The compound TB47 is highly bactericidal against *Mycobacterium ulcerans* in a Buruli ulcer mouse model

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Buruli ulcer (BU) is an emerging infectious disease that causes disfiguring skin ulcers. The causative agent, *Mycobacterium ulcerans*, secretes toxin called mycolactone that triggers inflammation and immunopathology. Existing treatments are lengthy and consist of drugs developed for tuberculosis. Here, we report that a pyrazolo[1,5-a]pyridine-3-carboxamide, TB47, is highly bactericidal against *M. ulcerans* both in vitro and in vivo. In the validated mouse model of BU, TB47 alone reduces *M. ulcerans* burden in mouse footpads by more than 2.5 log10 CFU compared to the standard BU treatment regimen recommended by the WHO. We show that mutations of ubiquinol-cytochrome C reductase cytochrome subunit B confer resistance to TB47 and the dissimilarity of CydABs from different mycobacteria may account for their differences in susceptibility to TB47. TB47 is highly potent against *M. ulcerans* and possesses desirable pharmacological attributes and low toxicity that warrant further assessment of this agent for treatment of BU.

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Burruli ulcer (BU)\(^1\)–\(^3\), caused by *Mycobacterium ulcerans*, is the third prevalent mycobacterial disease, after tuberculosis (TB) and leprosy. The World Health Organization (WHO) recommends a combination of rifampin and streptomycin for 2 months for treatment of BU following evaluation of this regimen in a murine model of BU\(^3\). Although this treatment is suboptimal as streptomycin needs to be injected daily for at least 2 months and may result in hearing loss\(^4\)–\(^5\), surgery that was previously the only possible therapeutic intervention became only adjunctive treatment for serious case. All potential oral regimens\(^6\)–\(^8\) repurposed from TB treatment arsenal are not obviously more potent against *M. ulcerans* in vivo.

It takes ~3 months for *M. ulcerans* to form a visible colony on agar supplemented with enriched media specific for mycobacterial growth. Traditional methods that depend on enumerating colony-forming units of bacteria to evaluate efficacy of a drug would significantly prolong the duration of preclinical studies required for development of anti-BU treatment\(^2\),\(^6\). To address this concern, an autoluminescent reporter *M. ulcerans* strain (ALMu), which spontaneously emits light without the addition of exogenous substrate was created using the luxCDABE operon from *Photorhabdus luminescens* and used for anti-BU drug discovery including for in vivo efficacy evaluation\(^9\).

That study assessed activities of a panel of drugs with different mechanisms of actions and drug combinations and demonstrated that relative light units (RLU) counts correlated well with colony-forming unit (CFU) of *M. ulcerans*. Use of this technology enabled rapid (requiring only 3 s) and serial-time monitoring of *M. ulcerans* in samples in vitro and in the same batch of small live animals for evaluation of drug activity. Therefore, this approach can drastically reduce the time, effort, animals, and resources necessary for in vitro and in vivo assessment that require monitoring of *M. ulcerans* growth. Here, we used ALMu to evaluate our small compounds library to discover agents to develop new anti-BU treatment.

We recently reported pyrazolo[1,5-a]pyridine-3-carboxamides exhibit activity against *Mycobacterium tuberculosis*\(^8\). The lead compound of this class, TB47, is now being developed as an anti-TB drug (http://www.newtbdrugs.org/pipeline/discovery). Its mechanism of action is unknown and agents with this pharmacophore have not been evaluated against non-tuberculous mycobacteria. In this study, we found TB47 to be highly potent against *M. ulcerans*\(^9\). We also identified the target inhibited and the mechanism by which resistance to TB47 may arise.

