophages (**Figure 2B**). Treatment with imipenem (96 µg/mL, 24 x MIC) also significantly reduced the intracellular *M. abscessus* load, similar to compound **12**. The intracellular activity of both compounds was further confirmed by counting the number of macrophages infected with *M. abscessus* expressing tdTomato at day 4 post-treatment. A significant decrease in the percentage of infected cells treated with either imipenem or compound **12** was found (**Figure 2C**). Overall, these results clearly indicate that compound **12** is able to enter human macrophages and to stop bacterial replication.

The A309P mutation in MAB_4508 confers high resistance levels to compound 12.

To search for the molecular mechanism of action of the inhibitor, we first selected a spontaneous mutant of *M. abscessus* resistant to compound **12**, designated CIP_OK4-10^{R}, which was subsequently found to exhibit high resistance levels to both compounds **12** and **6** (MIC values of 32 µg/mL) (**Table 3**). Gene sequencing identified a single g925c single nucleotide polymorphism in *MAB_4508*, encoding MmpL3, which results in an amino acid replacement at position 309 (A309P). Interestingly, we had previously identified the same mutation in a compound **1**-resistant mutant (CIP_PIPD1^{R}),[15] which showed cross-resistance with compounds **12** and **6**. As anticipated, the A309P mutant selected on compound **12** was found to be resistant to compound **1** (**Table 3**).

To further validate the A309P mutation as the mechanistic basis for resistance to the indolecarboxamides, both the wild-type *MAB_4508* gene and the *MAB_4508* allele harboring the A309P mutation were cloned in pMV261 under the control of the constitutive *hsp60* promoter to allow overexpression of the wild-type and mutated versions of the protein in *M. abscessus*. Whereas overproduction of the wild-type protein failed to result in increased resistance to compounds **6** or **12** as compared to the control drug-susceptible strain, overexpression of the mutated protein resulted in high resistance levels when plated on 7H10^{OADC} media **(Figure 3)**. This indicates that transferring the single point mutation identified in the spontaneously resistant mutant to a susceptible strain is sufficient to confer resistance to compounds **6** and **12**.

Together, these results implicate the A309P mutation as critical in conferring high resistance levels to indole-2-carboxamides in *M. abscessus* and present evidence for the involvement of MmpL3 in the mode of action of this family of inhibitors.

(Figure 3)

Lead compound 12 inhibits mycolic acid transport in *M. abscessus*.

Indolecarboxamides have previously been proposed to target the mycolic acid transporter MmpL3 in *M. tuberculosis*[18], and it has been reported that a mutation in *mmpL3* correlates with resistance in *M. abscessus*. To explore whether compound **12** may alter this pathway in *M. abscessus*, untreated and **12**-treated cultures were metabolically labeled with [^{14}C]acetic acid to monitor (glyco)lipid synthesis. When applied at concentrations of up to 50 x MIC (6.2 μg/mL), compound **12** had no effect on *de novo* mycolic acid biosynthesis (**Figure 4A**). However, following apolar lipid extraction and their subsequent separation by thin layer chromatography (TLC), a dramatic inhibition of trehalose dimycolate (TDM) synthesis was observed concomitantly with an accumulation of trehalose monomycolate (TMM) (**Figure 4B** and **4D**), the latter being the substrate of MmpL3 for the transport of mycolic acids onto the mycobacterial surface where TDM is formed. To test whether treatment with compound **12** also impacts mycolylation of arabinogalactan (AG), radiolabeled mycolic acids were extracted from delipidated bacteria.[15, 21] The dose-dependent inhibition of [^{14}C]acetic acid incorporation suggests that compound **12** inhibits AG mycolylation with a pronounced decrease of cell wall-bound α- and α'- mycolic acids (**Figure 4C** and **4D**). The two types of mycolic acids (i.e., α and α') correspond to long-chain (C_{77-79}) and short-chain (C_{62-64}) mycolic acids, respectively, as reported earlier.[22] Indeed, compound **12** behaved similarly to compound **1**, an unrelated compound that inhibits the transport of mycolic acid transport in *M. abscessus* by targeting MmpL3.[15] Importantly, the mutant strain carrying the *MAB_4508*(A309P) allele was found to be totally refractory to the inhibition of TMM transport and to obstruction of AG mycolylation as compared to the parental strain, even in the presence of high concentrations of compound **12** (**Figure 4E-G**). In addition, very similar TLC profiles were obtained when treating

M. abscessus cultures with increasing doses of compound **6** (Supporting Information, **Figure S1**), indicating that both analogs target the same biosynthetic pathway. To exclude the possibility that compounds **12** and **6** may inhibit the synthesis of TDM and mycolylated arabinogalactan directly rather than the mycolic acid transport by targeting the mycolyltransferase activity of the antigen 85 complex[23], both compounds were tested for their potential inhibitory activity in an *in vitro* enzymatic assay using purified Ag85C[24]. This assay is based on a fluorescent probe (resorufin butyrate) that produces a direct measurement of the Ag85C enzymatic activity. Concentrations ranging from 2.5 to 50 μM of compounds **6** or **12** failed to inhibit the Ag85C activity (Supporting Information, **Figure S2)**. In sharp contrast, ebselen inhibited the activity of Ag85C in a dose-dependent manner, as reported previously[24].

Taken collectively, these biochemical data underline a mode of action that results in the abolition of mycolic acid translocation from the cytoplasm where they are synthesized, to the periplasmic side of the plasma membrane for subsequent use in the biogenesis of TDM and transfer onto the essential AG component of the mycobacterial cell wall.

(Figure 4)

Preliminary ADME studies on compound 12.

Based on the encouraging biological results described above, selected ADME studies were conducted on compound **12** to assess its drug-like properties (**Table 4**). The cell permeability of compound **12** was measured in the PAMPA cell line assay system. Compound **12** showed a low permeability with 0.2 x 10^{-6} cm/sec at pH 7.4 and 0.3 x 10^{-6} cm/sec at pH 5.0, respectively. In mouse and rat liver microsomes, compound **12** showed a high intrinsic clearance of 4.8 mL/min/g liver and 6.3 mL/min/g liver, respectively. Compound **12** showed a low propensity to inhibit CYP

1A2, 2C8 2C9, 2C19, 2D6, and 3A4 with an IC_{50} value of ≤ 20 μM. The compound exhibited high plasma protein binding in mouse (98.9%) and rat (99.2%). Based on the AMES mutagenicity assay, compound **12** showed no mutagenic potential in the histidine auxotrophic strains of *S. typhimurium* TA98 and TA100 at 0.24–500 μg/well. Since the compound showed less than a 2-fold increase in the number of revertant colonies compared to the negative controls in either the presence or absence of S9, it can be inferred that it has no mutagenic potential under the test conditions. The compound showed precipitation at 500 and 250 μg/well.

