Assessment of Clofazimine and TB47 Combination Activity against *Mycobacterium abscessus* Using a Bioluminescent Approach

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**ABSTRACT** *Mycobacterium abscessus* is intrinsically resistant to most antimicrobial agents. The emerging infections caused by *M. abscessus* and the lack of effective treatment call for rapid attention. Here, we intended to construct a selectable marker-free autoluminescent *M. abscessus* strain (designated UAlMab) as a real-time reporter strain to facilitate the discovery of effective drugs and regimens for treating *M. abscessus*. The UAlMab strain was constructed using the *dif/Xer* recombinase system. In *vitro* and *in vivo* activities of several drugs, including clofazimine and TB47, were assessed using UAlMab. Furthermore, the efficacy of multiple drug combinations, including the clofazimine and TB47 combination, were tested against 20 clinical *M. abscessus* isolates. The UAlMab strain enabled us to evaluate drug efficacy both *in vitro* and in live BALB/c mice in a real-time, noninvasive fashion. Importantly, although TB47 showed marginal activity either alone or in combination with clarithromycin, amikacin, or roxithromycin, the drug markedly potentiated the activity of clofazimine, both *in vitro* and *in vivo*. This study demonstrates that the use of the UAlMab strain can significantly facilitate rapid evaluation of new drugs and regimens. The clofazimine and TB47 combination is effective against *M. abscessus*, and dual/triple electron transport chain (ETC) targeting can be an effective therapeutic approach for treating mycobacterial infections.

**KEYWORDS** *Mycobacterium abscessus*, cytochrome bc1, inhibitor, TB47, clofazimine, autoluminescent

*Mycobacterium abscessus*, a rapidly growing nontuberculous mycobacterium (NTM), is an important epidemiological agent causing opportunistic infections in human lungs, skin, and soft tissues, leading to significant morbidity and even mortality (1, 2). Notably, structural lung diseases such as cystic fibrosis and chronic obstructive pulmonary disease are important risk factors potentiating *M. abscessus* infection. Indeed, it has
been reported that between 16% to 68% cases of cystic fibrosis are further complicated by *M. abscessus* infection (3).

*M. abscessus* infection is difficult to treat due to its intrinsic resistance to most of the available antibiotics, including the classic antitubercular drugs (4). Current medical intervention of *M. abscessus* infection depends on combinational chemotherapy with drugs belonging to different groups, e.g., amikacin, clarithromycin or azithromycin, imipenem, and cefoxitin (4). Unfortunately, treatment failure occurs frequently despite prolonged administration of these antibiotics. Furthermore, current treatment regimens are often associated with severe side effects (4). Therefore, development of new drugs and regimens is urgently needed to facilitate the eradication of *M. abscessus* infection.

A series of drug candidates are currently under evaluation for tuberculosis treatment (5). This is exemplified by clofazimine, a riminophenazine drug that was originally prescribed for leprosy and now repurposed for tuberculosis treatment. Clofazimine was reported to compete with menaquinone by shuttling electrons from type II NADH dehydrogenase (NDH-2) to oxygen, leading to the generation of lethal levels of reactive oxygen species (ROS) (6). Possibly due to this unique mode of action, the drug is effective against multidrug-resistant *Mycobacterium tuberculosis* strains (7, 8). In addition to *M. tuberculosis*, clofazimine was also found to exert potent activity either alone or in combination with amikacin or clarithromycin against *M. abscessus in vitro* (9). Furthermore, a recent retrospective study also supported the use of clofazimine-containing regimens in the treatment of human *M. abscessus* infection (10). Thus, clofazimine seems to be a valuable drug for coping with *M. abscessus* infection. However, it should be noted that clofazimine has a delayed activity against *M. tuberculosis* both *in vitro* and *in vivo* (11). It is yet unknown whether the antimicrobial activity of clofazimine is also delayed against *M. abscessus*. Furthermore, long-term *in vivo* activity of clofazimine against this bacterium, to our knowledge, has not been reported.