**Results**

**Bactericidal activity against *M. ulcerans* in vitro.** We determined the minimum inhibitory concentrations (MICs) of TB47 against multiple non-tuberculous mycobacteria and a range of clinically important bacteria using standard broth dilution assay as per the Clinical and Laboratory Standard Institute Guidelines (Supplementary Table 1). While its MICs (\(\mu\text{g mL}^{-1}\)) were >128 for most of the clinical strains, the lowest MICs (0.0016) were observed for the two different *M. ulcerans* isolates. These data indicated that TB47 is specific to and highly potent against *M. ulcerans*. Next, we used the standard time kill assay to determine whether TB47 was bacteriostatic or bactericidal against *M. ulcerans*. In this assay, TB47 demonstrated bactericidal activity at concentration \(\geq 0.0016 \mu\text{g mL}^{-1}\) (Fig. 1a). The bactericidal activity of TB47 at 0.008 \(\mu\text{g mL}^{-1}\) or higher was more potent than that exhibited by 0.2 \(\mu\text{g mL}^{-1}\) rifampin, a drug currently used to treat BU. The bactericidal activity of TB47 was also verified by enumerating CFU (Supplementary Fig. 1). Its minimum bactericidal concentration (MBC) against *M. ulcerans* is 0.0064 \(\mu\text{g mL}^{-1}\).

**In vivo bactericidal activity of TB47 against *M. ulcerans*.** We evaluated activity of TB47 in a validated mouse model of BU\(^4\)–\(^6\) using ALMu generated from *M. ulcerans* 10596\(^9\). TB47 exhibited bactericidal activity even at \(\geq 0.4 \mu\text{g kg}^{-1}\), the lowest among doses we evaluated in the mouse model of BU. TB47 alone at 0.8 \(\mu\text{g kg}^{-1}\) exhibited efficacy superior to the current WHO recommended standard regimen to treat Burruli ulcer which includes a combination of 10 rifampin and 150 streptomycin (doses \(\mu\text{g kg}^{-1}\)) in the live mouse model (Fig. 1b, \(P < 0.001\)). At doses \(\geq 12.5 \mu\text{g kg}^{-1}\), the bioluminescence detected 3 days after treatment completion (at day 7) in live mice was indifferent from the background reading (Fig. 1b, \(P > 0.05\)). The classical disease presentation in the mouse model of BU includes swelling, redness and tenderness of the footpads (the site of *M. ulcerans* injection). In mice treated with \(\geq 3.1 \mu\text{g kg}^{-1}\) TB47, at day 7, we observed no swelling or redness in the footpads of mice with average lesion index \(\geq 10\) (ALI = 0), while those treated with the standard regimen containing rifampin and streptomycin exhibited ALI \(\geq 3\) (Fig. 2). Alternatively, we used bioluminescence and CFUs to quantify *M. ulcerans* burden in the footpad tissue at day 7. This assessment also showed \(> 2 \log_{10}\) reduction in RLU in the footpad tissue of mice treated with \(\geq 3.1 \mu\text{g kg}^{-1}\) TB47. The minimal bactericidal dose (MBD) was 1.6 \(\mu\text{g kg}^{-1}\) and at doses \(\geq 12.5 \mu\text{g kg}^{-1}\), RLUs were reduced to the background level. *M. ulcerans* burden (CFU per footpad) in mice treated with 50 mg kg\(^{-1}\) of TB47 was \(< 3.9 \log_{10}\) lower than at day 0 and \(< 2.5 \log_{10}\) lower than in mice treated with the standard regimen containing rifampin and streptomycin (Fig. 1c).

**Preliminary sterilizing activity.** For mice treated with \(\geq 25 \mu\text{g kg}^{-1}\) of TB47, RLU could not be detected at significant levels even up to 14 weeks post completion of treatment (Fig. 1d). However, *M. ulcerans* infection relapsed in 4 to 6 out of 10 mice at 20 to 24 weeks (ALI > 1, Supplementary Fig. 2). In a separate experiment, mice treated with 25 mg kg\(^{-1}\) TB47 for 1 or 2 weeks were compared with mice treated with the standard regimen containing rifampin and streptomycin for 5 or 6 weeks. RLUs from footpads of live mice treated with the standard regimen for 4 weeks were still above the background reading, while the RLUs from footpads of live mice treated with TB47 for 5 days reached the background noise levels (Fig. 1e). In comparison to the standard regimen for 5 weeks, 25 mg kg\(^{-1}\) TB47 for 1 week significantly extended the time-to-relapse (\(P < 0.01\); Fig. 1f). Also, longer duration of treatment of TB47 further prolonged time-to-relapse.

