Acta Pharmaceutica Sinica B 2021;11(3):738–749

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Acta Pharmaceutica Sinica B

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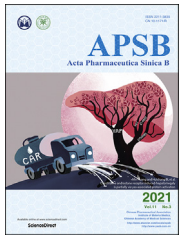

ORIGINAL ARTICLE

# Ultra-short-course and intermittent TB47-containing oral regimens produce stable cure against Buruli ulcer in a murine model and prevent the emergence of resistance for *Mycobacterium ulcerans*

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Received 9 June 2020; received in revised form 1 September 2020; accepted 7 September 2020

**KEY WORDS**

*Mycobacterium ulcerans*;
Buruli ulcer;
Electron transport chain;
QcrB;
Chemotherapy;
TB47;
Drug resistance;
Clofazimine

**Abstract** Buruli ulcer (BU), caused by *Mycobacterium ulcerans*, is currently treated with rifampin−streptomycin or rifampin−clarithromycin daily for 8 weeks recommended by World Health Organization (WHO). These options are lengthy with severe side effects. A new anti-tuberculosis drug, TB47, targeting QcrB in cytochrome bc1:aa3 complex is being developed in China. TB47-containing regimens were evaluated in a well-established murine model using an autoluminescent *M. ulcerans* strain. High-level TB47-resistant spontaneous *M. ulcerans* mutants were selected and their *qcrB* genes were sequenced. The *in vivo* activities of TB47 against both low-level and high-level TB47-resistant mutants were tested in BU murine model. Here, we show that TB47-containing oral 3-drug regimens can completely cure BU in ≤2 weeks for daily use or in ≤3 weeks given twice per week (6 doses in total).

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Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2020.11.007

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All high-level TB47-resistant mutants could only be selected using the low-level mutants which were still sensitive to TB47 in mice. This is the first report of double mutations in QcrB in mycobacteria. In summary, TB47-containing regimens have promise to cure BU highly effectively and prevent the emergence of drug resistance. Novel QcrB mutations found here may guide the potential clinical molecular diagnosis of resistance and the discovery of new drugs against the high-level resistant mutants.

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## 1. Introduction

Buruli ulcer (BU)[1−3], caused by *Mycobacterium ulcerans* (*M. ulcerans*), is the third most common mycobacterial infection worldwide after tuberculosis and leprosy. BU has become treatable by antibiotics following evaluation of regimens in a murine model of BU[3,4]. Now, two 8-week regimens are recommended by the World Health Organization (WHO): rifampin combined with either streptomycin or, more recently, clarithromycin[5]. Although both of them have good therapeutic effect, they have significant disadvantages, such as the treatment duration is relatively long, streptomycin needs injection and serious side effects occur such as hearing loss[6,7], clarithromycin has drawbacks in terms of gastrointestinal tolerability, and rifampin can cause drug−drug interaction with many drugs including clarithromycin[8]. In addition, surgery is still needed as the adjuvant treatment for serious cases. Some potential oral regimens repurposed from tuberculosis treatment arsenal are emerging, such as increasing the dose of rifampin, using longer half-life rifapentine and including clofazimine[5], which could possibly cure BU in 4 weeks. Recently, we reported TB47, a compound targeting the respiratory cytochrome bc1:aa3, exhibited highly bactericidal activity against *M. ulcerans* both *in vitro* and *in vivo*[9]. TB47 was dose-dependent and at a very low dose of 0.8 mg/kg was more effective than the standard regimen, rifampin 10 mg/kg−streptomycin 150 mg/kg, in reducing the *M. ulcerans* burden in a mouse footpad model of BU[9]. Meanwhile, TB47 at 25 mg/kg with expanding treatment duration, the time-to-relapse was prolonged and 80% mice treated for 2 weeks showed no relapse at least 23 weeks after treatment completion. A combination of new compounds and available antibiotics may enhance the therapeutic efficacy, such as D-serine and hypericin had synergistic activity in combination with $\beta$-lactams against methicillin-resistant *Staphylococcus aureus in vivo*[10,11]. So we speculated increasing TB47 dose and combining TB47 with 1 or 2 other drugs currently available could possibly cure BU quickly.