DISCUSSION AND CONCLUSIONS

M. abscessus is an emerging causative agent of chronic lung disease, particularly in patients with altered host defenses or disrupted airway clearance mechanisms, such as in CF. *M. abscessus* infection represents a threat to patients with CF with increased prevalence in recent years.[25,26] Among mycobacterial species, *M. abscessus* is regarded as one of the most drug-resistant species, and chemotherapeutic options are very limited.[8] The fact that most available treatments are often unsuccessful or poorly tolerated by patients, emphasizes the urgent need for developing more active and better-tolerated drugs. As originally conjectured, the notion of screening drugs that had previously demonstrated activity against *M. tuberculosis* for possible efficacy against *M. abscessus* has been shown to provide good lead candidates for combating *M. abscessus* infections.[15, 27] Following this cross-screen approach, we have screened a library of indole-2-carboxamides, reported to be highly active against *M. tuberculosis*.[16, 17] This allowed us to define a new structural class of compounds exhibiting promising activity against *M. abscessus*, further validating the value of utilizing data obtained from prior TB screens to directly identify new chemotypes with strong activity against *M. abscessus*. Our lead compounds **6** and **12** display very low MIC values against a

vast panel of clinical strains with different susceptibility profiles to antibiotics, regardless of whether they were isolated from CF or non-CF patients. Moreover, the efficacy of compounds **6** and **12** was not dependent on the smooth or rough morphotype of the *M. abscessus* subspecies, which is of particular interest since *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* infections can present various drug sensitivity test results, responses to antibiotics, and clinical symptoms.[28, 29] Additionally, compound **12** exhibited appreciable activity in infected macrophages.

To pinpoint the possible molecular mechanism of action of these inhibitors, we identified a spontaneous mutant which exhibited a high resistance phenotype to both lead compounds. Gene sequencing identified a single g925c single nucleotide polymorphism in *MAB_4508*, encoding MmpL3. This mutation resulted in an A309P amino acid replacement also previously identified in a compound **1**-resistant mutant,[15] which itself showed cross-resistance to compounds **6** and **12**. Consistent with these findings, compounds **6** and **12** inhibited the translocation of TMM, resulting in the abolition of both TDM formation and mycolylation of AG. Our analyses also revealed that compounds **6** and **12** were not capable to inhibit the activity of Ag85C *in vitro*. Together, our combined genetic and biochemical data reflect the in accessibility of the intracellularly generated TMM to the Ag85 family of mycolyltransferases,[23] similarly to other MmpL3 inhibitors, such as compound **1**,[15] the adamantyl-urea AU1235,[21,30] or *N*-geranyl-*N*'-(2-adamantyl)ethane-1,2-diamine (SQ109).[30]

Furthermore, sequence analysis indicates that the MmpL3 protein of *M. abscessus* (MAB_4508) and its counterpart in *M. tuberculosis* (Rv0206c)[18,31] show high similarity in multiple aspects. In addition to similar gene sizes, 3006 and 2835 base pairs, respectively, amino acid sequence blasting also revealed 56% identity and 69% similarity. Bioinformatic analysis indicates that both

MAB_4508 and Rv0206c are transmembrane proteins comprising 12 transmembrane helices. Currently, MmpL3 appears to be one of the most promising antimycobacterial pharmacological targets as multiple chemical entities have recently been shown to inhibit MmpL3 activity not only in *M. tuberculosis* but also in *M. abscessus*, thus opening a new field centered on the inhibition of mycolic acid transport.[15, 18, 21, 30-34] Interestingly, recent findings indicate that MmpL3 inhibitors can also act synergistically with other anti-tubercular drugs, which is of interest to efforts aiming at reducing the length of treatments.[35] Whether indole-2-caboxamides may also act in synergy with drugs used for the treatment of *M. abscessus* infections remains to be investigated.

Tetrahydropyrazo[1,5-*a*]pyrimidine-3-carboxamide (THPP) has recently been reported to inhibit the biogenesis of mycolic acids by targeting the enoyl-coenzyme A (CoA) hydratase EchA6 rather than the previously assumed MmpL3 target,[36] even though several mutations identified in *mmpL3* had been proposed to confer resistance to THPP[37]. It was subsequently demonstrated that THPP competes with CoA-binding so as to arrest mycolic acid production, leading to the conclusion that spontaneous resistance-conferring mutations in *mmpL3* can potentially obscure the actual target identification of small inhibitors. Our results failed to show an inhibition of mycolic acid biosynthesis, even in the presence of high concentrations of compounds **6** or **12**, and clearly demonstrated a dose-dependent inhibition of TDM synthesis with a concomitant accumulation of TMM. In addition, overexpression of the MmpL3(A309P) variant in a genetically susceptible strain correlated with high resistance levels to compounds **6** and **12** and rendered the strain refractory to TMM transport inhibition. Together, these results extend the results obtained previously in *M. tuberculosis* that indole-2-carboxamides act by altering the transport of TMM across the membrane in pathogenic, fast-growing non-tubercular mycobacteria.

How compound **1** and our indole-2-carboxamides inhibit MmpL3, and whether they have access to the same or different binding cavities in MmpL3, remains to be established. The design of further improved indolecarboxamides targeting mycolic acid transport would also greatly benefit from future structural and functional studies on MmpL3. Moreover, details as to whether the activity of the indolecarboxamides results from specific inhibition of the MmpL3 transporter functions or through indirect mechanisms, for example involving the dissipation of the proton-motive force as recently suggested[38], require additional investigations. In summary, our indole-2-carboxamides represent a very promising chemotype that is able to alter the mycolic acid profile in *M. abscessus*, a species that is naturally resistant to most mycolic acid biosynthesis inhibitors. Of particular interest for medicinal chemistry and drug development, these indole analogs are easy to prepare and generally show reasonably good ADME properties as reported earlier[16] and as further investigated herein for compound **12**. This work, along with previous findings using compound **1**, support the view that targeting the transport of mycolic acids rather than their biosynthesis, has significant translational potential for development of these chemotypes into real drugs for *M. abscessus* treatment and control. Research is currently underway to assess their efficacy in *M. abscessus* animal models.

EXPERIMENTAL SECTION

Chemistry.

General information. The tested 28 compounds were synthesized and characterized as reported in literature.[16, 17] Purities of final compounds were confirmed to be ≥ 95% by analytical HPLC, which was carried out using an Agilent 1100 HPLC system with a Synergi 4 μm Hydro-RP 80A column, and a variable wavelength detector G1314A; method 1, flow rate 1.4 mL/min, gradient elution over

20 min, from 30% MeOH–H_2O to 100% MeOH with 0.05% TFA added to both solvents; method 2, flow rate 1.4 mL/min, gradient elution over 20 min, from 50% MeOH–H_2O to 70% MeOH–H_2O with 0.05% TFA added to both solvents.