**Resistance to TB47 due to QcrB.** To explore its mechanism of action, *Mycobacterium marinum*, a close relative of *M. ulcerans* with >98% genome sequence identity\(^9\), was used at first as it takes only about 2 weeks for *M. marinum* to form a visible colony on the agar plate. We were able to select *M. marinum* spontaneous mutants in the presence of TB47 and confirmed that the isolates stably exhibited resistance to TB47 (Fig. 3a). The isolates exhibited a consistent increase in MICs for TB47 but remained susceptible to the control drug rifampin (Table 1). Similarly, nine TB47-resistant *M. ulcerans* colonies were isolated very recently only on plates containing 0.02 \(\mu\text{g mL}^{-1}\) TB47 but none on plates containing \(\geq 0.05 \mu\text{g mL}^{-1}\) TB47, although we attempted >10 screens at \(\geq 0.05 \mu\text{g mL}^{-1}\) of TB47. Based on three independent mutant-selection attempts using *M. marinum* and *M. ulcerans*, we determined spontaneous resistance mutation rates against TB47 to be 0.45 \(\times 10^{-8}\) for *M. marinum* at 1 \(\mu\text{g mL}^{-1}\) and 0.83 \(\times 10^{-9}\) for *M. ulcerans* at 0.02 \(\mu\text{g mL}^{-1}\).

We sequenced the genomes of seven independent TB47-resistant *M. marinum* isolates and the parent strain. We identified single-nucleotide polymorphisms (SNPs) at the same codon in
all resistant isolates ACC→GCC or CCC resulting in Thr323Ala or Thr323Pro substitution in QcrB (ubiquinol-cytochrome C reductase cytochrome subunit B) (Table 1). We amplified this locus in an additional 20 independent spontaneous M. marinum mutants, sequenced and found mutations only in the same codon that resulted in Thr323Ala or Thr323Pro substitutions (Table 1 and Fig. 3b). Alignment of amino acid sequences of QcrBs from M. ulcerans and M. marinum revealed 100% identity (Supplementary Fig. 3a). Similarly, we sequenced qcrB gene in 9 independent spontaneous M. ulcerans mutants. Eight out of nine mutants harbored the SNP ACC→GCC resulting in Thr323Ala while the remaining isolate harbored SNP ACC→ATC resulting in Thr323Ile. To verify if such substitutions truly lead to resistance to TB47, we overexpressed wild-type and mutated qcrB genes in M. marinum and M. ulcerans, and observed an increase in MICs of TB47 (Table 1). Based on these data, we

Fig. 1 Activity of TB47 (T) against M. ulcerans. 

*Data are expressed as mean ± SD from three independent biological repeats. The experiments were performed in triplicate (three independent experiments) and the representative results are shown. 

(b) RLU detected from left hind footpads of the same batch of mice treated for 5 days and stopped for 3 days. Statistical analysis was performed using unpaired Student's t-test. The linearity of the relationship of RLU and CFU for the in vivo experiments were 0.966 (including the positive) and 0.898 (TB47 treated groups only), respectively. Correlation analysis was performed using Pearson's correlation test. The linearity of the relationship of RLU and CFU for the footpad suspension is valid when the CFUs are from 2.5 to 6.2 log10 CFU mL−1.

(c) The dotted green lines indicated the base line (the limit of detection). Data are expressed as mean ± SD of 3 samples.

(d) Mice were treated from 12 days post infection of AlMu (AlMu were infected s.c. with 10^7 CFU) or TB47 (0.1 ml, 1.5 mL). Each group consisted of 5 mice. Mice were treated from 12 days post infection.