In addition to clofazimine, the current antituberculosis drug discovery pipeline (5) also includes other mycobacterial electron transport chain (ETC)-targeting drugs, such as Q203 inhibiting QcrB in the cytochrome *bc*$_1$ complex (12). We reported a series of pyrazolo[1,5-a]pyridine-3-carboxamide derivatives as active compounds against both drug-susceptible and multidrug-resistant *M. tuberculosis* strains (13). The lead compound of this series, i.e., TB47, showed excellent therapeutic activity in a mouse model of *Mycobacterium ulcerans* infection (14) and is now considered a promising antitubercular drug candidate (http://www.newtbdrugs.org/pipeline/discovery). Similar to Q203, TB47 also targets the QcrB protein, thereby inhibiting the activity of the cytochrome *bc*$_1$-*aa*$_3$ supercomplex (14, 15).

Interestingly, combination treatment of clofazimine with cytochrome *bc*$_1$ inhibitors (e.g., Q203) was demonstrated to deliver a markedly enhanced killing of both replicating and nonreplicating *M. tuberculosis* bacteria (16, 17). Furthermore, inhibitors of menaquinone biosynthesis also have enhanced activity when administered in combination with other ETC-targeting drugs, such as clofazimine and QcrB inhibitors (18). These findings emphasize dual-ETC targeting as a novel antitubercular strategy. This strategy has not been evaluated against *M. abscessus*. In this study, we, for the first time, constructed a selectable marker-free autoluminescent *M. abscessus* strain. Autoluminescent strains of *M. tuberculosis*, *M. ulcerans*, and *M. abscessus* have been constructed previously (19–21), allowing for more facile, real-time, and noninvasive assessment of antibiotic activity both *in vitro* and *in vivo*. Notably, the previously described autoluminescent *M. abscessus* strain harbors a selection marker (21). Using the selectable marker-free autoluminescent *M. abscessus* strain (designated strain UAAMab) constructed in this study, we further evaluated the efficacy of various drugs and combinations, with a focus on the clofazimine and TB47 combination, against *M. abscessus* both *in vitro* and *in vivo*.
RESULTS

Engineering of UAlMab suitable for antibiotic screening. Using the Xer/dif system, we first constructed a selectable marker-free, autoluminescent M. abscessus strain expressing the luxCDABE operon for facile and real-time evaluation of antibiotic efficacy, as described earlier in M. tuberculosis (19, 22). The construction and further stability assessment of the UAlMab strain are detailed in the supplemental material.

We compared the growth of UAlMab with that of its parental strain. It was observed that under in vitro conditions, the growth of UAlMab, judged by the optical density at 600 nm (OD600) (Fig. 1A) and CFU counts (Fig. 1B), was similar to that of the parental strain. Furthermore, the growth pattern of UAlMab was also comparable to that of the parental strain in immunocompromised nude mice (Fig. 1C). We also monitored the growth kinetics of the UAlMab culture by measuring the relative light units (RLU). The result showed that the RLU curve of UAlMab fitted well with its CFU curve (Fig. 1B). Therefore, RLU counts can be used as an indicator of bacterial growth for UAlMab.

The comparable growth of UAlMab rendered this reporter strain suitable for antibiotic evaluation. However, prior to its application, it was necessary to determine if general antibiotic susceptibility was altered in this reporter strain. We therefore compared the MICs of various drugs with distinct modes of action (i.e., clarithromycin, amikacin, clofazimine, and cefoxitin) against both UAlMab and the parental strain using the conventional agar proportion method. As shown in Table 1, the MICs of these drugs determined for UAlMab were identical to those determined for the parental strain, suggesting that the general antibiotic susceptibility is not altered in the UAlMab strain.

Given that the use of autoluminescent M. tuberculosis and M. ulcerans in antibiotic susceptibility testing can offer considerable advantages, such as real-time detection and cost efficiency (19, 20), we interrogated if RLU measurement using the UAlMab strain could be applied as a surrogate methodology for antibiotic evaluation in M. abscessus. Thus, RLU counts in UAlMab culture in the presence of various concentrations of clarithromycin, amikacin, clofazimine, or cefoxitin were monitored. Represen-
The luminescence-based antibiotic susceptibility testing using UAlMab demonstrated that MICs obtained by measuring RLU were comparable to those by conventional assay (Table 1), suggesting that RLU measurement of UAlMab is both valid and reliable for antibiotic susceptibility testing. However, considering that the MICs could be obtained as early as 6 h postinoculation in a small volume (e.g., 200 μl) for the luminescence-based assay (Fig. 2), in sharp contrast to the 4 days required for conventional agar-based method, we propose that the UAlMab strain be used as a surrogate reporter strain in future screening and evaluation of new anti-M. abscessus drug candidates.

