Piperidine-4-Carboxamides Target DNA Gyrase in *Mycobacterium abscessus*

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*ABSTRACT*  New, more-effective drugs for the treatment of lung disease caused by nontuberculous mycobacteria (NTM) are needed. Among NTM opportunistic pathogens, *Mycobacterium abscessus* is the most difficult to cure and intrinsically multidrug resistant. In a whole-cell screen of a compound collection active against *Mycobacterium tuberculosis*, we previously identified the piperidine-4-carboxamide (P4C) MMV688844 (844) as a hit against *M. abscessus*. Here, we identified a more potent analog of 844 and showed that both the parent and improved analog retain activity against strains representing all three subspecies of the *M. abscessus* complex. Furthermore, P4Cs showed bactericidal and antibiofilm activity. Spontaneous resistance against the P4Cs emerged at a frequency of $10^{-8}$/CFU and mapped to *gyrA* and *gyrB* encoding the subunits of DNA gyrase. Biochemical studies with recombinant *M. abscessus* DNA gyrase showed that P4Cs inhibit the wild-type enzyme but not the P4C-resistant mutant. P4C-resistant strains showed limited cross-resistance to the fluoroquinolone moxifloxacin, which is in clinical use for the treatment of macrolide-resistant *M. abscessus* disease, and no cross-resistance to the benzimidazole SPR719, a novel DNA gyrase inhibitor in clinical development for the treatment of mycobacterial diseases. Analyses of P4Cs in *recA* promoter-based DNA damage reporter strains showed induction of *recA* promoter activity in the wild type but not in the P4C-resistant mutant background. This indicates that P4Cs, similar to fluoroquinolones, cause DNA gyrase-mediated DNA damage. Together, our results show that P4Cs present a novel class of mycobacterial DNA gyrase inhibitors with attractive antimicrobial activities against the *M. abscessus* complex.

*KEYWORDS*  *Mycobacterium abscessus*, NTM, nontuberculous mycobacteria, MMV688844, DNA gyrase

While the incidence of tuberculosis has declined, infections by nontuberculous mycobacteria (NTM) are increasing (1–3). NTM lung disease is the most common clinical presentation and is primarily caused by members of the *Mycobacterium abscessus* and *Mycobacterium avium* complexes (4). Although they are close relatives of *Mycobacterium tuberculosis*, these NTM species exhibit differential pathogenesis due to expression of novel surface lipids, adaptation to both host and environmental niches, and acquisition of novel virulence factors (5). In addition, NTM demonstrate intrinsic resistance to a broad range of antibiotics (3, 6). The current treatments for NTM lung disease vary by species and differ from the standard four-drug TB chemotherapy (7, 8).
*M. abscessus* next to *M. avium* presents the most difficult to cure NTM disease (9, 10). Its treatment regimen typically consists of a macrolide (azithromycin or clarithromycin) combined with parenteral drugs (amikacin, imipenem-cefoxitin, tigecycline) and may include other oral antibiotics (clofazimine, linezolid) for a minimum of 12 months (8). However, with cure rates of less than 50%, treatment outcomes for patients with *M. abscessus* infections are unsatisfactory (11, 12). Contributing to the poor treatment outcomes may by the ability of *M. abscessus* to grow as biofilms in patients (13, 14). Most clinically used anti-*M. abscessus* drugs are bacteriostatic and do not show antibiofilm activity, which may contribute to the ineffectiveness of current regimens (15). Thus, new, more- efficacious drugs are needed to curb the rise of NTM infections, including “incurable” *M. abscessus* lung disease (16–18).

Over the past 2 decades, a large number of novel hits active against *M. tuberculosis* were identified through successful whole-cell screening campaigns, leading to a reenergized tuberculosis drug discovery and development pipeline (https://www.newtbdrugs.org/). We and others have shown that focused libraries of compounds active against *M. tuberculosis* provide an attractive source of hits active against NTM (17–21). In one such screen, using the Pathogen Box collection from Medicines for Malaria Venture (https://www.mmv.org/mmv-open/pathogen-box), we identified the piperidine-4-carboxamide (P4C) MMV688844 (844) as a hit active against our *M. abscessus* screening strain *M. abscessus* subsp. *abscessus* Bamboo (21). This compound was originally identified as the noncytotoxic hit TCMDC-143649 in GlaxoSmithKline’s whole-cell screen against *M. tuberculosis* (22). Based on in silico analyses, it was proposed that 844 targets mycobacterial ABC transporters (23).

Here, we show that 844 and a more potent analog are broadly active against a collection of strains representing the three subspecies of the *M. abscessus* complex. We demonstrate that the P4Cs show attractive bactericidal and antibiofilm activity and determine their mechanism of action. Genetic, biochemical, and reporter strain analyses indicate that P4Cs target mycobacterial DNA gyrase, rather than ABC transporters as proposed earlier (23). Thus, this work identified a novel mycobacterial DNA gyrase inhibitor.

**RESULTS AND DISCUSSION**

**844 and improved analog 844-TFM are active against *M. abscessus* subspecies.** We previously reported the activity of 844 (Fig. 1) against the clinical isolate *M. abscessus* subsp. *abscessus* Bamboo (21). To determine the potential usefulness of this new
whole cell active for the treatment of *M. abscessus* disease, we measured its activity against reference strains representing the three subspecies of the *M. abscessus* complex as follows: *M. abscessus* subsp. *abscessus* ATCC 19977, *M. abscessus* subsp. *bolletii* CCUG 50184T, *M. abscessus* subsp. *massiliense* CCUG 48898T, and a collection of clinical isolates. 844 retained activity against the *M. abscessus* complex strains with MICs ranging from 6 to 14 μM (Table 1).