(e) Footpad RLUs detected after completion of treatment for different regimens. Data are expressed as mean ± SD of 3 samples.

(f) Time to footpad swelling after completion of antibiotic treatment. Time to footpad swelling in mice treated with either rifampin + streptomycin for 5 weeks (gray circles) or 6 weeks (black triangles), or TB47 for 1 week (red square) or for 2 weeks (purple triangle). Statistical differences were determined by Log-rank (Mantel-Cox) test with 15 mice in each group. T, TB47; BL, base line; Ui, uninfected (for RLU detection, Ui is the base line); Ut, untreated; R, rifampin, 25; S, streptomycin, 150; Dosage (mg kg−1).

(g) Percent without lesion. 

(h) Percent without lesion.
conclude that mutations of QcrB Thr323 confer M. marinum and M. ulcerans resistance to TB47.

Two terminal oxidases in electron transport chain. The electron transport chain of mycobacteria usually contains two terminal oxidases, the cytochrome bc1:aa3 containing QcrCAB and the cytochrome bd oxidase (Cyt-bds) containing CydAB (Fig. 4). Inhibition of the bc1:aa3 complex is only bacteriostatic because the alternate Cyt-bds is capable of maintaining a membrane potential and menaquinol oxidation and is therefore sufficient to maintain respiration to protect M. tuberculosis from death. If TB47 inhibits QcrB, it should be more powerful against the strain lacking of functional Cyt-bds. The cydAB genes were deleted in the model organism M. smegmatis to generate a ΔcydAB strain (Msm ΔcydAB). The synthetic lethal interaction between the Cyt-bc1:aa3 and the Cyt-bds was evaluated by treating the Msm ΔcydAB with TB47. A synthetic lethal interaction is a well-described phenomenon where simultaneous inactivation of two genes that confer an essential activity results in cell death but inactivation of either gene alone does not affect cell viability. Similar to a synthetic lethality described in M. tuberculosis, deletion of cydAB in M. smegmatis did not impact significantly on bacterial growth (Supplementary Fig. 4) but sharply increased the inhibitory potency of TB47 (Fig. 5a) as its MIC was reduced from 50 to 3.12 μg L⁻¹.

Activities of Cyt-bds from different mycobacteria. The amino acid sequences of QcrB, CydA, and CydB from different mycobacteria were aligned (Supplementary Fig. 3a, b, c). The QcrB as shown before, is highly conserved among mycobacteria, but...
the similarity of CydA/B between M. tuberculosis and that of M. smegmatis or M. ulcerans was low. It is interesting that M. leprae genome altogether lacks genes that encode CydA/B. This led us to hypothesize that the Cyt-bds from different mycobacteria may not have identical activities, which potentially explained the distinct susceptibilities of mycobacteria to TB47.

To verify this hypothesis, we created a series of recombinant strains by introducing cydAB genes from different mycobacteria into MsmΔcydAB. The MICs of TB47 against MsmΔcydAB::cydABMsm, MsmΔcydAB::cydABMtb, MsmΔcydAB::cydABMu, and MsmΔcydAB were 50, 37.5, 6.5, and 3.2 μg mL⁻¹, respectively (Fig. 5a), while MICs of isoniazid were 4 μg mL⁻¹ for all four strains. In the presence of TB47, these strains exhibited different in vitro growth profiles (Fig. 5b). M. ulcerans cydAB could not sufficiently complement the function of M. smegmatis Cyt-bds. This indicated that intrinsic activity of Cyt-bds of M. ulcerans could be too weak, which may explain why TB47 had surprising bactericidal activity against M. ulcerans.