**TB47 and clofazimine combination is effective against M. abscessus in vitro.** The treatment of M. abscessus infections is now challenged by limited options of effective drugs and by the emergence of drug-resistant strains (4, 23). Clofazimine, clarithromycin, and amikacin were active against UAlMab (Table 1 and Fig. 2). We were interested to know whether their activity could be further potentiated by the QcrB-targeting TB47, discovered and reported recently by us (14, 15). In particular, given that combinations of mycobacterial ETC-targeting drugs were shown to yield markedly enhanced efficacy against M. tuberculosis (16–18), we wondered whether the TB47 and clofazimine

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<th>UAlMab</th>
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*Results were recorded at 4 days postinoculation.*

**FIG 2** Luminescence-based antibiotic susceptibility testing using UAlMab. Precultures of UAlMab were diluted to 10^6 CFU/ml in 7H9 medium containing 0.2% glycerol prior to the addition of various concentrations (mg/liter) of amikacin (A), cefoxitin (B), clarithromycin (C), and clofazimine (D). The luminescence was recorded at 0, 3, 6, and 9 h post-drug treatment. Representative data from one experiment performed in triplicate are shown. Error bars represent the standard deviations. The curves indicating the MICs of these drugs are shown in blue.
combination could be effective against *M. abscessus*. We noted that TB47 had no obvious effect on the RLU values of UAlMab in vitro even at 50 mg/liter (Fig. 3A and data not shown), suggesting that TB47 alone had no discernible activity under this condition. Unlike TB47, clofazimine alone inhibited the increase in the RLU counts during growth (Fig. 3A), suggesting that this drug is effective against *M. abscessus* under this condition. Importantly, the combination of clofazimine with only 0.02 or 0.08 mg/liter TB47 gradually decreased the RLU counts (Fig. 3A), implying that this drug combination may kill *M. abscessus* efficiently. In contrast to activity of clofazimine, the activity of amikacin and two macrolides (clarithromycin and roxithromycin) was not potentiated by TB47 (data not shown).

**Assessment of drug efficacy in a murine model of acute *M. abscessus* infection.**

The growth of the UAlMab strain in mice was similar to that of the parental strain (Fig. 1C), allowing us to evaluate the efficacy of drug combinations in a mouse model through a noninvasive approach, as described earlier in *M. tuberculosis* (19). Acute infection...
M. abscessus infection was initiated by infecting nude mice via tail vein with 7.5 log_{10} CFU of the parental strain or the UAlMab strain. We found that RLU counts in lungs of mice infected with UAlMab were 1.5 to 2 log_{10} greater than background values (i.e., data obtained with mice infected or not with the parental strain [data not shown]). Therefore, we could apply this noninvasive approach to evaluate drug efficacy in mice.

We observed that although treatment with clofazimine or TB47 had a marginal effect on the RLU curve relative to that of the control group, the combination of the two drugs markedly decreased the RLU value, particularly at 4 days postinfection (Fig. 3B). Therefore, the clofazimine and TB47 combination appears to be effective against M. abscessus in vivo.

**Assessment of drug efficacy in a murine model of M. abscessus pulmonary infection.** To further corroborate that the combination of clofazimine and TB47 is effective against M. abscessus, we applied a murine model of M. abscessus pulmonary infection using immunocompromised nude mice. Both CFU and RLU counting revealed an initial increase of bacterial burden in the lungs of the infected mice within the first week of infection and, thereafter, a decrease (Fig. 3C). In this model, treatment with roxithromycin slightly decreased bacterial burden in mouse lung (Fig. 3D). Clofazimine was found to decrease the CFU count only after 2 and 3 weeks of treatment (Fig. 3D), suggesting that clofazimine has a delayed effect against M. abscessus. In addition, bacterial burden in mice treated with TB47 was lower than that of the untreated controls (Fig. 3D), suggesting that TB47 alone has activity against M. abscessus in vivo.