Next, we generated a set of analogs, demonstrating a dynamic structure activity relationship (A. Beuchel, D. Robaa, D. A. Negatu, A. Madani, N. Alvarez, M. D. Zimmerman, A. Richter, L. Mann, S. Hoenke, R. Csuk, T. Dick, and P. Imming, unpublished data). By substituting the chlorine in position 4 of the phenyl moiety of 844 with a trifluoromethyl group (Fig. 1), we identified an ~8-fold more potent analog (844-TFM). Similar to 844, 844-TFM retained activity across the *M. abscessus* complex with MICs ranging from 1.5 to 6 μM (Table 1).

P4Cs are bactericidal and active against *M. abscessus* biofilm cultures. We previously reported reduced viability of *M. abscessus* subsp. *abscessus* Bamboo treated with 844 in broth culture, suggesting bactericidal activity of the compound (21). To confirm and characterize the bactericidal activity of the class, we determined time-concentration kill for 844 and 844-TFM against the reference strain *M. abscessus* subsp. *abscessus* ATCC 19977. Both compounds were bactericidal in planktonic cultures (Fig. 2A) and against *M. abscessus* grown as biofilm (Fig. 2B).

Resistance against P4Cs is caused by missense mutations in *M. abscessus* DNA gyrase. Based on *in silico* analyses, 844 was proposed to act as an inhibitor of mycobacterial ABC transporters (23). To determine the mechanism of action of 844 experimentally, we isolated spontaneous resistant mutants in *M. abscessus* subsp. *abscessus* Bamboo on 844-containing agar. Resistant colonies emerged at a frequency of 10^{-8} CFU. Three randomly selected resistant strains were further characterized, showing 2- to more than 8-fold increases in 844 MIC. Whole-genome sequencing, confirmed by targeted sequencing, revealed missense mutations in *gyrA* and *gyrB*, the genes encoding the subunits of DNA gyrase (Table 2). We repeated the mutant selection experiment for 844-TFM using *M. abscessus* subsp. *abscessus* ATCC 19977, again yielding resistant colonies at a frequency of 10^{-4} CFU. Characterization of five randomly selected resistant colonies revealed 3- to more than 66-fold increased 844-TFM MICs and, again, missense mutations in *gyrA* and *gyrB* (Table 2). MIC determinations of the 844-resistant strains for 844-TFM and of the 844-TFM-resistant strains for 844 revealed cross-resistance of all strains to both compounds (Table 2). To

### Table 1

| M. abscessus subspecies | erm(41) sequevar | CLR susceptibility | MIC (μM) |
|-------------------------|-----------------|-------------------|----------|
|                         |                 |                   | 844      | 844-TFM | MXF   | SPR719 | CLR   |
| Reference strains       |                 |                   |          |         |       |        |       |
| *abscessus* ATCC 19977  | T28             | Resistant         | 8.0      | 1.5     | 3     | 0.6    | 2.0   |
| *bolletii* CCUG 50184T  | T28             | Resistant         | 14.0     | 2.0     | 6.3   | 1.5    | 1.2   |
| *massiliense* CCUG 48898T | Deletion      | Sensitive         | 14.0     | 2.0     | 6.3   | 3.0    | 0.2   |
| Clinical isolates       |                 |                   |          |         |       |        |       |
| *abscessus* Bamboo      | C28             | Sensitive         | 12.0     | 1.5     | 4.0   | 0.8    | 0.4   |
| *abscessus* K21         | C28             | Sensitive         | 14.0     | 3.5     | 3.0   | 1.5    | 0.6   |
| *abscessus* M9          | T28             | Resistant         | 6.3      | 3.1     | 1.5   | 1.5    | 3.2   |
| *abscessus* M199        | T28             | Resistant         | 6.3      | 6.3     | 2.5   | 1.5    | 3.2   |
| *abscessus* M337        | T28             | Resistant         | 6.3      | 3.1     | 1.5   | 0.8    | 3.2   |
| *abscessus* M404        | T28             | Resistant         | 6.3      | 1.6     | 3.0   | 1.5    | 0.8   |
| *abscessus* M421        | T28             | Resistant         | 6.3      | 1.6     | 1.5   | 0.8    | 3.2   |
| *bolletii* M232         | T28             | Resistant         | 6.3      | 1.6     | 1.5   | 1.5    | 3.2   |
| *bolletii* M506         | C28             | Sensitive         | 6.3      | 3.1     | 2.5   | 1.5    | 0.8   |
| *massiliense* M111      | Deletion        | Sensitive         | 12.5     | 6.3     | 3.2   | 6.3    | 0.2   |

*M. abscessus* Bamboo (42), K21 (43), and the M strains (44) have been described previously.

erm(41), ribosome methylase gene responsible for inducible clarithromycin (CLR) resistance. C28 and deletion sequevars of the gene are susceptible to clarithromycin, while the T28 sequevar confers inducible resistance against CLR (55).

The DNA gyrase inhibitors moxifloxacin (MXF) and SPR719 are included for comparison and the ribosome inhibitor CLR as assay control. MIC determinations were carried out three times independently in technical duplicates, and mean values are shown.
confirm that the observed polymorphisms are indeed causing resistance, one representative resistant strain (M. abscessus subsp. abscessus 19977 844-TFM-1), harboring a D91N missense mutation in \( \text{gyrA} \) associated with high-level P4C resistance (Table 2), was complemented with a copy of \( \text{M. abscessus} \) subsp. abscessus ATCC 19977 wild-type (wt) \( \text{gyrAB} \), which

![FIG 2 Bactericidal and antibiofilm activity of 844 and 844-TFM against M. abscessus. (A) Exponentially growing suspension cultures of M. abscessus subsp. abscessus ATCC 19977 were treated with 1×, 4×, and 8× MIC of 844 or 844-TFM, moxifloxacin (MXF), SPR719, or clarithromycin (CLR), and CFU were enumerated by plating samples on agar after 2 and 3 days. (B) Exponentially growing biofilm cultures were treated with 1×, 4×, and 8× MIC of 844, 844-TFM, MXF, SPR719, or CLR, and surface-attached CFU were enumerated by suspending bacteria and plating on agar after 2 and 3 days. MXF, SPR719, and CLR are included for comparison (MXF, SPR719) or as control (CLR). Experiments in panels A and B were carried out three times independently, and the results are represented as mean values with the standard deviations displayed as error bars. A two-way ANOVA multiple comparison test was performed using GraphPad Prism 8 software to compare treated groups with untreated day 0 CFU.]