Bacterial strains.

M. abscessus subsp. *abscessus* CIP104536^T, *M. abscessus* subsp. *bolletii* CIP108541^T, and *M. abscessus* subsp. *massiliense* CIP108297^T reference strains were used along with a series of clinical isolates as reported previously.[27] Strains were routinely grown and maintained at 30 °C in Middlebrook 7H9 broth (BD Difco) supplemented with 0.05% Tween 80 (Sigma-Aldrich) and 10% oleic acid, albumin, dextrose, and catalase (OADC enrichment; BD Difco) (7H9$^{T/OADC}$), or on Middlebrook 7H10 agar (BD Difco) containing 10% OADC enrichment (7H10^{OADC}), and in the presence of antibiotics when required. For drug susceptibility testing, bacteria were grown in cation-adjusted Mueller-Hinton broth (CaMHB; Sigma-Aldrich).

Drug susceptibility testing.

The minimal inhibitory concentrations (MIC) were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[39] The broth microdilution method was used in CaMHB with an inoculum of $5x10^6$ CFU/mL in the exponential growth phase. Briefly, the bacterial suspension was seeded in 100 µL volumes in all of the wells of a 96-well plate, except for the first column to which 198 µL of the bacterial suspension was added to each well. In the first column, 2 µL of compound at its highest concentration was added in six wells (the solvent used to dissolve the compound was added to the two outermost wells as a control). Two-fold serial dilutions were then carried out by transferring 100 µL from the wells in the first column to the next column and

repeating this for each successive column. Plates were subsequently incubated at 30 °C for 3-5 days. MICs were recorded by visual inspection and by absorbance at 560 nm to confirm visual recordings. Experiments were done in triplicate on three independent occasions.

Time-kill assay.

Microtiter plates were set up as for the MIC determination. Serial dilutions of the bacterial suspensions from these microtiter plates were plated after 0, 24, 48, 72, and 96 h of exposure to different drug concentrations. Colony-forming units (CFU) were counted after 4 days of incubation at 30 °C.

Intramacrophage killing assay.

THP-1 cells were grown in RPMI medium containing 10% fetal bovine serum ($RPMI^{FBS}$) and differentiated with 20 ng/mL phorbol myristate acetate (PMA) in 12-well flat-bottom tissue culture microplates ($1x10^5$ cells/well) and incubated for 48 h at 37 °C. Cells were infected with a multiplicity of infection (MOI) of 1:2 and incubated at 37 °C in the presence of 5% CO_2 for 2 h. Wells were carefully washed three times with 1 x PBS, refed with $RPMI^{FBS}$ supplemented with 200 μg/mL amikacin, re-incubated at 37 °C in the presence of 5% CO_2 for 1 h, and washed again three times with PBS prior to the addition of 1 mL $RPMI^{FBS}$ supplemented with either 3 μg/mL compound **12** or 96 μg/mL imipenem. Media were changed on a daily basis with fresh drug preparations. At various time points (0, 48, and 96 h), cells were washed three times with PBS and lysed by adding 100 μL 1% Triton X100. Cell lysis was stopped after 2 min by adding 900 μL PBS, and serial dilutions were plated to monitor the intracellular bacterial counts. Experiments were done two times independently. For determining the percentage of infected macrophages, THP-1 cells

were prepared and infected with *M. abscessus* expressing tdTomato and treated with drugs, as described above. Untreated and drug-treated infected cells (96 h time point) were observed under a fluorescence microscope to count the number of bacteria-containing macrophages, as previously described.[15]

Selection of a compound 12-resistant mutant.

Exponentially growing *M. abscessus* cultures were plated on $7H10^{OADC}$ containing 6 μg/mL of compound **12**. After one week of incubation at 30 °C, a single colony was selected and grown in liquid medium, assessed by MIC determination, and subsequently scored for resistance to compound **12**. Identification of putative SNPs in the *MAB_4508* (*MmpL3*) gene was carried out by PCR amplification using the *MAB_4508seq1* (5'-GGCCACCGTCTTTACCAATA-3') and *MAB_4508seq7* (5'- GTGCCGTACCTGAATGTCCT-3') primers to produce a 3549 bp amplicon to gain full coverage sequencing of *MAB_4508*, as done previously.[15] The SNP was confirmed by at least two independent sequencing reactions. In order to over-express the MAB_4508 A309P allele, the *MAB_4508* open reading frame was PCR-amplified using as template purified genomic DNA of CIP_OK4-10^{R}, Phusion DNA polymerase (Finnzymes, Finland) and the primers *MAB_4508f* and *MAB_4508r*, as previously described.[15] The obtained PCR product was subsequently digested with HindIII and ligated to MscI/HindIII linearised pMV261.

Whole-cell radiolabeling experiments and lipid analysis.

For visualizing drug-induced changes in the lipid profile, increasing concentrations of compound **12** were added to exponentially-growing cultures for 1 h, and metabolic labeling of lipids was performed by adding 1 μCi/mL of [^{14}C]acetate (56 mCi/mmol) for an additional 2 h at 37 °C. Cells

were harvested and either delipidated or used immediately to extract mycolic acids, as previously described.[40,22] The apolar lipid fraction containing trehalose monomycolate (TMM) and trehalose dimycolate (TDM) was separated on a one-dimensional thin layer chromatography (TLC) plate using chloroform/methanol/water (40:8:1, v/v/v) and revealed after exposure to a film. Delipidated cells were further processed to extract the mycolic acids[41], which were analyzed by TLC/autoradiography using petroleum ether/acetone (95:5, v/v) and exposure to a film to reveal ^{14}C-labeled mycolic acid methyl esters (MAME).

Cell Permeability.

The permeability of compound **12** was measured at two pH values (pH 7.4 and pH 5.0) in Prisma^{TM} HT aqueous buffer (pION Inc.). The pH was adjusted with 0.5 N NaOH. The test compound in buffer solutions (25 μM) was added to the PAMPA donor plate (in triplicate). The acceptor plate was painted with 4 μL of GIT-0 lipid solution (pION Inc.), and ASB (acceptor sink buffer, pION Inc.) was added. The acceptor and donor plates were stacked, sealed with a rubber plate, and incubated for 4 h at 25 ± 2 °C. The donor and acceptor plate solutions were analyzed by an LC-MS (UPLC) system.