Pharmacological attributes and low toxicity of TB47. There is no obvious toxicity of TB47 observed until now. TB47 did not display obvious cytotoxicity with IC₅₀ of >100 μM for VERO cell and 50 μM for THP-1 cell. TB47 did not inhibit hERG (IC₅₀ > 30 μM), suggesting a low risk for cardiotoxicity. A single oral administration of 2000 mg kg⁻¹ of TB47 or oral 200 mg kg⁻¹ daily for 4 weeks, the highest dosage we tested, was well tolerated by mice. No genotoxicity or obvious CYP inhibition was associated with this compound, and its metabolic stability in different animal species and permeability are high (Supplementary Table 1).
Table 2. Results of pharmacokinetic parameters of TB47 in BALB/c mice.

| Drug delivering | Parameters       | Unit       | Mean   | SD    |
|-----------------|-----------------|------------|--------|-------|
| **Intravenous injection** | AUC(0-1) | µg L⁻¹ x h | 10,409 | 139   |
|                  | AUC(0-∞) | µg L⁻¹ x h | 11,951 | 565   |
|                  | MRT(0-1) | h          | 14.7   | 1.7   |
|                  | MRT(0-∞) | h          | 22.3   | 3.7   |
|                  | t½/2 | h          | 17.7   | 2.6   |
|                  | CL   | L h⁻¹ kg⁻¹ | 0.17   | 0.01  |
|                  | V    | L kg⁻¹     | 4.27   | 0.44  |
|                  | Cmin | µg L⁻¹      | 37.09  | 1805  |
|                  | AUC(0-1) | µg L⁻¹ x h | 19,823 | 1665  |
|                  | AUC(0-∞) | µg L⁻¹ x h | 33,144 | 5164  |
| **Oral administration** | MRT(0-1) | h          | 20.2   | 0.3   |
|                  | MRT(0-∞) | h          | 52.0   | 4.2   |
|                  | t½/2 | h          | 35.6   | 2.7   |
|                  | Tm | h          | 3.2    | 2.8   |
|                  | CL   | L h⁻¹ kg⁻¹ | 0.30   | 0.02  |
|                  | V    | L kg⁻¹     | 15.49  | 0.38  |
|                  | Cmax | µg L⁻¹      | 626    | 282   |

Fig. 6. The ratios of footpad/plasma and lung/plasma of TB47 concentrations. BALB/c mice were given a dose of 10 mg kg⁻¹ TB47 by oral gavage. Data are expressed as mean ± SD of five samples.

Table 2). Furthermore, in a long-term administration study, TB47 was well tolerated without any meaningful clinical signs of toxicity when administered daily at a dose of 10, 30, or 100 mg kg⁻¹ body weight orally for 30 days using both male and female rats. These data demonstrate that TB47 is well tolerated even during prolonged use.

Pharmacokinetic parameters of TB47 in BALB/c mice are summarized in Table 2. After intravenous administration of 2 mg kg⁻¹ TB47, the AUC (0–t) and t½/2 were 10,409 ± 139 µg L⁻¹ x h and 17.7 ± 2.6 h, respectively. Whereas the AUC (0–t), Cmax and t½/2 were 19,823 ± 1665 µg L⁻¹ x h, 0.63 ± 0.28 µg mL⁻¹ and 35.6 ± 2.7 h, respectively, after oral administration of 10 mg kg⁻¹ TB47, and the bioavailability was 38.1%. The long half-life and high blood concentration of TB47 are likely significant contributors to its potent in vivo activity against M. ulcerans. Furthermore, the concentrations in mice foot tissue or lungs at 6, 12, 24, and 48 h were > 4 times higher than in the plasma (Fig. 6).

Discussion

Despite the efficacy of the standard regimen of streptomycin and rifampin for 2 months for treatment of BU, it has significant disadvantages including daily parenteral administration and potential risk of side effects, such as hearing loss. An all-oral and less toxic treatment regimen of Buruli ulcer has been sought after and encouraged by WHO. However, most of newly developed treatments were based on rifampin although it is well known that rifampin causes interactions with many drugs, including anti-retroviral agents and rifampin-resistance in M. ulcerans isolates from patients and infected animals have been reported. New powerful drugs, especially those with new mechanisms of action could address the current need for novel regimens to treat BU.