### TABLE 2 Characterization of 844- and 844-TFM-resistant \( \text{M. abscessus} \) strains

| Strain          | MIC (\( \mu \text{M} \)) | \( \text{gyrA} \) | \( \text{gyrB} \) |
|-----------------|--------------------------|-------------------|-------------------|
|                 | 844| 844-TFM| MXF*| SPR719*| CLR*| Nuc | AA | Nuc | AA |
| Mab Bamboo WT** | 12 | 1.5 | 4 | 0.8 | 0.4 | G217C | A73P | A1322C | K441T |
| 844-TFM-1       | 25 | 12 | 2 | 0.2 | 0.3 | G271A | D91N | C1418G | S473W |
| >100            | >100 | >100 | 30 | 0.1 | 0.3 | G271A | D91N |
| 844-TFM-2       | 90 | 5 | 2 | 0.05 | 0.4 | G136T | V46F |
| Mab ATCC 19977 WT** | 8 | 1.5 | 3 | 0.6 | 2 | G271A | D91N |
| >100            | >100 | >100 | 20 | 0.06 | 2 | G271A | D91N |
| >100            | >100 | >100 | 6 | 0.04 | 1 | G136T | V46F |
| 844-TFM-3       | 40 | 20 | 1.5 | 0.05 | 2 | G1422A | M474I |
| 844-TFM-4       | 40 | 6 | 2 | 0.3 | 2 | G1486T | V496L |
| 844-TFM-5       | 25 | 5 | 2 | 0.02 | 2 | G286T | D96Y |
| MXF-1*          | 40 | 6 | 100 | 0.8 | 2 | G286A | D96N |
| MXF-2           | 15 | 2 | 80 | 0.2 | 1 | A287G | D96G |
| MXF-3           | 15 | 1 | 80 | 0.3 | 1 | G217C | A73P |

*\( \text{M. abscessus} \) subsp. abscessus Bamboo wild type. Used as parental strain for the isolation of spontaneous 844-resistant mutants 844-TFM-1 to -3.

*\( \text{M. abscessus} \) subsp. abscessus ATCC 19977 wild type. Used as parental strain for the isolation of spontaneous 844-TFM-resistant mutants 844-TFM-1 to -5.

*Strain used for complementation (Fig. 3), generation of GyrA D91N mutant recombinant DNA gyrase (Fig. 4), and for recA promoter DNA damage reporter analysis in P4C-resistant background (Fig. 5).

*\( \text{M. abscessus} \) subsp. abscessus ATCC 19977 was also used to isolate spontaneous moxifloxacin-resistant mutants MXF-1 to -3. High-level MXF resistance causing GyrA D96 mutations (30) caused low-level or no cross-resistance to P4Cs.

*Moxifloxacin (MXF) and SPR719 MICs are included for determination of cross-resistance to other DNA gyrase inhibitors and CLR as negative control.

*Nuc, nucleotide substitution; AA, amino acid substitution.
partially restored sensitivity to both 844 and 844-TFM (Fig. 3). These results suggest that missense mutations in *M. abscessus* DNA gyrase genes cause resistance to P4Cs and that the compounds target this enzyme.

**P4Cs inhibit activity of recombinant *M. abscessus* wild-type DNA gyrase but not mutant enzyme harboring a P4C resistance mutation.** To provide direct evidence that the P4Cs indeed target DNA gyrase, we tested whether the molecules inhibit the supercoiling activity of recombinant *M. abscessus* DNA gyrase. The two DNA gyrase inhibitors, moxifloxacin and SPR719, inhibited supercoiling activity as expected, whereas the negative control, the ribosome inhibitor clarithromycin, did not affect the activity of the enzyme (Fig. 4). Both P4Cs inhibited activity of DNA gyrase (Fig. 4). Consistent with its improved whole-cell activity, the 50% inhibitory concentration (IC₅₀) of 844-TFM (1.9 μM) was 2.4-fold lower than the IC₅₀ of the parental 844 (4.6 μM) (Fig. 4). To confirm the mechanism of resistance, we tested activity of the P4Cs against recombinant *M. abscessus* DNA gyrase harboring the resistance mutation D91N in *gyrA* (*M. abscessus* subsp. *abscessus* ATCC 19977 844-TFMr-1) (Table 2; Fig. 3). The mutant version of DNA gyrase was not inhibited by P4Cs (Fig. 4).

**P4C resistance causing DNA gyrase mutations show limited cross-resistance to moxifloxacin and no cross-resistance to SPR719.** Taken together, the genetic and biochemical analyses indicate that P4Cs target *M. abscessus* DNA gyrase, an essential and clinically validated target in mycobacteria (24). The type II A DNA topoisomerase is a GyrA₂GyrB₂ heterotetrameric protein that regulates DNA topology (25). The unwinding of DNA during replication and transcription introduces positive supercoils that, left unaddressed, would affect DNA function. This problem is resolved by DNA gyrase, which introduces negative supercoils into DNA in an ATP-dependent fashion. To do this, the enzyme generates a DNA double-stranded break, passes a segment of double-stranded DNA through the break, and subsequently reseals the DNA molecule (25).