***In vitro* study - CYP inhibition.**

Inhibition of CYP by compound **12** was evaluated using pooled human liver microsomes (Cat# 452118, BD-Biosciences). 10 μM of compound **12** or the positive control was preincubated with human liver microsomes for 5 min at 37 °C. The reaction was initiated by addition of an NADPH regenerating system and incubated at 37 °C with gentle agitation for the specified time recommended for each of the CYP's (5 min for CYP3A4, 10 min for CYP1A2, 2C8, 2C9, and 2D6,

and 40 min for CYP2C19). The samples were vacuum-filtered using Captiva 96 well plates followed by LC-MS/MS analysis. The formation of standard metabolite (concentration/peak area ratio) was estimated. The percentage of inhibition of the test compound and the positive control in comparison to a negative control (DMSO) were determined using the equation:

$$\% \text{ Inhibition} = 100 - \left[\left(\frac{\text{Average metabolite concentration of test compound/positive control}}{\text{Average metabolite concentration of no inhibitor control}}\right) \times 100\right]$$

Plasma Protein Binding.

Plasma protein binding studies were performed using an ultracentrifuge method. A 5 μM solution of compound **12** was prepared in mouse or rat plasma using 500 μM stock solution (in DMSO) and incubated for 10 min at 37 °C. For determination of total plasma concentration, 5 μL of incubated plasma was mixed thoroughly with 50 μL of saline, 50 μL of acetonitrile, and 100 μL of 1 μM niflumic acid. For determination of unbound concentrations, the incubated plasma sample was centrifuged at 200,000 x *g* for 4.5 h, and 50 μL of supernatant from the middle layer was taken after removing the upper layer with a Beckmann coulter slicer. To this solution, 5 μL blank plasma, 50 μL acetonitrile, and 100 μL of 1 μM niflumic acid were added. The samples were filtered into a 96-deep well storage plate with a Captiva 0.45 μm 96-well filter plate, and appropriate volumes of all samples were analyzed by LC-MS/MS.

Metabolic stability.

Compound **12** (0.5 μM) was incubated in a reaction mixture consisting of mouse or rat liver microsomes and an NADPH regenerating system. Aliquots were withdrawn at 3 min intervals for 30 min and analyzed for the parent compound by LC-MS/MS. The loss of parent was expressed as the percentage of the test compound remaining, and the rate of decay was estimated by mono-

exponential decay kinetics. The rate of decay was normalized to microsomal protein expressed as mL/min/g liver.

AMES mutagenicity assay.

Compound **12** was evaluated for its mutagenic potential in the presence and absence of the S9 fraction by investigating its ability to induce reverse mutations at the histidine locus in the genome of *Salmonella typhimurium* strains. Two histidine auxotrophic strains of *Salmonella typhimurium* were used, namely the TA98 strain carrying a frame shift mutation, and the TA100 strain carrying a base-pair substitution. Plates were incubated at 37 ± 2 °C for 72 h, and the experiments were carried out in triplicates. DMSO was used as vehicle control. Sodium azide, 2-nitrofluorene (2NF), and 2-aminoanthracene (2AA) were used as positive controls. A compound was considered positive if the number of revertants increased by ≥ 2-fold over the negative control in a dose-dependent manner.

ASSOCIATED CONTENT

SUPPORTING INFORMATION

The supporting information contains 2 supplementary figures. Material is available free of charge via the Internet at http://pubs.acs.org. Molecular formula strings and some data (CSV) are also available as supporting information.

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The authors declare no conflict of interest.

ABBREVIATIONS USED:

CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; CFTR, cystic fibrosis transmembrane conductance regulator; TB, tuberculosis; NTM, non-tubercular mycobacteria;

CaMHB, cation-adjusted Mueller-Hinton broth; MIC, minimal inhibitory concentration; CLSI, Clinical Laboratory Standards Institute; CFU, colony-forming units; PMA, phorbol myristate acetate; SAR, structural activity relationship; TMM, trehalose monomycolate; TDM, trehalose dimycolate; TLC, thin layer chromatography; MAME, mycolic acid methyl ester; DMSO, dimethyl sulfoxide; 2NF, 2-nitrofluorene; 2AA, 2-aminoanthracene; AG, arabinogalactan; THPP, tetrahydropyrazo[1,5-*a*]pyrimidine-3-carboxamide.

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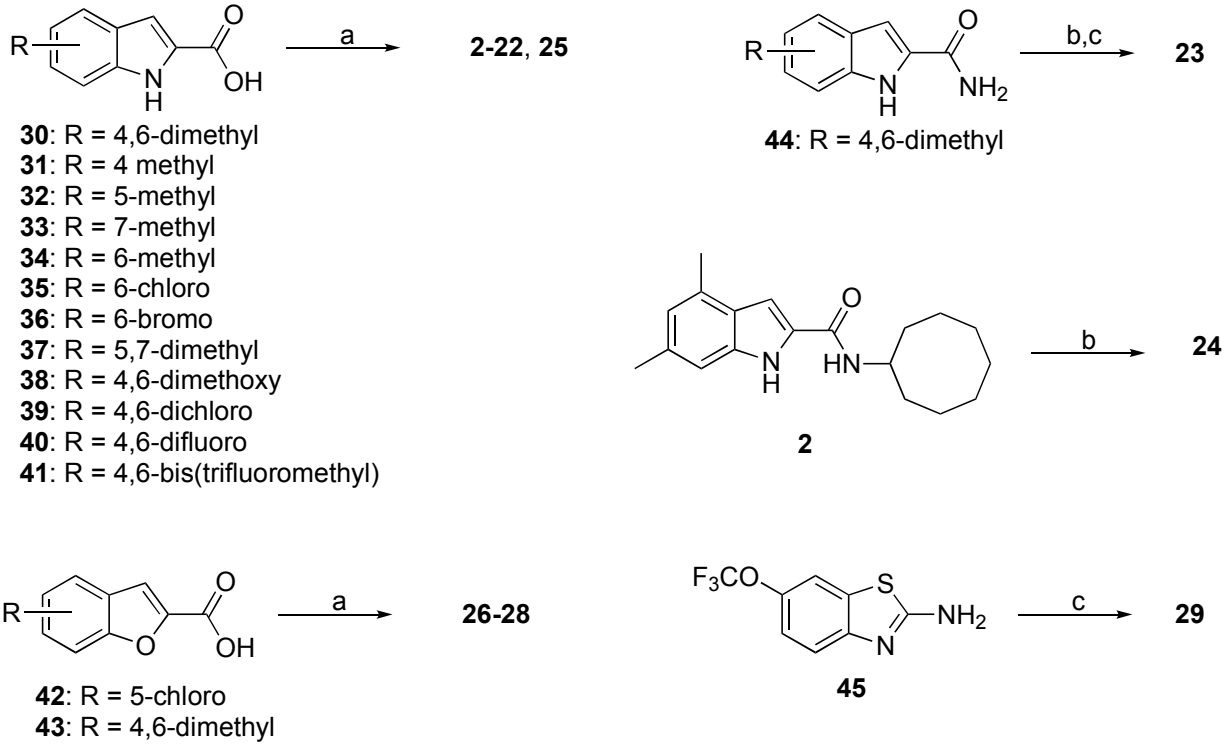
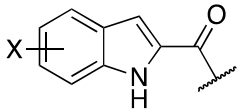
Scheme 1. Synthesis of compounds **2-29**.[a]



[a]**Reagents and conditions**: (a) EDC·HCl, corresponding amines, Et_3N, CH_2Cl_2, rt, 12-16 h; (b) $LiAlH_4$, THF, reflux, overnight; (c) cycloheptanecarbonyl chloride, CH_2Cl_2, rt, overnight.