We further tested various drug combinations (Fig. 3D) and observed that the combination of roxithromycin and clofazimine was more potent in decreasing bacterial burden than either drug alone. In contrast, the combination of roxithromycin and TB47 did not show any enhanced activity. Consistent with the data obtained from our in vitro study and the acute infection model (Fig. 3A and B), the combination of clofazimine and TB47 demonstrated augmented activity relative to that of either drug alone (Fig. 3D), emphasizing the efficacy of this combination in the treatment of M. abscessus infection. Since the combination of clofazimine with TB47 or roxithromycin had better activity (Fig. 3D), we further tested the efficacy of a clofazimine-TB47-roxithromycin triple combination. Relative to the activity of the clofazimine-TB47 combination, the triple combination delivered a slightly but significantly enhanced activity. Treatment with the triple combination was found to decrease bacterial burden in lung by >1 log_{10} CFU compared to that of the control (Fig. 3D).

In the applied pulmonary infection model, bacterial burden in untreated mice began to decrease after 1 week of infection (Fig. 3C and D). This auto-clearing of bacterial loads may complicate the interpretation of our results. To further demonstrate the efficacy of treatment, we calculated and compared the rates of decrease in lung bacterial burden. As shown in Fig. S1 in the supplemental material, treatment with TB47 slightly boosted bacterial clearance relative to the level in the control. Treatment with clofazimine, the clofazimine-roxithromycin combination, the TB47-clofazimine combination, and the triple combination led to bacterial clearance 1.7-, 1.7-, 1.4-, and 1.5-fold faster than that of the solvent control. Thus, these drug combinations are effective against M. abscessus.

**Assessment of efficacy against 20 M. abscessus clinical isolates.** We further assessed the efficacy of the drug combinations against 20 new M. abscessus clinical isolates. These isolates were identified to be M. abscessus subsp. abscessus based on the sequences of the 16S rRNA gene, DNA gyrase subunit B, and DNA-directed RNA polymerase subunit β. The results obtained with these clinical isolates are shown in Fig. 4. The growth inhibition rate by the combination of clofazimine and TB47 was 68.09\% ± 17.95\%, which was significantly higher than that of either drug alone. The clofazimine-TB47-roxithromycin triple combination further increased the inhibition rate to 85.24\% ± 4.97\% (Fig. 4).
**DISCUSSION**

*M. abscessus* requires 3 to 6 days to form visible colonies on solid medium, rendering the evaluation of drugs time-consuming using the conventional agar proportion method. In this study, we constructed a selectable marker-free, stable autoluminescent *M. abscessus* strain using an integration-proficient plasmid harboring two *dif* sequences, the *apr* gene as the selection marker, and the *int* gene and the *attP* site from mycobacteriophage Tweety. Our initial attempts with plasmids derived from mycobacteriophage L5 or Giles were not successful, suggesting that these integrative plasmids may not be suitable for integration into the genome of the *M. abscessus* GZ002 strain used in the study. Interestingly, when we performed a BLAST search in NCBI using the core *attP* sites of L5 (5′-TCTTCCAAACGCTAGCGCGGGTCGATTCCCGTCGCCCGCTCC-3′), Giles (5′-TGACTGGGGGTCAAGGGGTCGCAGGTTCAAATCCTGTCAGCCTCACGACC-3′), and Tweety (5′-GCTGACTCTTAATCAGCGGGTCGGGGGTTCGAAACCCTCACGCGCAC-3′) against genome sequences of *M. abscessus*, the *attP* Tweety shared 100% identity to some *M. abscessus* strains, but the other two sites affirmed only ~98% identity since 1 base (underlined) was different. The underlined G residues in *attP* L5 and *attP* Giles were A and T, respectively, in *M. abscessus* genomes. Thus, this could possibly explain why only a Tweety-derived vector worked and why a high degree of identity may be strictly necessary for such an integrative vector to work. The *int* Tweety and *apr* in the same cassette were resolved efficiently, which meant the *dif* used works efficiently not only in *M. tuberculosis*, *Mycobacterium bovis* BCG, and *Mycobacterium smegmatis* (24) but also in *M. abscessus*. Several luminescent *M. abscessus* strains have been reported. However, they all contained selectable markers (21, 25), making them less suitable for subsequent drug screening/evaluation due to the potential cross-resistance. Here, we report for the first time the construction of a selectable marker-free and stable autoluminescent *M. abscessus* strain, UAlMab, based on the *dif*/*Xer* recombinase system (26). Application of the UAlMab strain as a reporter allowed us to assess drug activity in vitro in ≤9 h in larger scale. Thus, the development and evaluation of anti-*M. abscessus* drugs, which are of urgent need, can be greatly facilitated.