The DNA gyrase inhibitor moxifloxacin (Fig. 1) is a pillar of the treatment of multidrug-resistant tuberculosis (26) and is used less widely for the treatment of macrolide-resistant *M. abscessus* disease (27, 28). The fluoroquinolone targets the catalytic core of the enzyme comprised of the C-terminal TOPRIM domains of two GyrB subunits and the N-terminal breakage-and-reunion domains of two GyrA subunits (29). Consequently, acquired fluoroquinolone resistance involves missense mutations within these domains (30). P4C resistance mutations (Table 2) are also located in the TOPRIM and breakage-and-reunion domains. Interestingly, D91 missense mutations causing high-level P4C resistance have been reported to also confer resistance to moxifloxacin in *M. tuberculosis* (31). To determine whether the P4C resistance
mutations in \textit{M. abscessus} confer cross-resistance to moxifloxacin, we measured the MICs of moxifloxacin for the P4C-resistant strains. The strains harboring D91 missense mutations in \textit{gyrA} showed low level cross-resistance with a 2- to 8-fold increase in MIC (Table 2). None of the other P4C resistance mutations altered the fluoroquinolone MIC (Table 2).

A novel DNA gyrase inhibitor, SPR719 (Fig. 1), is in clinical development for the treatment of mycobacterial lung diseases. This benzimidazole acts as an inhibitor of the ATPase activity of DNA gyrase located in the N-terminal domain of Gyrb (32). SPR719-resistant mutants in \textit{M. tuberculosis} have been mapped to the ATPase domain (32). As expected, P4C resistance mutations did not cause cross-resistance to SPR719 (Table 2). Interestingly, several P4C-resistant mutations conferred hypersusceptibility to SPR719. The mechanistic basis for this phenotype remains to be determined.
Thus, P4C-resistant mutations caused limited or no cross-resistance to moxifloxacin or SPR719, suggesting a novel on-target mechanism of this new DNA gyrase inhibitor. **P4Cs trigger induction of recA DNA damage reporter in wild-type but not in P4C-resistant M. abscessus.** Fluoroquinolones arrest DNA gyrase—DNA complexes in their double strand broken state. This mechanism of action results in DNA damage, which contributes to the bactericidal activity of the class (33). Similar to moxifloxacin, P4Cs are bactericidal and resistance mutations map to the catalytic core of DNA gyrase. To determine whether P4Cs also cause DNA damage, we constructed a DNA damage reporter strain by introducing the DNA damage inducible recA promoter controlling expression of the bioluminescence LuxCDABE operon into M. abscessus subsp. abscessus ATCC 19977. The positive control moxifloxacin strongly induced recA promoter-dependent reporter expression (Fig. 5), similar to what has been described for M. tuberculosis (34, 35). SPR719, inhibiting gyrase’s ATPase activity, caused only a weak increase, and treatment of M. abscessus with the protein synthesis inhibitor clarithromycin as negative control did not cause any increase in reporter expression (Fig. 5). Similar to moxifloxacin, treatment of reporter cultures with P4Cs strongly induced expression of the reporter gene (Fig. 5), suggesting that the compounds damage bacterial DNA. If P4C-mediated recA promoter induction is due to interaction of the compounds with DNA gyrase, induction should not occur in P4C-resistant gyrA8 mutant background. To test this prediction, we introduced the recA reporter operon into the P4C-resistant M. abscessus subsp. abscessus ATCC 19977 844-TFM-1 strain harboring the D91N allele of gyrA (Table 2; Fig. 3 and 4). In this background, induction of recA promoter activity was strongly reduced, suggesting that the increase of recA promoter activity in the wild-type background is DNA gyrase dependent (Fig. 5). Together, these results suggest that inhibition of DNA gyrase by P4Cs results in DNA damage.

**In vivo and in vitro pharmacokinetic properties of 844-TFM.** To identify the pharmacokinetic (PK) liabilities of 884-TMF, we determined its concentration-time profile in mice and measured basic PK properties in established in vitro assays. 884-TMF was
not orally bioavailable in mice with a calculated bioavailability of 0.05 to 0.2% (Fig. 6A and Table 3). To determine whether this was due to poor solubility, low permeability, or rapid metabolism, these properties were evaluated in standard in vitro PK assays (Table 3). 844-TFM exhibited adequate solubility at both pH 2.0 (simulated gastric fluid) and pH 7.4 (standard physiological conditions). In the parallel artificial membrane permeability assay (PAMPA), 844-TFM showed modest permeability with a

![Graph](image-url)

**TABLE 3** In vitro and in vivo pharmacokinetic properties of 844-TFM

| Pharmacokinetic property | Value               |
|--------------------------|---------------------|
| Solubility (µM)          |                     |
| pH 2                     | 446.5               |
| pH 7.4                   | 390.0               |
| PAMPA, pH 7.4            |                     |
| Mean Pe (10⁻⁶cm/s)       | 2.0                 |
| LogPe                    | −5.7                |
| Mouse Hepatic Microsomes Stability (in vitro) |  |
| k_e (min⁻¹)              | 0.1                 |
| t_1/2 (min)              | 5.2                 |
| In vitro Cl_{int} (µL/min/mg protein) | 267.3              |
| Cl_{int} (mL/min/kg)     | 1,052.4             |
| Cl_{hepatic} (mL/min/kg) | 82.9                |

Mouse pharmacokinetic parameters (in vivo)⁶

| Intraduction route | AUC i.v. 5 mg/kg (h * ng/ml) | elimination half-life (h) | k_e (h⁻¹) | V (liter/kg) | Clearance (mL/kg * h) | Other routes of administration |
|------------------|-------------------------------|--------------------------|------------|-------------|----------------------|-------------------------------|
|                  |                               |                          |            |             |                      | AUC p.o. 25 mg/kg (ng * h/ml) | p.o. bioavailability (%) | s.c. bioavailability (%) |
|                  | 5,627 (2,181)                 | 1.03 (0.17)              | 0.69 (0.12) | 0.811 (0.221) | 638 (231)            | 58 (54)                      | 0.15 (0.14)               | 3.0 (1.5)                |

⁴PAMPA, parallel artificial membrane permeability assay; Pe, effective permeability coefficient; k_e, elimination rate constant; t_1/2, half-life; Cl_{int}, intrinsic clearance; Cl_{hepatic}, hepatic clearance; AUC, area under the concentration-time curve; V, volume of distribution; i.v., intravenous; p.o., oral; s.c., subcutaneous.