Table 1. MIC values of indole-2-carboxamides against *M. tuberculosis* and the *M. abscessus* 104536 reference strain, and the compounds' CLogP values. Data for compounds **6** and **12** are in bold.



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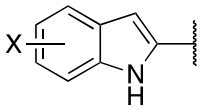
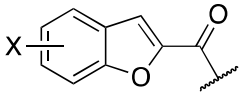
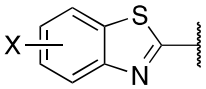


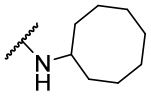
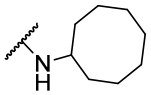
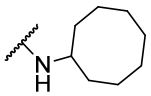
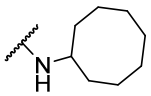
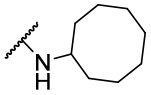
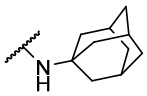
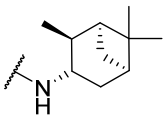
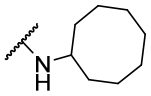
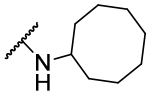
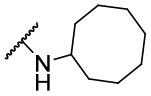
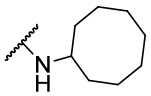
23-24

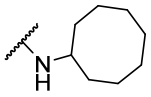
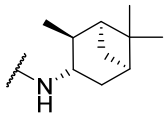
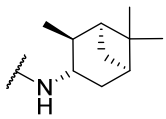
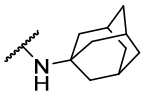
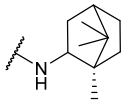
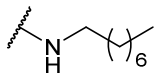
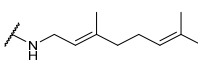
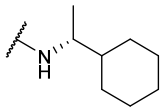
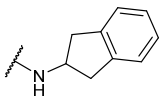
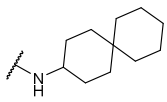
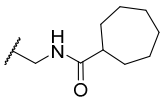
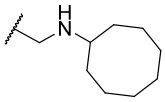
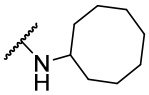
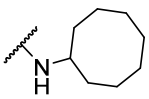
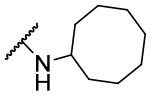


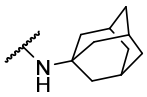
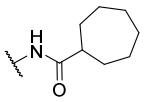
26-28



29

Compd.	X	R	$MIC_{M.\ tb.}$[a] (μg/mL)	$MIC_{M.\ abs.}$[b] (μg/mL)	$IC_{50\ VERO}$ (μg/mL)	SI[c] *M. tb.*	SI[d] *M. abs.*	CLogP[e]
2	4,6-dimethyl		0.0039	0.125	16	4100	128	5.59
3	4-methyl		0.0313	0.25				5.10
4	5-methyl		0.250	0.25				5.10
5	6-methyl		0.0313	0.25				5.10
6	**6-Br**		**0.0313**	**0.125**	**≥64**	**≥2050**	**≥512**	**5.56**
7	6-Br		0.0156	16.0	≥64	≥4100	≥4	5.07
8	6-Br		0.0039	0.125				6.28
9	7-methyl		1.00	16.0				5.10
10	5,7-dimethyl		0.0625	0.250				5.59
11	4,6-dimethoxy		0.0625	1.00				4.57
12	**4,6-dichloro**		**0.0039**	**0.125**	**8-16**	**2050-4100**	**64-128**	**6.16**

13	4,6-difluoro		0.031	0.125				5.03
14	4,6-dichloro		0.0078	128	16	2050	0.125	6.88
15	4,6-difluoro		0.0039	32.0	≥64	≥16400	≥2	5.74
16	4,6-dichloro		0.0039	>32.0	≥32	≥8210	≥1	5.67
17	4,6-dichloro		0.0156	>32.0	8	513	0.25	6.88
18	4,6-dichloro		128	>32.0				6.72
19	4,6-dichloro		4.00	>32.0				6.75
20	4,6-dichloro		2.00	0.50				5.97
21	4,6-dichloro		0.250	>32.0				5.00
22	4,6-dichloro		0.001953	>32.0	≥64	≥32800	≥2	7.04
23	4,6-dimethyl		16.0	>32.0				4.53
24	4,6-dimethyl		0.125	0.50				5.65
25	4,6-bis(trifluoro-methyl)		0.0156	0.50				6.64
26	5-chloro		8.00	0.50				5.43
27	4,6-dimethyl		8.00	0.50				5.57

28	4,6-dimethyl		1.00	1.00			5.54
29	5-trifluoro-methoxy		128	>32.0			5.99
INH			0.04		>256	>6400	
RIF			0.125		>256	>2050	

[a]The lowest concentration of drug leading to at least a 90% reduction of bacterial growth signal by the microplate AlamarBlue assay (MABA), as previously reported.[16, 17] MIC values are reported as an average of three individual measurements; [b]MIC values (μg/mL) were determined using the microdilution method and cation-adjusted Mueller-Hinton broth according to the *Clinical and Laboratory Standards Institute* guidelines.[39] [c]SI = $IC_{50\ VERO}$/ $MIC_{M.\ tb.}$; [d] SI = $IC_{50\ VERO}$/ $MIC_{M.\ abs.}$; [e]Calculated using ChemBioDraw Ultra 13.0. INH, isoniazid; RIF, rifampicin.

Table 2. MICs (μg/mL) of compounds **12** and **6** in cation-adjusted Mueller-Hinton broth for 30 clinical isolates from CF and non-CF patients belonging to the *M. abscessus* complex.