In the previous study, TB47-resistant spontaneous *M. ulcerans* mutants were only obtained at very low TB47 concentrations and their minimum inhibitory concentrations (MICs) were relatively low (0.2−0.4 μg/mL)[9]. If TB47 is active against them or not *in vivo* in mice is unknown. If yes, the doses of TB47 used should be able to prevent the emergence of TB47 resistance. We screened high-level TB47-resistant *M. ulcerans* mutants using the low-level TB47-resistant mutants we already had. In the one hand, we were interested in whether the high-level TB47-resistant *M. ulcerans* mutants could be obtained and if yes, where the new gene mutation(s) could occur, in other words, if there were other mechanisms of action of TB47 except for targeting the ubiquinol−cytochrome *c* reductase cytochrome subunit B, QcrB, in the electron transport chain. On the other hand, we wanted to investigate whether TB47 could be still active against high-level TB47-resistant *M. ulcerans* mutants *in vivo* at higher doses used alone or in combination.

In this study, we found TB47 was the main driver in all regimens containing TB47, and TB47-containing 3-drug regimens can completely cure BU in $\leq$2 weeks for daily use or in $\leq$3 weeks given twice per week (only 6 doses in total) in a well-established BU murine model. All high-level TB47-resistant *M. ulcerans* mutants showed double mutations in QcrB which could be divided into ten types with eight novel mutations distributing in five novel mutation sites. TB47 showed anti-BU activity against the low-level TB47-resistant mutants but not the high-level ones *in vivo*. TB47-containing regimens may prevent the emergence of drug resistance.

## 2. Materials and methods

### 2.1. Ethical statement

All animal procedures were conducted in accordance with national and international guidelines. All animal care and experimental protocols were approved by the committee on Laboratory Animal Ethics of Guangzhou Institutes of Biomedicine and Health (GIBH), Chinese Academy of Sciences (#2017077). GIBH is in compliance with the Animal Welfare Act regulations and Public Health Service Policy.

### 2.2. Bacteria

*M. ulceran*s 1059 is an isolate originating from a clinical specimen from a patient in Ghana and the autoluminescent *M. ulcerans* (AlMu) was created based on this isolate[12]. The TB47-resistant *M. ulceran*s mutants were obtained from AlMu in one or two steps. A uniform homogenous suspension was prepared from the colonies by suspending them in sterile phosphate buffered saline (PBS, GENOME, Hangzhou, China) and vortexing them with sterile glass beads before injection into the mouse footpads. All *M. ulceran*s strains were grown in Middlebrook 7H9 broth medium (Becton, Dickinson and Company, New Jersey, USA) with 10% 2-oxo-acid dehydrogenase complexes (OADC, Becton, Dickinson and Company)+0.2% glycerol (Shanghai Macklin Biochemical, Shanghai, China)+0.05% Tween 80 (Amresco, USA) for culture or on Middlebrook 7H11 plates (Becton, Dickinson and Company) supplemented with 0.2% glycerol and 10% OADC. Plates were incubated at 30 °C for 12 weeks before counting colony-forming units (CFUs).

**Table 1** TB47 susceptibility testing and analysis of QcrB polymorphism in resistant mutants.

| Strain No. | Amino acid change in QcrB[a] | Codon change in *qcrB* gene | No. of mutant obtained | MIC (μg/mL) |
|---|---|---|---|---|
| $Mu^{Sm1}$ | T323A | ACC→GCC | / | 0.2−0.4 |
| $Mu^{Sm2}$ | T323I | ACC→ATC | / | 0.2−0.4 |
| $Mu^{Dm1}$ | T323A; I183T | ACC→GCC; ATC→ACC | 2 | >50 |
| $Mu^{Dm2}$ | T323A; M352V | ACC→GCC; ATG→GTG | 4 | >50 |
| $Mu^{Dm3}$ | T323A; G325D | ACC→GCC; GGC→GAC | 1 | >50 |
| $Mu^{Dm4}$ | T323A; M352T | ACC→GCC; ATG→ACG | 1 | >50 |
| $Mu^{Dm5}$ | T323I; L185P | ACC→ATC; CTG→CCG | 5 | >50 |
| $Mu^{Dm6}$ | T323I; F158V | ACC→ATC; TTC→GTC | 1 | >50 |
| $Mu^{Dm7}$ | T323I; F158L | ACC→ATC; TTC→CTC | 1 | >50 |
| $Mu^{Dm8}$ | T323I; G325D | ACC→ATC; GGC→GAC | 2 | >50 |
| $Mu^{Dm9}$ | T323I; M352T | ACC→ATC; ATG→ACG | 1 | >50 |
| $Mu^{Dm10}$ | T323I; G325S | ACC→ATC; GGC→AGC | 1 | >50 |