⁶Average (SD).
logPe of $-5.7$ cm/s ($\log Pe$ of $-5.0$ cm/s is considered the border between high and low permeability). In mouse liver microsomes, 844-TFM was highly unstable with a half-life of 5 min and a high rate of clearance (Table 3), nearly as high as the mouse hepatic blood flow of 90 ml/min/kg (36), predicting an extraction ratio of $\sim 1$, consistent with rapid first-pass liver metabolism and in line with poor oral bioavailability. To circumvent first-pass metabolism, we administered 844-TFM via the subcutaneous route to CD-1 mice, leading to improved exposure compared to that of the oral route (Fig. 6A). However, the compound was still rapidly eliminated, and bioavailability remained modest at 3%. Indeed, 844-TFM was rapidly degraded in mouse plasma (Fig. 6B). Identification of the two major breakdown products in mouse plasma revealed cleavage of the central amide bond of 844-TFM (see Fig. S1 and S2 in the supplemental material). Interestingly, the compound was markedly more stable in rabbit, monkey, and human plasma, with approximately 80% remaining after 24 h at 37°C (Fig. 6B). Taken together, characterization of the PK properties of 844-TFM revealed limited permeability and metabolic instability as the major liabilities of the lead compound in the mouse model.

**Conclusion.** Fluoroquinolones are used successfully as second-line agents for the treatment of multidrug-resistant tuberculosis (37). Variable in vitro susceptibilities due to (unknown) intrinsic resistance mechanisms limit the therapeutic utility of this drug class against *M. abscessus* (5). Thus, moxifloxacin is used only rarely as a second-line drug for the treatment of macrolide-resistant infections, and there is no effective DNA gyrase inhibitor available for the treatment of *M. abscessus* disease. Recently, the benzimidazole SPR719, an inhibitor of ATPase activity of the mycobacterial DNA gyrase, entered early clinical development for tuberculosis and NTM lung diseases, bringing new hope for patients suffering from mycobacterial infections (38). Here, we have identified a novel class of mycobacterial DNA gyrase inhibitors with attractive bactericidal and antibiofilm activity against *M. abscessus* complex. We characterized the PK properties of the lead compound to enable medicinal chemistry programs. A novel DNA gyrase inhibitor would be a welcomed addition to the anti-*M. abscessus* drug pipeline.

**MATERIALS AND METHODS**

**Bacterial strains, culture media, and drugs.** *M. abscessus* subsp. *abscessus* ATCC 19977, harboring the inducible clarithromycin resistance-conferring *erm(41) T28* sequevar (39), was purchased from the American Type Culture Collection. *M. abscessus* subsp. *boletii* CCUG 50184T, harboring the inducible clarithromycin resistance-conferring *erm(41) T28* sequevar (40), and *M. abscessus* subsp. *massiliense* CCUG 48898T, harboring the nonfunctional *erm(41)* deletion sequevar (41), were purchased from the Culture Collection University of Goteborg. *M. abscessus* subsp. *abscessus* Bambooo (42) was provided by Wei Chang Huang (Taichung Veterans General Hospital, Taichung, Taiwan). *M. abscessus* subsp. *abscessus* K21 (43) was provided by Sung Jae Shin (Department of Microbiology, Yonsei University College of Medicine, Seoul, South Korea) and Won-Jung Koh (Division of Pulmonary and Critical Care Medicine, Samsung Medical Center, Seoul, South Korea). The clinical M isolates of *M. abscessus* (44) were provided by Jeanette W. P. Teo (Department of Laboratory Medicine, National University Hospital of Singapore).

*Mycobacterium abscessus* was grown in complete Middlebrook 7H9 broth (271310; BD Difco, Spark, MD, USA) supplemented with 0.05% Tween 80, 0.2% glycerol, and 10% albumin-dextrose-catalase. 7H10 agar (262710; BD Difco, Sparks, MD, USA) was used as solid medium.

844 was obtained from the Medicines for Malaria Venture’s (Geneva) compound archive. 844-TFM was synthesized as described below. Moxifloxacin (MXF, SML1581) and clarithromycin (CLR, C9742) were purchased from Sigma-Aldrich, USA. SPR719 (HY-12930) was purchased from MedChemExpress LLC, USA. MXF, SPR719, and CLR were dissolved in 100% dimethyl sulfoxide (DMSO) (MP Biomedicals, USA) at 10 mM. 844 and 844-TFM were dissolved in ethanol (BP2818100; Fisher Scientific, USA) at 5 mM and 10 mM, respectively. All compounds were stored in aliquots at $-20^\circ$C until use.