More importantly, UAlMab offers considerable advantages for *in vivo* drug efficacy assessment. In this study, we applied a murine model of acute *M. abscessus* infection. The combination of this model with our autoluminescent strain allowed us to perform a rapid, facile, and noninvasive evaluation of antibiotic activity *in vivo* in the same batch of live mice. On one hand, the simplicity of detecting light production of live mice infected with UAlMab obviated the need for CFU measurement, making it possible to

![FIG 4] Efficacy against 20 clinical *M. abscessus* isolates. Clinical isolates of *M. abscessus* were treated with various drugs and combinations, as indicated, for 24 h prior to the measurement of growth inhibition by alamarBlue assay. The inhibition rates were calculated by comparing the fluorescence of a drug-treated group to that of the drug-free control. The concentrations of TB47, clofazimine (CLF), and roxithromycin (RTM) were 1 mg/liter, 1 mg/liter, and 2.5 mg/liter, respectively. * P < 0.05 (unpaired t test).
assess in vivo drug activity in ≤4 days with more powerful statistical significance. Furthermore, this noninvasive approach also allowed real-time monitoring of drug activity, as is the case for autoluminescent M. tuberculosis and M. ulcerans (19, 20), thereby providing a more detailed image of drug activity profiles. Facilitated by this noninvasive approach, we identified that the combination of two mycobacterial ETC-targeting drugs, i.e., clofazimine and TB47, has superior activity against M. abscessus, which was further verified in the pulmonary infection model.

It is important to note that in our pulmonary infection model, M. abscessus loads in lungs were found to undergo a decline after 1 week of infection even in the immunocompromised nude mice, suggestive of residual immunity capable of controlling the infection. A previous study demonstrated the successful use of nude mice to produce stable chronic M. abscessus infection in lungs (27). This inconsistency may be due to the different M. abscessus strains used and/or different doses of infection applied.

The ETC of mycobacteria has emerged as the target of many recently developed or repurposed antitubercular drugs. This is exemplified by clofazimine (6), TB47 (14, 15), and Q203 (12). Among these drugs, only clofazimine has been introduced for clinical treatment of M. abscessus infection (10) and evaluated for efficacy against M. abscessus. A previous report showed that clofazimine was effective against M. abscessus under in vitro conditions when it was administered alone or in combination with amikacin or clarithromycin (9), making clofazimine a potential drug for treatment of M. abscessus. However, the delayed effect of clofazimine reported in M. tuberculosis (11) indicates that long-term administration of the drug is necessary, which will, inevitably, lead to more severe adverse effects, such as skin pigmentation (6). It is thus important to know whether clofazimine also has delayed activity against M. abscessus. Here, we found that the obvious in vivo bactericidal activity of clofazimine occurred only after 2 and 3 weeks of treatment but not within the first week of treatment, suggesting that the drug may have a delayed effect against M. abscessus.

Similar to Q203, TB47 targets mycobacterial QcrB, a conservative component of the energy-efficient cytochrome bc_{1}-aa_{3} supercomplex in mycobacteria (12, 14, 15). We reported previously that TB47 is highly bactericidal against M. ulcerans (14). In stark contrast, we found that TB47, when administered alone, did not show any effect in vitro, suggesting either that TB47 cannot inhibit M. abscessus QcrB or that other pathways could alleviate the inhibitory effect of TB47 in vitro. Differentiation between these possibilities warrants further investigation. However, we noted that M. abscessus QcrB shares only 78% identity with that of M. tuberculosis, which is sensitive to TB47 (15). Moreover, QcrB from Mycobacterium marinum and M. ulcerans, both of which are susceptible to TB47 (14), shares 89% identity with that of M. tuberculosis. Mutation of Thr313 in M. tuberculosis QcrB (12) and of Thr323 (Thr313 in M. tuberculosis QcrB) in QcrB of M. marinum and M. ulcerans (14) confers resistance to Q203 and TB47, respectively. Importantly, amino acid polymorphism was observed directly after the Thr site, i.e., aspartic acid, in M. abscessus QcrB, in contrast to the glutamic acid in the QcrB of M. tuberculosis, M. marinum, and M. ulcerans. Based on these observations, it may be argued that the polymorphism of QcrB could affect the susceptibility of M. abscessus to TB47. However, TB47 alone showed in vivo activity and synergistic activity with clofazimine, which indicated that TB47 may inhibit the QcrB of M. abscessus and affect its growth when the compensatory pathway(s), such as the cytochrome bd oxidase (Cyt-bd) containing CytDAB (14), could not work well.