Strain	Morphotype	Source	Cpd. 12	Cpd. 6	AMK*
M. abscessus subsp. *abscessus*					
CIP104536	R	Non-CF	0.125	0.0625	12.5
2524	R	CF	0.125	0.125	25
2648	R	CF	0.125	0.125	12.5
3022	R	Non-CF	0.125	0.125	12.5
CF	R	CF	0.125	0.125	12.5
CIP104536	S	Non-CF	0.125	0.125	25
3321	S	Non-CF	0.125	0.125	25
1298	S	CF	0.125	0.125	12.5
2587	S	CF	0.125	0.25	25
2069	S	Non-CF	0.125	0.125	25
CF	S	CF	0.125	0.125	12.5
M.abscessus subsp. *massiliense*					
CIP108297	R	Addison Disease	0.125	0.125	12.5
210	R	CF	0.125	0.125	12.5
CIP108297	S	Addison Disease	0.125	0.25	25
111	S	CF	0.125	0.125	25
212	S	CF	0.125	0.125	25
185	S	CF	0.125	0.125	25
140	S	CF	0.125	0.125	25
100	S	CF	0.125	0.125	>100
107	S	CF	0.25	0.25	12.5
122	S	CF	0.125	0.125	12.5
120	S	CF	0.125	0.125	12.5
M. abscessus subsp. *bolletii*					
19	R	Non-CF	0.125	0.125	50
108	R	CF	0.25	0.5	12.8
112	R	CF	0.125	0.0625	>100
CIP108541	S	Not reported	0.125	0.125	25
17	S	Non-CF	0.125	0.125	12.5
97	S	CF	0.125	0.25	12.5
114	S	CF	0.125	0.125	12.5
116	S	CF	0.25	1	12.5

*AMK, amikacin; MIC values are from the literature.[27]

Table 3. MIC of a spontaneous resistant strain carrying the A309P mutation in MAB_4508. MIC values (µg/mL) were determined in cation-adjusted Mueller-Hinton broth. The resistant strain, designated CIP_OK4-10^{R}, was derived from the *M. abscessus* CIP104536 (S) parental strain. The CIP_PIPD1^{R} mutant was reported previously.[15]

Strain	MIC Cpd. 1	MIC Cpd. 12	MIC Cpd. 6	Mutation in *MAB_4508*	
				SNP	AA change
CIP104536 (S)	0.125	0.125	0.125	-	-
CIP_OK4-10^{R}	8	32	32	g925c	A309P
CIP_PIPD1^{R}	8	32	32	g925c	A309P

Table 4. ADME data for compound **12**.

Assay	Values
Permeability (PAMPA)	Low ($0.2x10^{-6}$ cm/s and $0.3x10^{-6}$ cm/s at pH 7.4 and 5.0, respectively)
Plasma Protein Binding (%), Mouse/Rat	98.9/99.2
CYP inhibition (% inhibition at 10 μM) CYP1A2 CYP2C8 CYP2C9 CYP2C19 CYP2D6 CYP3A4	 19 20 6 4 10 1
CL_{int} in LM*, mL/min/g liver (Mouse/Rat)	High (4.8/6.3)
Ames	Negative

*LM: Liver microsomes

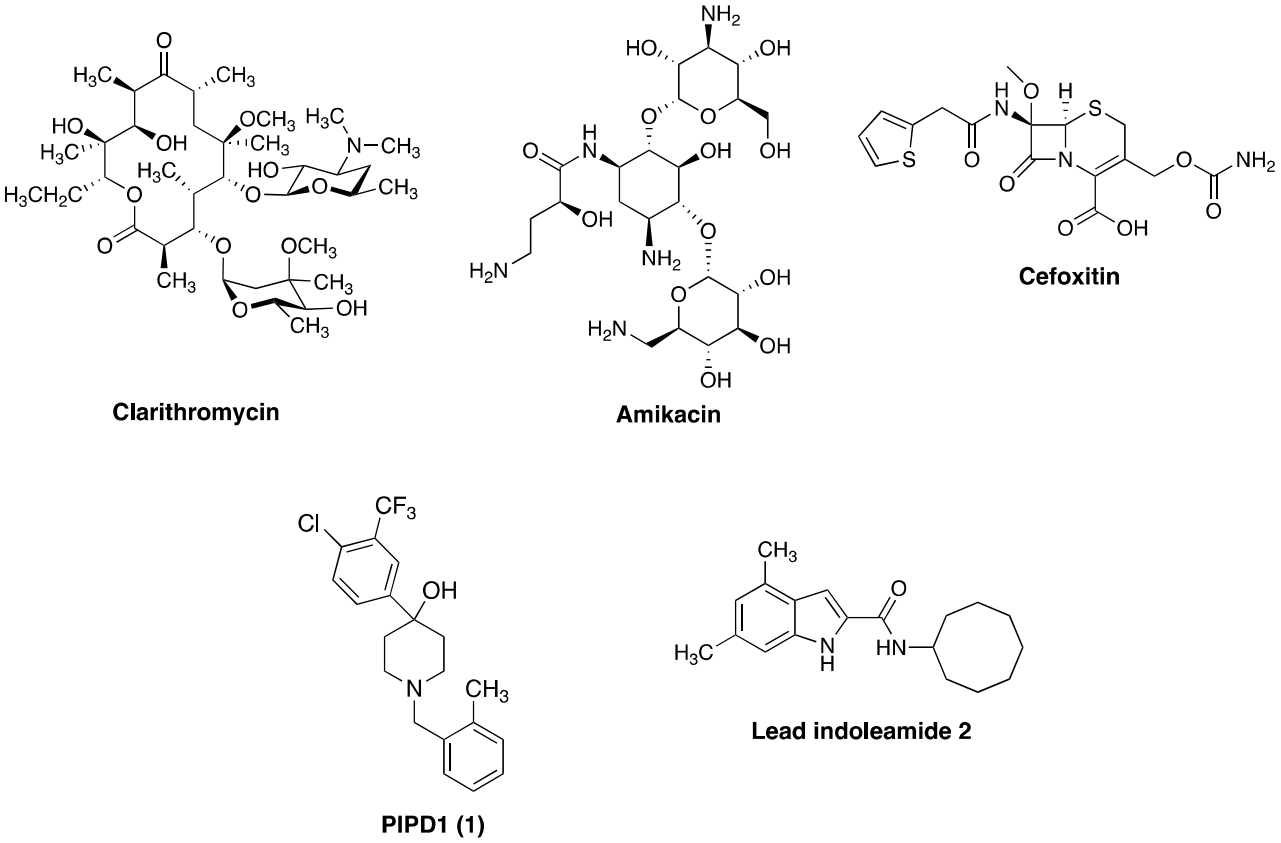


Figure 1. Structures of clarithromycin, amikacin, cefoxitin, PIPD1 (**1**), and our lead indolecarboxamide **2**.