TB47 exhibits potent bactericidal activity with very low MIC and MBC against M. ulcerans. Detection of MICs of TB47 against M. ulcerans on agar plates was repeated only once (duplicate but not triplicate), which may hinder potential reproducibility. However, We (1) used two strains (M. ulcerans 1059 and M. ulcerans 1615) and they showed the same MICs of TB47 and the MIC detection was duplicated for each strain; (2) repeated the liquid MIC detection more than four times by different persons; (3) repeated more than ten times to select TB47-resistant M. ulcerans mutants on agar containing as low as 0.05 to 0.1 µg mL⁻¹ TB47 but failed. This observation further validates the very low MIC observed for TB47; (4) repeated the MBC determination three times (independent repeats) and confirmed it to be very low. Moreover, it is striking that even at 0.8 mg kg⁻¹ of TB47, once daily, it exhibited bactericidal activity and overall superior efficacy than the currently used standard regimen containing rifampin and streptomycin. While mice treated with the standard regimen sustained clinical indications such as swelling of the footpad, such indications were nonexistent in the footpads of mice that received TB47 at doses of ≥ 3.1 mg kg⁻¹. In addition, in comparison to the standard regimen, TB47 prolonged the time-to-relapse. This indicated that TB47 has a quick therapeutic effect and could possibly shorten the duration of the BU treatment. Although TB47 alone showed very good activity against M. ulcerans, it did not produce 100% cure at 25 mg kg⁻¹ daily, 5 days per week, for 2 weeks.

We demonstrate that TB47 targets mycobacterial QcrB because QcrB mutations cause TB47 resistance and deletion of cytochrome bd oxidase (Cyt-bds) makes mycobacteria more susceptible to TB47 (Fig. 4). The differences in Cyt-bds from different bacteria potentially explain their differential susceptibilities to TB47. The M. smegmatis ΔcydAB can be a good system to test the function of cydAB introduced from other mycobacteria and could be used as a model for screening inhibitors of Cyt-bds. It is valuable to test activity of Cyt-bds in different mycobacteria, such as M. leprae. There are no significant similar CydAB proteins in M. leprae, which indicated that Cyt-bc1:aa3 could be the only terminal oxidase in the electron transport chain in this mycobacterium. So it is very attractive to test TB47 against leprosy caused by M. leprae as it is still a public health concern in many countries despite sustained efforts to eliminate it over the last 30 years. Depending upon the leprosy patient’s disease presentation, they are currently treated for 6, 12, or 24 months with a combination of rifampin, dapsone and with or without clofazimine. Many patients experience side effects, toxicity, and potential drug resistance. Our study also supports that the combination of the potential Cyt-bds inhibitors and TB47 may shorten the duration of further for both drug-susceptible and drug-resistant TB. None of atomic structures of mycobacterial bd oxidases has been reported yet but that from Geobacillus thermodenitrificans was resolved recently. The structures of mycobacterial CydAB complexes and the rational design of inhibitors of them are highly needed in the discovery and development of antismycobacterial drugs.

In summary, our study demonstrates that TB47 acts by a different mechanism compared to drugs considered to treat BU thereby reducing the chances of development of cross-resistance. In addition, the rate of spontaneous resistance of M. ulcerans to
TB47 is very low. Oral bioavailability, relatively long half-life and lack of toxicity are additional appealing features of TB47. To leverage these clinically desirable attributes, we propose that a multi-drug combination regimen containing TB47 has promise to produce a stable cure while also potentially shortening the duration of treatment. Further preclinical assessments of dose, frequency and duration of administration would be necessary to harness the optimal potential of TB47 against BU.

Methods

Drugs formulation. TB47 was synthesized in a batch and supplied by Guangzhou Eggbio Co. Ltd with purity 98.67% by High Performance Liquid Chromatography (Batch number: TB47160616). It was formulated in 0.05% CMC-Na (sodium carboxy methyl cellulose) for in vivo use. Rifampin and streptomycin were both bought from TCI (Japan) and dissolved in distilled water for in vivo use. Streptomycin and isoniazid (Macklin, Shanghai) were dissolved in distilled water, and rifampin and TB47 were dissolved in dimethyl sulfoxide (DMSO) for in vitro use. All drugs prepared weekly for in vivo studies. Hygromycin was bought from Roche (Swiss) in liquid form and kanamycin was bought from Sigma (USA). They were dissolved in distilled water for in vitro study.