Dual- and even triple-ETC targeting proved to be an effective approach in M. tuberculosis. In particular, combination treatment of clofazimine with cytochrome bc_{1} inhibitors (e.g., Q203) was found to exert a markedly enhanced killing of both replicating and nonreplicating M. tuberculosis bacteria (16, 17). Furthermore, inhibitors of menaquinone biosynthesis also have enhanced activity when administered in combination with other ETC-targeting drugs, such as clofazimine and QcrB inhibitors (18). These observations led us to investigate in M. abscessus the combination activity of clofazimine and TB47, a representative cytochrome bc_{1} inhibitor. Interestingly, although TB47 did not inhibit the growth of M. abscessus in vitro, it showed an inhibitory
effect on bacterial growth in mice. Furthermore, TB47 also markedly potentiated the activity of clofazimine both in vitro and in murine models. In contrast, TB47 did not potentiate the activity of non-ETC-targeting drugs such as clarithromycin, amikacin, and roxithromycin (data not shown). These findings seem to support the idea that *M. abscessus* QcrB can be inhibited by TB47. The lack of growth inhibition phenotype under in vitro conditions may be the result of the activation of an unidentified pathway(s) that could exempt the bacteria from TB47-mediated growth inhibition. Another interesting finding is that the combination of clofazimine and TB47 delivered a much earlier effect, suggesting that TB47 also accelerated the activity of clofazimine. This is rather interesting, given the delayed effect of clofazimine. Altogether, our findings seem to emphasize the notion that dual/triple-ETC targeting is an effective therapeutic approach for treating mycobacterial infections. Finally, we found that the addition of roxithromycin, a macrolide drug, to the clofazimine-TB47 combination further enhanced the efficacy. In particular, this triple combination was also effective against 20 clinical isolates of *M. abscessus* subsp. *abscessus*, which is the most frequently found *M. abscessus* subspecies in hospital (28). In addition to the efficacy, addition of a macrolide to the TB47-clofazimine combination may provide a further benefit by limiting the emergence of resistant strains. Indeed, resistance of *M. abscessus* to clofazimine was recently shown to be caused by overexpression of a putative efflux pump, which also mediated coresistance to bedaquiline (29). Whether the same mechanism could affect susceptibility to macrolides remains to be investigated. Based on these results, we propose that regimens based on clofazimine, TB47 (or other cytochrome *bc*$_1$ inhibitors), and a macrolide should be further exploited to obtain efficient anti-*M. abscessus* regimens.

In conclusion, we have successfully constructed a selectable marker-free autoluminescent *M. abscessus* strain for rapid, convenient, real-time, and noninvasive antibiotic evaluation both in vitro and in vivo. The noninvasive approach for antibiotic assessment using the autoluminescent strain allowed us to identify that the combination of clofazimine and TB47 is effective against *M. abscessus*. Furthermore, the addition of a macrolide to the combination further enhanced the efficacy.

**MATERIALS AND METHODS**

**Bacterial strains, culture conditions, and drug preparations.** *Escherichia coli* DH5$_\alpha$ was grown at 37°C in Luria-Bertani (LB) medium. *M. abscessus* G2002, a clinical isolate described previously (30), for which the whole genome and DNA methylome have been sequenced and analyzed (31), was used as the parental strain to construct the UAlMab strain. In addition, 20 new clinical strains of *M. abscessus* subsp. *abscessus* isolated from Guangzhou Chest Hospital (Guangzhou, China), as identified by comparison of sequences of the 16S rRNA gene, the DNA gyrase subunit gene, and the DNA-directed RNA polymerase subunit $\beta$ gene (Table 2 gives primer information), with their corresponding homologous genes in the

### TABLE 2 Primers used in the study

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$^a$Restriction sites are underlined.