**Chemicals and physical methods.** Starting materials were purchased and used as received. Solvents were of reagent grade and distilled before use. N-(6-methoxy-1,5-naphthyridin-4-yl)-4-piperidinecarboxamide was prepared according to the literature (45, 46). The melting point (uncorrected) was determined on a Bürkle melting-point apparatus (VEB Kombinat NAGEMA, Dresden, GDR). $^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectra were recorded at room temperature on an Agilent Technologies VNMRS 500 NMR spectrometer. The residual solvent signals of methanol-$d_4$ ($\delta_{\text{H}} = 3.31$ ppm; $\delta_{\text{C}} = 49.00$ ppm) were used to reference the spectra (abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, td = triplet of doublets, m = multiplet). The mass spectrum was recorded on a Q Exactive Plus Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) using methanol as solvent.
Synthesis of 844-TFM. A mixture of N-(6-methoxy-1,5-naphthyridin-4-yl)-4-piperidinecarboxamide (50 mg, 0.17 mmol), triethylamine (35 µL, 0.26 mmol, 1.5 eq), and 1-(2-bromomethyl)-4-(trifluoromethyl) benzene (58 µL, 0.34 mmol, 2 eq) in dimethyformamide (DMF) (5 ml) was stirred at 50°C for 16 h. The reaction was quenched by the addition of water (20 ml). The mixture was extracted with ethyl acetate (3 × 10 ml), and the combined organic phases were washed with water (10 ml) and brine (10 ml). The solution was dried over Na₂SO₄, filtered, and evaporated under reduced pressure. The residue was chromatographed on silica gel with methanol/ethyl acetate (0 to 5%) to afford the product as a white solid (40 mg, 0.09 mmol, 51%). M.p. 433-435 K; 1H NMR (500 MHz, methanol-<i>d</i>₄) δ 8.61 (d, <i>J</i> = 2.5 Hz, 1H), 8.50 (d, <i>J</i> = 5.2 Hz, 1H), 8.19 (d, <i>J</i> = 9.1 Hz, 1H), 7.58 (d, <i>J</i> = 8.0 Hz, 2H), 7.43 (d, <i>J</i> = 8.0 Hz, 2H), 7.26 (d, <i>J</i> = 9.1 Hz, 1H), 4.16 (s, 3H), 3.14 (dt, <i>J</i> = 12.1, 3.5 Hz, 2H), 2.96 to 2.91 (m, 2H), 2.74 to 2.66 (m, 3H), 2.27 (td, <i>J</i> = 11.8, 2.5 Hz, 2H), 2.09 (d, <i>J</i> = 1.26 Hz, 2H), 1.93 (qd, <i>J</i> = 11.7, 4.3 Hz, 2H) (see Fig. S3 in the supplemental material). 13C NMR (126 MHz, methanol-<i>d</i>₄) δ 174.7, 161.8, 148.3, 144.7, 144.7, 140.4, 140.3, 139.2, 132.0, 129.0, 128.2, 128.0, 125.5, 124.9, 124.9, 124.8, 117.1, 110.8, 59.6, 53.3, 52.4, 43.5, 32.4, 28.1, 19.3 (see Fig. S3); electrospray ionization-high-resolution mass spectrometry (ESI-HRMS): <i>m/z</i> 459.2 calculated for C₂₄H₂₆F₃N₄O₄ 459.2007, found 459.1995.

Determination of MICs. MIC was determined using the broth microdilution method in 96-well plates as described previously (47). Briefly, a 10-point 2-fold serial dilution of compounds was performed in 96-well plates (Costar 3370; Corning, Corning, USA) starting at twice the desired highest concentration. Exponentially growing M. abscessus cultures (optical density at 600 nm [OD₆₀₀] = 0.4 to 0.8) were diluted to a final OD₆₀₀ of 0.01 in Middlebrook 7H9 broth (Becton, Dickinson). One hundred microliters of the bacterial suspension was seeded onto the 96-well plates containing the same concentration used for selection of resistant colonies. Genomic DNA was extracted as described previously using the phenol-chloroform method (50). Whole-genome sequencing, including library construction and bioinformatics analyses, was performed by Genewiz (Genewiz, Inc., South Plainfield, NJ, USA) using primers for gyrb (Fw-gyrb 5’-CTGAAACTAGTCCGCTGGTGTC-3’; Rv-gyrb 5’-TTGAACCTACCGGGCGGTTACC-3’).

Time-concentration kill assay. To determine the bactericidal activity of 844 and 844-TMF, exponentially growing M. abscessus subsp. abscessus ATCC 19977 cultures (OD₆₀₀ = 0.4 to 0.8) were diluted to a final OD₆₀₀ of 0.005, and 1 ml of the culture suspension was exposed to 1 ×, 4 ×, and 8 × MIC of 844 (MIC = 1.5 µM), 844-TMF (MIC = 3 µM), MXF (MIC = 3 µM), SPR719 (MIC = 0.6 µM), and CLR (MIC = 2 µM) in 14-ml vented, round-bottom tubes (150268; Fisher Scientific, USA) and incubated at 37°C with orbital shaking at 90 rpm. Absorbance at 600 nm was measured using a TECAN Infinite Pro 960 plate reader after resuspension. Absorbance values at day 3 were subtracted from the day 0 readout. Percent growth inhibition was calculated by dividing the absolute absorbance value of treated cells with untreated control and multiplying by 100. CLR was included in all MIC experiments as a positive control to monitor assay reproducibility. MIC was defined as 90% of growth inhibition relative to untreated controls.

Determination of antibiofilm activity. The antibiofilm activity of 844 and 844-TMF was determined employing the MBEC 96-well Biofilm assay kit (1911; Innovotech, Edmonton, AB, Canada) as described previously (15) with minor modifications. Briefly, exponentially growing M. abscessus subsp. abscessus ATCC 19977 cultures were spun down at 3,200 g for 10 min and washed with 7H9 broth without Tween 80 (‘7H9’). Bacteria were resuspended in ‘7H9’ to an OD₆₀₀ of 0.125 and 150 µl was dispensed into the wells of Innovotech 96-well plates. Pegs (protruding from the specialized lids of the Innovotech multiwell plates) were inserted into the bacterial suspensions in the wells, and the Innovotech cultures were grown 24 h with shaking at 110 rpm at 37°C to allow attachment and growth of the bacteria on the surface of the pegs. Then, the lids with the pegs were transferred to a new multitriter plate containing 150 µl fresh ‘7H9’ broth (without bacteria; time zero) (Fig. 2B) with no drug or with 1 ×, 4 ×, or 8 × MIC of 844, 844-TMF, MXF, SPR719, or CLR and incubated for 0, 2, and 3 days. Biofilm growth on the pegs was measured by CFU determination. The pegs were washed in 200 µl ‘7H9’ medium, removed, and placed in 1.7 ml microcentrifuge tubes (87003-294; VWR, Radnor, PA, USA) containing 500 µl phosphate-buffered saline (PBS) (10010023; Thermofisher, USA) with 0.025% Tween 80 (PBS/Tween 80) for 10-fold serial dilution. To prevent compound carryover effects, we plated out serially diluted samples onto 7H10 agar supplemented with 0.4% activated charcoal (C9154; Sigma-Aldrich, USA) for all time points as described (48). CFU were enumerated after 4 days of incubation at 37°C. A two-way analysis of variance (ANOVA) multiple comparison test was performed using GraphPad Prism 8 software to compare treated groups with untreated day 0 CFU.