Strains and growth conditions. M. ulcerans strains 1059 and 1615, M. marinum, M. smegmatis C7-155, and derivative strains were grown in Middlebrook 7H9 broth medium and used for in vitro tests with 0.05% tween80 for culture and without tween80 for drug susceptibility testing or 10% OADC. M. ulcerans and M. marinum strains were grown at 30 to 32 °C and others at 37 °C. Other clinical important bacteria were grown according to the Clinical and Laboratory Standard Institute Guidelines.

RLU-based MIC and CU-based MBC determination. Serial dilutions of drug-containing solutions and autoluminescent mycobacteria broth culture (OD560 of 0.3 to 1.0) were prepared8. RLU counts from the same batch of triplicate samples were measured according to the designed time points. Ninety-six-well plates were measured as rendering the amount of light by using a Veritas Microplate Luminometer Operating Manual (Turner Bionetics). MIC(MBC) was determined as the lowest concentration that can inhibit > 90% RLUs compared with that from the untreated controls4,8. The time-killing curves and MIC(MBC) of autoluminescent strains were determined by detecting RLUs from these cultures. For M. ulcerans and M. marinum strains, the incubation time for MIC(MBC) reading was 7 days and 3 days, respectively. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of drug that killed ≥99% of the CFU compared with the CFU count of the initial inoculum used for the liquid activity assays, which was adopted from reference9. In our case, the liquid media in the culture tubes that showed the RLU at 1 log10 lower than that at day 7 were defined as the control plates on the MIC(MBC) plates for CFU counts. The concentrations used were twofold series of dilutions for this purpose. The incubation time for RLU reading was 14 days for the MBD experiment and the light from each 1.5 mL tube containing total 500 μL culture was detected using Promega GloMax 20/20. Triplicate tubes for each concentration were plated onto the CFU plates at d0, 3, and 7 to detect the quick bactericidal activity of TB47 and to show the correlation of RLU and CFU counts. The concentrations used were fivefold series of dilutions for this purpose. Each above experiment was performed in triplicate (three independent experiments) or more by independent persons and from three or more independent cultures. The representative results are shown.

MIC determination. The serial tenfold diluted mid-log-phase cultures were plated on 7H11 plates containing different concentrations of drugs. The MICs of TB47 to M. ulcerans strains 1059 and 1615 were defined as the lowest concentration that can inhibit at least 99% growth observed from drug-free control plates. The experiments detecting such MICs using agar plates were only repeated once (in independent experiments were plated for CFU counts at d0 and d7 for MBD detection. The representative results are shown in Fig. 1c.

Selection of spontaneous-resistant mutants to TB47. Broth cultures (OD600 from 0.6 to 1.3) of autoluminescent M. ulcerans 1059, wild-type M. ulcerans 1615, M. ulcers 1059, and autoluminescent M. marinum were plated on 7H11 plates containing 0.02, 0.05, 0.1, 0.5, 1, 2, 10, or 40 μg mL−1 TB47. The colonies grown upon the TB47-containing plates were picked up to confirm the drug resistance phenotype by real-time in vitro drug susceptibility testing in liquid media or agar method as described above. We repeated about ten times using M. ulcerans and each time we plated at least 20 plates (0.5 mL per plate, so > 10 mL culture per plate) at concentrations ≥0.1 μg mL−1 but three times only at 0.02 and 0.05 μg mL−1. And we tried different M. ulcerans strains and with tenfold concentrated broth culture. The light from each well reached 100-1000 RLU mL−1, which indicated more than 104 CFU mL−1. The series of tenfold diluted culture was diluted and plated on drug-free plates for detecting the bactericidal density.