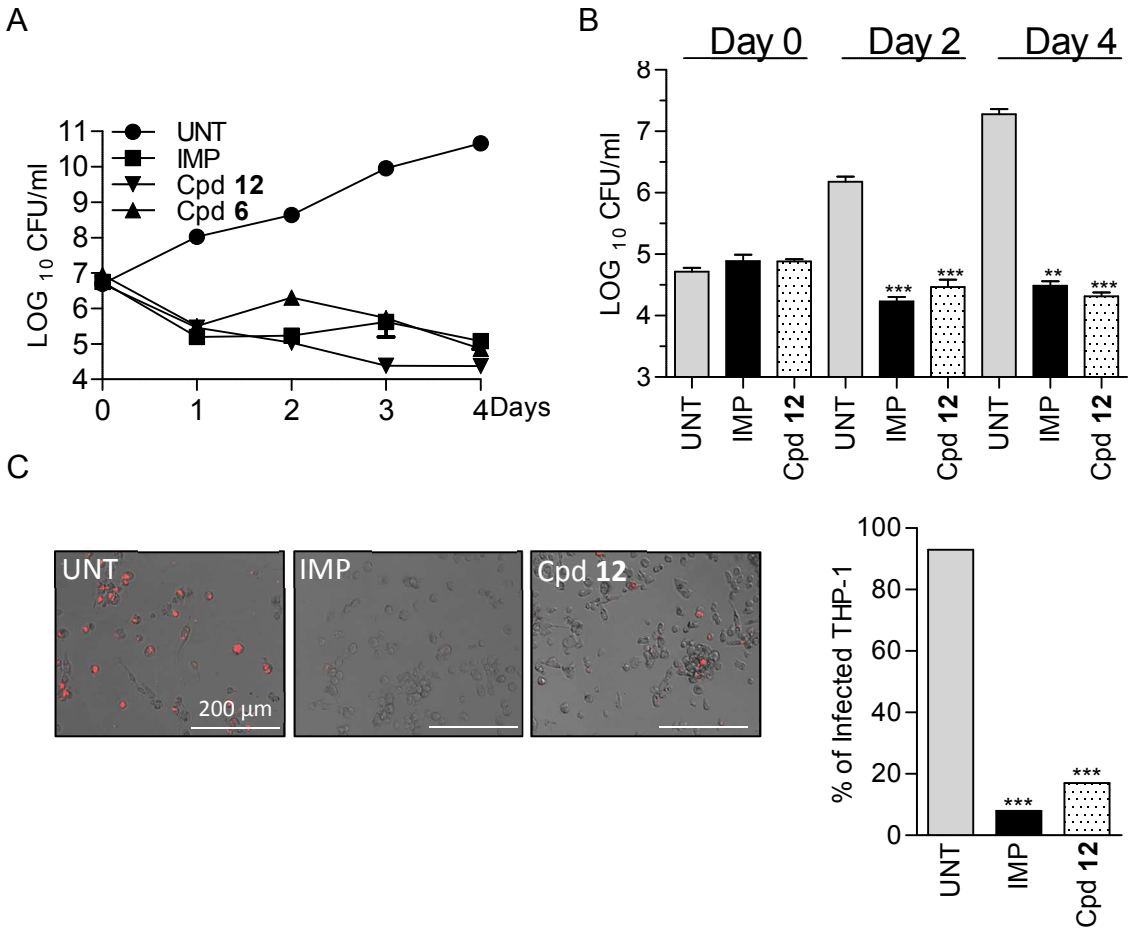


Figure 2. *In vitro* and intracellular activity of indole-2-carboxamides. (A) *In vitro* killing curves of *M. abscessus* CIP 104536 (smooth strain) exposed to 8 µg/mL imipenem (IMP) (2 x MIC), 0.125 µg/mL compound **12** (1 x MIC), or 0.125 µg/mL compound **6** (1 x MIC) in CaMH broth at 37 °C. Results are expressed as mean of triplicate Log10 values ± SEM. UNT, untreated cultures. The results for each drug concentration are representative of three independent experiments. **(B)** THP-1 macrophages were infected with *M. abscessus* CIP 104536. After removal of the extracellular bacteria by extensive washing and treatment with amikacin, cells were treated with IMP at 96 µg/mL (24 x MIC) or compound **12** at 3 µg/mL (24 x MIC). Cells were washed with PBS, and fresh medium containing the different drugs was renewed every 24 h. CFUs were determined at 0, 2, and 4 days post-treatment. Results are expressed as mean of triplicates from two independent experiments, and error bars represent the SEM. One-tailed Mann-Whitney's *t* test comparing the values with the day 0 was applied with $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. **(C)** Following infection with *M. abscessus* expressing tdTomato (red fluorescence), macrophages were treated with either IMP or compound **12** (each at 24 x MIC) and observed under the microscope at day 4 post-treatment (4297 total cells were counted) to determine the percentage of infected cells. Corresponding microscopy captures are also shown. Fisher exact test, $^{***}P < 0.001$.