$^b$These primers were used for identifying the clinical *M. abscessus* isolates.
reference strain *M. abscessus* ATCC 19977 (30), were investigated in this study. *M. abscessus* strains were grown at 37°C in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween 80 and 10% oleic acid-albumin-dextrose-catalase (OADC; Difco), or on Middlebrook 7H11 agar (Difco) supplemented with 10% OADC. Apramycin was used at a concentration of 100 mg/liter for *E. coli* and 240 mg/liter for *M. abscessus*, and hygromycin was used at 200 mg/liter for *E. coli*. Stock solutions of apramycin, gentamicin, kanamycin, and amikacin were prepared in water, and stock solutions of clarithromycin, cefoxitin, and roxithromycin were formulated freshly in 0.05% sodium salt of carboxymethyl cellulose (CMC-Na) for oral delivery, and amikacin was formulated in distilled water for subcutaneous injection.

Construction of the UAlMab strain. We initially tried to use mycobacteriophage L5-derived vectors (i.e., pMH94 [32] and pOPHI [33]) to transform *M. abscessus* GZ202, but the attempts failed. Subsequently, mycobacteriophage Tweety-derived pTTp1B (34) was used. The procedure for constructing the UAlMab strain is in accordance with our previous work (33). We took advantage of a previously described plasmid, pUCDhmk1e, which harbors a dif sequence (24). The *int* gene from pTTp1B (34) was cloned and inserted into pUCDhmk1e between Smal and XbaI to create pUCD11B containing *int* flanked by *dif* sequences. The *apr* gene, cloned from pUCDA11B, was inserted into pUCD11B at the XbaI site to generate pUCDA11B. In this plasmid, both *apr* and *int* are located between the two *dif* sequences. The *attP* fragment cloned from pTTp1B was inserted into pOPHI (33), expressing the *luxCDABE* operon under the control of the *hsp60* promoter (36), to create the plasmid pOP1B. The pOP1B plasmid was linearized by Xhol digestion, followed by ligation with Xhol-digested pUCDA11B to generate pOPA1B (see Fig. S2 in the supplemental material). The pOPA1B plasmid was sequenced and then electroporated into the parental *M. abscessus* GZ202. The primers used are listed in Table 2.

After electroporation, individual colonies were selected on apramycin-containing 7H11 plates and suspended in 200 μl of 7H9 medium, followed by measuring relative light units (RLU) using a luminometer (Promega). The colonies yielding >1,000 RLU/colony, considered to be autoluminescent, were subcultured in drug-free 7H9 medium to allow for the removal of the *dif-flanked int-apr* sequence (Fig. S2) by endogenous recombinases XerC and XerD (26). The cultures were then diluted and plated onto apramycin-free 7H11 plates, and resultant colonies were streaked on both apramycin-free and apramycin-containing plates. Colonies grown on apramycin-free but not apramycin-containing plates were further examined by PCR using primers AI1Bf and AI1Br (Table 2) to verify the loss of the *int-apr* sequence. The PCR products were sequenced.

Assessment of UAlMab bioluminescence stability. UAlMab colonies were subcultured in 50 ml of 7H9 medium to an OD600 of 0.5 to 0.7. Subsequently, 0.5 ml of culture was subgrown in 50 ml of 7H9 medium to mid-exponential phase. This was step repeated for a total of 5 rounds (∼30 to 35 generations). Then, cultures were diluted and plated on 7H11 agar. A total of 200 colonies, together with 5 parental *M. abscessus* colonies, were randomly picked and resuspended in 100 μl of LB broth for RLU measurement. The percentage of autoluminescent colonies was calculated as follows: number of positive colonies between 30 to 35 genera-

Growth comparison of UAlMab and the parental *M. abscessus* GZ202. The growth comparison was performed by culturing the two strains separately and parasynchronously. Log-phase UAlMab and wild-type (WT) *M. abscessus* cultures were diluted to an OD600 of 0.01, followed by measuring the OD600 at 3-h intervals. Alternatively, log-phase cultures were diluted by 10^4-fold, followed by measuring CFU at time points as indicated in Fig. 1B. RLU counts of the UAlMab culture were also recorded.

For assessing *in vivo* growth, mid-log-phase cultures of UAlMab and the parental *M. abscessus* strain were centrifuged and resuspended in phosphate-buffered saline (PBS). Four- to 6-week-old female BALB/c nude mice were infected with 7.5 log_{10} CFU via tail vein. Mice were sacrificed for CFU measurement at days 1, 7, and 14 postinfection.