[a]Total five novel mutation sites at F158, I183, L185, G325, M352 and total eight novel mutations F158V, F158L, I183T, L185P, G325D, G325S, M352V and M352T were found.

### 2.3. *MIC determination*

The serial tenfold diluted mid-log phase cultures containing $\sim 10^2$ or $\sim 10^4$ CFU/mL were plated on 7H11 plates containing different concentrations of TB47 (Guangzhou Eggbio, Guangzhou, China). The MIC was defined as the lowest concentration that can inhibit at least 99% growth observed from drug-free control plates[9]. The experiment detecting such MICs using agar plates was only repeated once (in duplicate) and each time with two different clones.

### 2.4. *Selection of high-level TB47-resistant spontaneous M. ulcerans mutants*

Broth cultures ($OD_{600}$ from 0.8 to 1.2) of low-level TB47-resistant *M. ulcerans* strains $Mu^{Sm1}$ and $Mu^{Sm2}$ (Table 1) were plated on 7H11 plates containing 0.8, 1, 4, 10 or 20 μg/mL TB47. The colonies grown up on the TB47-containing plates were picked up to confirm the drug resistance phenotype by detecting their MICs using agar method as described above. We repeated 2 times for screening. The series of 10-fold diluted cultures were plated on drug-free plates for detecting the bacterium density.

### 2.5. *Identification of mutation site(s) causing TB47 resistance*

The *qcrB* genes were amplified from the genomic DNAs of all the selected resistant *M. ulcerans* mutants by PCR using primer pairs of Mu_qcrB1-F1/Mu_qcrB1-R1 and Mu_qcrB1-F2/Mu_qcrB1-R2 (Table 2; Sangon Biotech, Shanghai, China). The PCR products were sequenced by Sangon Biotech. The sequences were aligned with both the *qcrB* genes of their parent low-level TB47-resistant strains and the *qcrB* gene of *M. ulcerans* 1059 strain.

### 2.6. *Animal studies*

Serial, non-invasive, real-time monitoring of drug activity in a murine model of BU[13] using AlMu, was used for testing earlier bactericidal activities of 2- or 3-drug regimens and later on, the sterilizing activities were assessed by using the classical murine model. The mice were purchased from Charles River (Beijing, China). The activities of TB47 against TB47-resistant *M. ulcerans* mutants were evaluated in the murine model of BU by detecting CFUs from the footpads of mice. Colony suspensions were made by vortex using fresh colonies in 10 mL PBS and the resulting suspensions were used to inject the right hind footpads of six-week old, 19 ± 1 g, female BALB/c mice with the left ones as controls. The inoculum volume was 0.05 mL, containing approximately 6−7 lgCFU in each experiment. The lesion index was defined as follows: index 0 = normal footpad; index 1 = noninflammatory footpad swelling; index 2 = inflammatory footpad swelling; index 3 = inflammatory hind foot swelling[14]. The animal experimental schemes for testing activities of TB47 or new regimens were demonstrated in Table 3 for TB47 alone or in 2-drug regimens, in Table 4 for TB47 in 3-drug regimens and Table 5 for TB47 against drug-resistant mutants, respectively. Subcutaneous route for streptomycin and oral gavage for others were used. Rifampin was purchased from Sigma−Aldrich (Missouri, USA). Rifapentine was purchased from APExBIO