Selection of spontaneous resistant mutants, whole-genome, and targeted sequencing. Spontaneous resistant mutants against 844 and 844-TMF were selected using M. abscessus subsp. abscessus Bamboo and M. abscessus subsp. abscessus ATCC 19977 as described previously (49). Briefly, 10⁴ to 10⁷ CFU of exponentially growing cultures were plated on 7H10 agar containing 8 × MIC of 844 or 844-TMF (or 32 × MIC of MXF for the selection of MXF-resistant strain). The plates were sealed with paraffin and incubated for 7 days at 37°C. Apparent resistant colonies were purified and confirmed by restreaking on 7H10 agar containing the same concentration used for selection of resistant mutants. Genomic DNA was extracted as described previously using the phenol-chloroform method (50). Whole-genome sequencing, including library construction and bioinformatics analyses, was performed by Genewiz (Genewiz, Inc., South Plainfield, NJ, USA). Polymorphisms detected in gyra (MAB_0019) and gyrb (MAB_0006) by whole-genome sequencing were confirmed by Sanger sequencing (Genewiz, Inc., South Plainfield, NJ, USA) using primers for gyrb (Fw-gyrb 5’-GCATCTAAAGCCGTAGAGACG-3’; Rv-gyrb 5’-GGTCACGGGCGCTGTTGTC-3’) and gyrb (Fw-gyrb 5’-CTGAAACTAGTCCGCTGGTGTC-3’; Rv-gyrb 5’-TTGAACCTACCGGGCGGTTACC-3’).
Complementation of P4C-resistant _M. abscessus_. To complement the P4C-resistant _M. abscessus_ subsp. _abscessus_ ATCC 19977 844-TFM-1 strain harboring a D91N allele of gyrA (Table 2), wild-type gyrA and gyrB were cloned into pMV262 (S1) and expressed from the plasmid’s constitutive hsp60 promoter as described previously (50). The coding sequences of gyrB and gyrA were amplified from plasmid pET28a gyrBA wt, which had been constructed to express recombinant wild-type DNA gyrase (see below) using primers Fw-gyrBA (HindIII, underlined) 5'-gcagcagctagcTTACTCGCCTGGCGGTGTCGACGATGCGAAGAAGAGTG-3' and Rv-gyrBA (HindIII, underlined) 5'-gcagcagctagcTTACTCGCCTGGCGGTGTCGACGATGCGAAGAAGAGTG-3' (lowercase sequences indicate added sequences with restriction enzyme recognition sites underlined) and Phusion high-fidelity DNA polymerase (F5305, Fisher Scientific, USA). To allow in-frame cloning of the gyrBA coding sequences to the N-terminal hsp60 coding sequence via pMV262’s HindIII site, pMV262’s Dral-HindIII segment (carrying the hsp60 promoter and the hsp60 N-terminal coding sequence) was replaced by a shortened Dral-HindIII fragment amplified using the primers Fw-hsp60 (Dral, underlined) 5'-gcgttcctaaCTCTCGCCGACCAACGACG-3' and Rv-hsp60 (HindIII, underlined) 5'-gcgacacattgTGCTGCCATTTG-3' and pMV262 as template. The HindIII-digested gyrA insert was ligated into HindIII-digested modified pMV262 plasmid and transformed into *Escherichia coli* DH5α. pMV262-psy60-gyrBA harboring transformants was identified by PCR, and the plasmid was electroporated _M. abscessus_ subsp. _abscessus_ ATCC 19977 844-TFM-1 as described (52). Transforms were selected on 7H10 agar containing 400 μg/ml kanamycin and verified by PCR to harbor pMV262-psy60-gyrBA. _M. abscessus_ subsp. _abscessus_ ATCC 19977 844-TFM-1 harboring pMV262-psy60-gyrBA or modified pMV262-psy60 plasmid without gyrBA insert (pMV262-empty) were treated with 844 or 844-TFM or the negative control CLR at 1× MIC in 14-mL vened, round-bottom tubes (150268; Fisher Scientific, USA) and incubated at 37°C on an orbital shaker at 200 rpm. After 2 days of incubation, tubes were vortexed and 200 μL of the samples from each tube transferred into a clear bottom 96-well plate for absorbance measurement at 600 nm using a TECAN Infinity Pro 200 plate reader. A two-way ANOVA with Sidak’s multiple comparisons test was performed using GraphPad Prism 8 software to compare the two groups.

**Cloning, expression, and purification of recombinant _M. abscessus_ DNA gyrase**. To clone and express recombinant _M. abscessus_ DNA gyrase gyrA and gyrB (MAB_0019, MAB_0006), we adapted the strategy previously used for expression of recombinant _M. tuberculosis_ DNA gyrase (53). The coding regions for residues 2 to 675 of GyrB and 2 to 839 of GyrA were amplified from genomic DNA of wild-type _M. abscessus_ subsp. _abscessus_ ATCC 19977 using primers Fw-gyrA (NdeI, underlined) 5’-gcagcagctagcATGACTGACACAACGCTGCCCCCC-3’ and Rv-gyrA (HindIII, underlined) 5’-gcagcagctagcTTACTCGCCTGGCGGTGTCGACGATGCGAAGAAGAGTG-3’ using Genewiz and then treating the cultures with 1 mM isopropyl-