Drug susceptibility testing. As defined earlier (14), the MIC determined by RLU measurement was the lowest drug concentration which decreased RLU counts by 90% compared the level of the untreated controls. The assay was performed in 96-well plates. In each well, 196 μl of the diluted UAlMab culture (∼10^3 CFU/ml) and 4 μl of drug were added. RLU values were recorded at 3-h intervals using a plate reader (Envision Multilabel). The MIC determined by the 7H11 agar method was defined as the lowest drug concentration inhibiting at least 99% of bacterial growth observed for drug-free controls (37). The plates were incubated for 4 days before they were read.

Antibiotic efficacy was also tested for 20 clinical *M. abscessus* strains using a microplate alamarBlue assay (38). Log-phase cultures were diluted to an OD600 of 0.001 (approximately 10^6 CFU/ml). Fixed concentrations of antibiotics were added into 0.2 ml of the diluted culture, followed by the addition, after 24 h of incubation, of 20 μl of 10× alamarBlue solution (Alamar Biosciences/Accumed, Westlake, Ohio). Twenty-four hours later, fluorescence units (FU) were recorded using a plate reader (Envision Multilabel) with excitation at 530 nm and emission at 590 nm. The inhibition rate was calculated as follows: 

\[
\text{FU}_{\text{treated}}/\text{FU}_{\text{untreated}} \times 100\%.
\]

RLU and CFU measurement in mice. Mice were first anesthetized by isoflurane inhalation, and RLU counts were determined, in a noninvasive fashion, by laying the breast of the mouse over the detection hole of a luminometer (Promega) (19). Uninfected mice or mice infected with the WT strain showed a baseline value of approximately 100. In some experiments, mice were euthanized to aseptically harvest lungs and spleens. Organs were homogenized in 2 ml of PBS, followed by serial 10-fold dilutions and plating on 7H11 agar plates. CFU counts were recorded after 6 days of incubation at 37°C. To prevent
possible drug carryover, tissue homogenates of clofazimine-treated mice were plated on 7H11 agar containing 0.4% charcoal, as described previously (11).

**Assessment of drug efficacy in murine model using UAI-Mab.** Four- to 6-week-old, female, BALB/c nude mice were infected via tail vein with 7.5 log_{10} CFU of UAI-Mab. RLU counts of the breast of the live mice were examined 4 h after infection to exclude any mice giving abnormal RLU counts. The mice were administered either 0.05% CMC-Na (negative control) or clofazimine (20 mg/kg/day) and/or TB47 (25 mg/kg/day) freshly prepared in 0.05% CMC-Na once daily via oral gavage. The RLU counts were recorded daily for 4 days.

Alternatively, 4- to 6-week-old, female, BALB/c nude mice were infected by aerosol with 4.6 log_{10} CFU UAI-Mab using the Middlebrook inhalation exposure system (Glas-Col). Treatment was initiated after day 1 of infection for 5 consecutive days. Mice were treated with 0.05% CMC-Na, clofazimine (50 mg/kg/day), roxithromycin (120 mg/kg/day), and TB47 (25 mg/kg/day). At time points indicated in Fig. 3D, mice were sacrificed for CFU determination in lungs and spleens. The rate of decrease in the lung bacterial loads was calculated based on viability data (CFU count) of weeks 1, 2, and 3. A linear trend line was made for each group, and the associated slope (i.e., the $k$ value of the linear line) was obtained and compared with that of the solvent (CMC-Na) control.

**Ethics statement.** Animals were housed and handled in accordance with guidelines set by the Association for the Assessment and Accreditation of Laboratory Animal Care. This study (no. 2016011) was approved by the Ethics Committee of Animal Experiments at Guangzhou Institutes of Biomedicine and Health.

**Statistical analysis.** Data are expressed as means ± standard deviations (SD). Student’s t test was used to analyze differences between two groups. P values of <0.05 were considered to be statistically significant.

**Data availability.** The sequence of pOPA1B was deposited in GenBank under accession number MG680211.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.2 MB.

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We declare that we have no conflicts of interest.

An application for a China invention patent for the selectable marker-freeautoluminescent *Mycobacterium abscessus* and the techniques for its construction was filed in March 2015 and it was authorized on 7 August 2018 (39). A China invention patent for the new application of pyrazolo[1,5-a]pyridine compounds and a combination of drugs to treat *M. abscessus* infections was filed in 2017 (40).

**REFERENCES**


32. Zhang T, Li SY, Converse PJ, Grosset JH, Nuernberger EL. 2013. Rapid, serial, non-invasive assessment of drug efficacy in mice with autolumi-