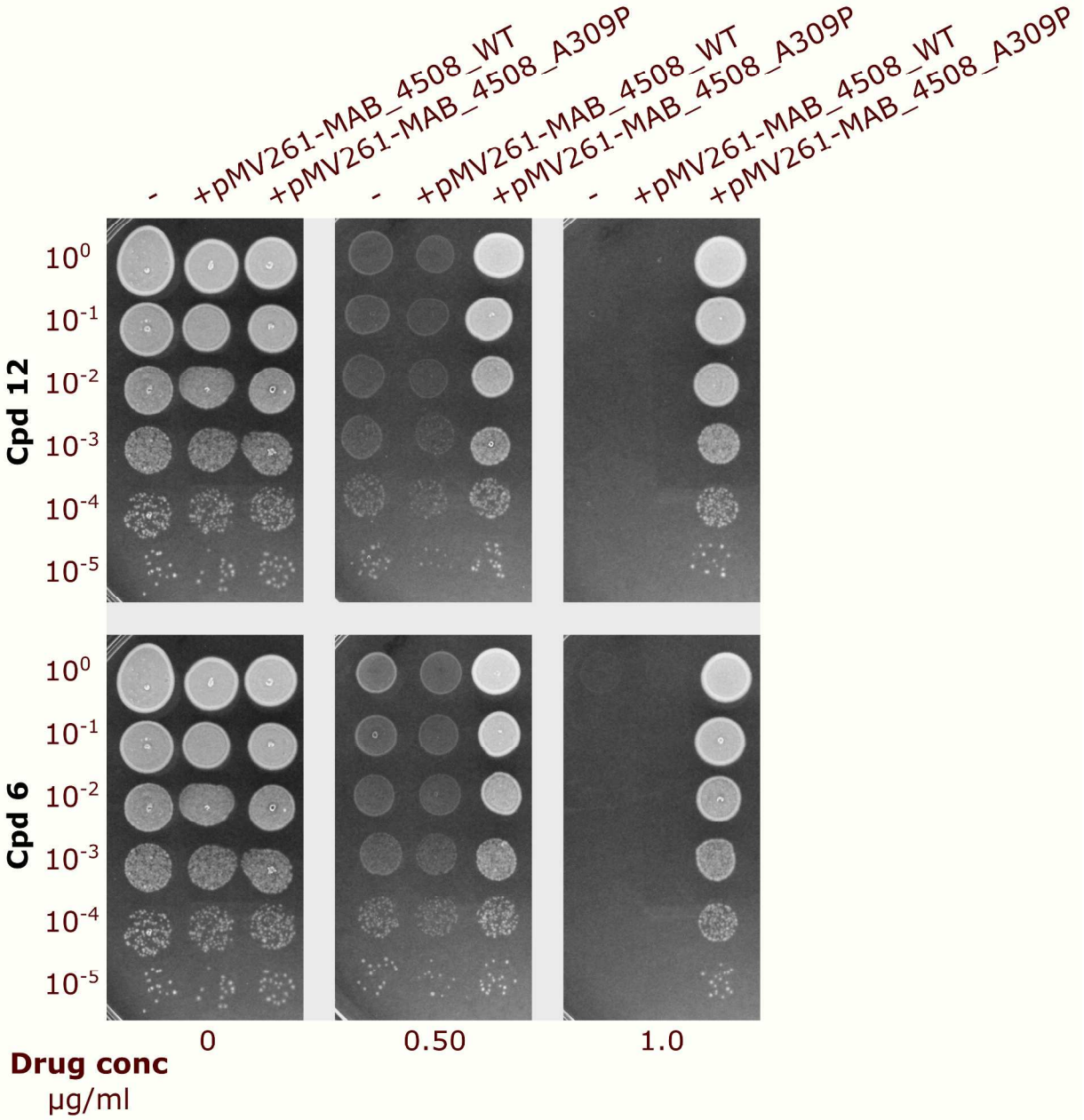


Figure 3. Over-expression of the *MAB_4508* A309P mutated allele in *M. abscessus* renders the bacterium resistant to compounds 12 and 6. Exponentially growing wild-type *M. abscessus*, or *M. abscessus* carrying either the pMV261_*MAB_4508*_WT or the pMV261_*MAB_4508*_A309P constructs, were grown in $7H9^{OADC}$ and serially diluted and spotted (5 µL) on $7H10^{OADC}$ media containing different concentrations of compounds **12** and **6**. Plates were incubated at 30 °C for 3 days prior to growth inspection.

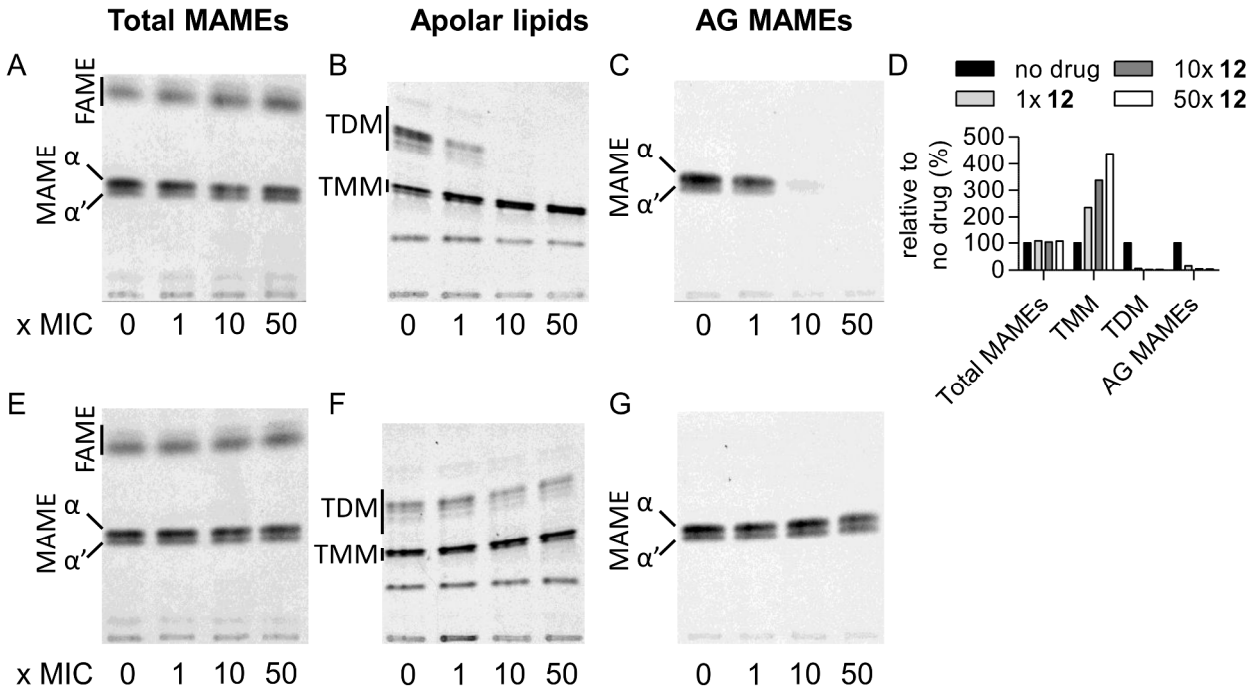


Figure 4. Treatment with compound 12 prevents TMM transport in *M. abscessus* and involves the Ala309 residue in MmpL3. Exponentially growing *M. abscessus* - wild-type **(A-D)** or carrying the A309P mutation in *mmpL3* **(E-G)** - were incubated with increasing concentrations of compound **12** in $7H9^{OADC}$ at 37 °C with agitation for 1 h. Subsequently, bacteria were labeled with [^{14}C]acetate for 2 h at 37 °C with agitation. The cultures were split, and from the first volume were extracted the total methyl esters of mycolates (MAME) and methyl esters of fatty acids (FAME). From the second volume, apolar and polar fractions were obtained prior to derivatization of arabinogalactan (AG) mycolate methyl esters. A and E: Equal counts (50,000 cpm) of the MAME and FAME fraction were loaded on a TLC plate and resolved once using the solvent system petroleum ether/acetone (95:5, v/v). B and F: The apolar fraction was loaded (50,000 cpm), and TMM/TDM were visualized on a 1D TLC plate using the solvent system chloroform/methanol/water (40:8:1, v/v/v). C and G: Equal volumes of the arabinogalactan MAME fractions were loaded, and α- and α'-mycolic acids were visualized on a 1D TLC plate using the solvent system petroleum ether/acetone (95:5, v/v). D: Densitometric analysis of the TLC plates shown in A-C.

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