Piperidine-4-Carboxamides Target _M. abscessus_ DNA Gyrase

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 AAC (Addgene, 26159) with the _M. abscessus_ subsp.
abscessus ATCC 19977 recA promoter (34). The M. abscessus recA promoter was amplified with primers Fw-PreA (NotI, underlined) 5'-gcccggccgcATTGGGAAAGCGCTTAC-3’ and Rv-PreA (EcoRI, underlined) 5’-cagagctagcCGTCTCCGTTTGCGT-3’ using Phusion high-fidelity DNA polymerase (F5305; Fisher Scientific, USA). The resulting amplicon was digested with NotI (no. R31189L; New England Biolabs) and EcoRI (no. R31015S; New England Biolabs), gel purified (Qiagen, Hilden, Germany), and ligated into NotI-EcoRI-digested pMV306 to transform the reporter plasmid pMV306recA-LUX (54). The ligated product was transformed into E. coli DH5α and plated on LB agar medium containing 25 μg/ml kanamycin. The plasmid was verified by PCR and transformed into wild-type M. abscessus subsp. abscessus ATCC 19977 and the M40-resistant M. abscessus ATCC 19977 844-TFM-1 strain harboring a D91N allele of gyrA (Table 2) via electroporation (52). To measure the effect of compounds on recA promoter activity, exponentially growing reporter cultures (OD600) = 0.4 to 0.8) were added to 10 μl of drug-free plasma (CD-1 K2EDTA Mouse; Bioreclamation IVT). Twenty microliters of control, standard, or sample were added to 200 μl of ACN/methanol (MeOH) 50:50 protein precipitation solvent containing internal standard (10 ng/ml verapamil). Extracts were vortexed for 5 min and centrifuged at 4,000 rpm for 5 min. One hundred microliters of supernatant was transferred into a 96-well plate and stored at −80°C.

**HPLC-MS analysis.** Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed on a Sciex Applied Biosystems Qtrap 6500+ triple-quadrupole mass spectrometer coupled to a Shimadzu Nexera 2 high-pressure liquid chromatography (HPLC) system to quantify each drug in plasma. Neat 1 mg/ml DMSO stocks for 844-TFM were serial diluted in 50:50 acetonitrile (ACN)/water to create standard curves and quality control (QC) spiking solutions. Standards and QCs were created by adding 10 μl of drug-free plasma (CD-1 K2EDTA Mouse; Bioreclamation IVT). Twenty microliters of control, standard, QC, or study sample were added to 200 μl of ACN/methanol (MeOH) 50:50 protein precipitation solvent containing internal standard (10 ng/ml verapamil). Extracts were vortexed for 5 min and centrifuged at 4,000 rpm for 5 min. One hundred microliters of supernatant was transferred for HPLC-MS/MS analysis and diluted with 100 μl of Milli-Q deionized water.

Chromatography was performed on an Agilent Zorbax SB-C8 column (2.1 x 30 mm; particle size, 3.5 μm) using a reverse-phase gradient. Milli-Q deionized water with 0.1% formic acid was used for the aqueous mobile phase and 0.1% formic acid in ACN for the organic mobile phase. Multiple-reaction monitoring of precursor/product transitions in electrospray positive-ionization mode was used to quantify the analytes. The following multiple reaction monitoring (MRM) transitions were used for 844-TFM (459.10/133.00) and verapamil (455.4/165.2). Sample analysis was accepted if the concentrations of the quality control samples were within 20% of the nominal concentrations. Data processing was performed using Analyst software (version 1.6.2; Applied Biosystems Sciex).

**Plasma stability analysis and metabolite identification.** The plasma stability assays were carried out using plasma from female CD-1 mice, New Zealand white rabbits, rhesus monkeys, and humans containing K2EDTA anticoagulant (Bioreclamation). Stability samples consisted of 5 μl of stock compound solution in 50:50 ACN/water and 95 μl of plasma to a final concentration of 1 μg/ml. The samples were incubated at 37°C with shaking; 10 μl of plasma was removed at each time point and combined with 100 μl of ACN/methanol 50:50 protein precipitation solvent containing internal standard (10 ng/ml verapamil). Chromatography for metabolite identification was performed the same as specified for pharmacokinetic sample analysis. Full scan total ion chromatograms (TIC) of plasma extracts were acquired using a Q Exactive high-resolution mass spectrometer (QE-HRMS) at 70,000 mass resolution. Thermo Fisher Compound Discoverer software was used to assist in identifying the 844-TFM metabolites from the QE-HRMS TIC mass spectrum. Figure S2 in the supplemental material illustrates the extracted ion chromatograms (XIC) of 844-TFM and the metabolites using 5 ppm mass accuracy before and after 12 h of incubation in mouse plasma.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.5 MB.

**ACKNOWLEDGMENTS**

We are grateful to Wei Chang Huang (Taichung Veterans General Hospital, Taichung, Taiwan) for providing M. abscessus Bamboo, to Jeanette W. P. Teo (Department of Laboratory Medicine, National University Hospital, Singapore) for providing the...
collection of *M. abscessus* clinical M isolates, and to Sung Jae Shin (Department of Microbiology, Yonsei University College of Medicine, Seoul, South Korea) and Won-Jung Koh (Division of Pulmonary and Critical Care Medicine, Samsung Medical Center, Seoul, South Korea) for providing *M. abscessus* K21.

Research reported in this work was supported by the Shared Instrumentation Grant S10-OD023524 to V.D. and the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award number R01AI132374 to T.D.

Conceptualization, D.A.N., A.B., P.I., and T.D.; investigation, D.A.N., A.B., A.M., N.A., C.C., W.W.A., M.D.Z., B.L., and M.G.; writing – original draft, D.A.N., A.B., V.D., P.I., and T.D.; writing – review and editing, all authors; supervision, M.G., V.D., P.I., and T.D.; funding acquisition, V.D. and T.D.

We declare no commercial or financial relationships that could be construed as a potential conflict of interest.

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