Whole-genome sequencing. The genome DNAs of the parent M. marinum and the confirmed TB47-resistant mutants were whole-genome sequenced by Beijing Genomics Institute. The resulting reads were aligned to the M. marinum genome sequence and compared with that of the parent strain.

Over-expression of qcrB genes in M. marinum and M. ulcerans. Three types (qcrBwild type, qcrBThr323Pro, and qcrBThr323Ala) of qcrB genes were amplified from genome DNAs of wild-type and spontaneous-resistant M. marinum mutants by PCR using primers qcrBmrEf- qcrBmrEr (Table S5) and inserted into the p60luxn plasmid under the control of the hsp60 promoter. The three plasmids were transformed into the wild-type autoluminescent M. marinum or M. ulcerans. The MICmin of the recombinant strains to TB47 were determined as described above.

Pharmacokinetics. BALB/c mice were given 2 mg kg−1 TB47 intravenously or 10 mg kg−1 orally. Blood samples were taken from the eyeballs of five mice per time point at 0.5, 1, 2, 6, 12, 24, and 48 h post-dose for intravenous group or at 15, 30 min, 1, 2, 6, 12, 24, and 48 h for oral group. Footpads and lung tissues were collected at 6, 12, 24, and 48 h post-dose for oral group. After extraction and centrifuge at 4 °C, TB47 concentrations were determined by Liquid Chromatograph-Mass Spectrometer (LC-MS).

Construction of the M. smegmatis cydAB knockout mutant. The recombinating method was used22. The DNA fragments of upstream of cydA and downstream of cydB of M. smegmatis were amplified using primers cydAF-cydAR and cydBF- cydBR (Supplementary Table 5) and cloned into a plasmid with three fragment ligation and verified by sequencing. Then the fragment dif-Hyg-dif was inserted in between the two fragments and the resulting ArmCyDA-cydA-Hyg-dif-ArmCydB was excised from the plasmid and transformed into induced M. smegmatis TS53 (M. smegmatis containing pV53Ts) competent cells22. The cydAB genes were replaced by the Hyg gene through allelic replacement. To remove the Hyg gene, the mutants were cultured in 7H9 broth without hygromycin for 3 days. To remove vector pV53Ts, the mutants were cultured in 7H9 broth at 42 °C for 3 days, serially tenfold diluted and plated onto 7H11 plates containing 10% sucrose, and incubated at 42 °C for 72 h. The loss of the vector pV53Ts in the mutants was subsequently confirmed by plating 100 colonies in 7H11 plates containing 10% sucrose and kanamycin or in 7H11 plates containing 10% sucrose at 42 °C. The M. smegmatis cydAB mutant (MsmΔcydAΔcydB) was verified PCR similar to the published design22 using primers cydA-d (Supplementary Table 5) and sequencing.

CydAB complementation and growth curves comparison. Complementation plasmids were created by amplifying the cydAB genes from genome DNAs of M. tuberculosis H37Rv, M. ulcerans, M. marinum, and M. smegmatis by PCR using primers indicated in Supplementary Table 5 and inserting them into the pβlux plasmid containing the hsp60 promoter, resulting in the series of plasmids pβlux−cydAB. A series of recombinant M. smegmatis strains were constructed as: MsmΔcydAΔcydB from M. tuberculosis, MsmΔcydAΔcydB from M. ulcerans,
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Competing interests: All authors declare competing interests: TB47 was synthesized in a batch and supplied by Guangzhou Eggbio Co. Ltd, which has been developing TB47 as a therapeutic agent against tuberculosis and other potential diseases and had no role in study design, data collection and analysis, decision to publish the manuscript. Guangzhou Institutes of Biomedicine and Health (GIBH), Chinese Academy of Sciences (CAS) has filed a Chinese application (filing # 201810106538.9) and a PCT application (filing # CN2018/077992), entitled ‘New use of a set of pyridine compounds’, listing T.Z. and Y.L. as inventors.

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