**Table 2** Primers used in this study.

| Primer | Primer sequences (5′−3′) | Purpose |
|---|---|---|
| Mu_qcrB1-F1 | GCGCAGTTGCCTATCACA | To amplify *qcrB* genes from TB47-resistant *M. ulcerans* colonies for checking mutation. |
| Mu_ qcrB1-R1 | GGTGTGGTGCCAGAAGTAG | |
| Mu_qcrB1-F2 | GTCTGGTGCGTTCTTCGCGGC | |
| Mu_ qcrB1-R2 | GTGCCGGTGGCCATGATGGG | |
| Mu_rpoB-F1 | GTTCGGTTGCGTGCGTGAG | To amplify *rpoB* genes from *M. ulcerans* colonies from relapse mice for checking mutation. |
| Mu_rpoB-R1 | GTGTTCCTCGATGTGGATC | |
| Mu_ropB-F2 | GTACGTGCCCTCGTCAGAG | |
| Mu_rpoB-R2 | CTTCTCGCAGAACAGGCCG | |
| Mu_rpsL-F | CGCAGGCGGGTATTGTGGT | To amplify *rpsL* genes from *M. ulcerans* colonies from relapse mice for checking mutation. |
| Mu_rpsL-R | GGATCGGTGCCGGTGTTGT | |

**Table 3** Original experimental scheme to compare activities of rifampin−streptomycin, TB47 alone and 2-drug regimens containing TB47 in *M. ulcerans*-infected footpads of BALB/c mice.

| Drug regimen (mg/kg) | Contents/number of mice for CFU counts or for RLU detection from live mice[a] or for relapse in brackets at the following time points | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | CFU | RLU-L, CFU | RLU-L | RLU-L, relapse | RLU-L, relapse | RLU-L, relapse | Relapse | Total |
| | Day −11 | Day 0 | Day 2, Day 4 | Week 1 | Week 2 | Week 5 | Week 6 | |
| Uninfected | | 5[a] | 5[a] | 5[a] | 5[a] | 5[a] | | 5 |
| Untreated | 5 | 5[a], 5 | 5[a] | 5[a] | | | | 15 |
| R10/S150 | | 10[b] | 10[b] | 10[b] | 10[b] | 10[b], (15) | (15) | 30 |
| T25 | | 10[b] | 10[b] | 10[b](15) | (15) | | | 30 |
| T25/R10 | | 10[b] | 10[b] | 10[b] (15) | (15) | | | 30 |
| T25/Cl100 | | 10[b] | 10[b] | 10[b] (15) | (15) | | | 30 |
| T25/Cf25 | | 10[b] | 10[b] | 10[b] (15) | (15) | | | 30 |
| Total (170) | 5 | 5, 60[a] | 60[a] | 60[a], (60) | 15[a], (60) | 15[a], (15) | (15) | 170 |

Drugs were given daily, 5 days/week.
Dosage (mg/kg, as indicated). R, rifampin; S, streptomycin; T, TB47; Cl, clarithromycin; Cf, clofazimine.
Mice were infected on Day −12 and treatment was initiated on Day 0.
CFU, colony-forming unit; RLU, relatively light unit; RLU-L, RLUs detected from foot pads of live mice.
[a]The same batch of 5 live mice were detected for the RLU-Ls and then sacrificed.
[b]The same batch of 10 live mice were detected for the RLU-Ls and then included in the relapse evaluation.

**Table 4** Original experimental scheme to compare activities of rifampin−streptomycin and 3-drug regimens containing TB47 in *M. ulcerans*-infected footpads of BALB/c mice.

| Drug regimen (mg/kg) | Contents/number of mice for CFU counts or for RLU detection from live mice[a] or for relapse in brackets at the following time points. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CFU | RLU-L, CFU | RLU-L | Relapse | | | | | Total |
| | Day −13 | Day 0 | Days 2, 4, 7 | Week 2 | Week 3 | Week 4 | Week 6 | Week 8 | |
| Uninfected | | 5[a] | 5[a] | | | | | | 5 |
| Untreated | 5 | 5[a], 5 | 5[a] | | | | | | 15 |
| R10/S150; 5/7 | | 10[b] | 10[a] | | | | (15) | (15) | 30 |
| T50/R20/Cf25; 5/7 | | 10[b] | 10[a] | | (16) | (15) | | | 31 |
| T50/R20/Cf25; 7/7 | | | | (16) | | | | | 16 |
| T50/Cf25/Cl100; 5/7 | | 10[b] | 10[b] | | | (15) | | | 15 |
| T50/R20/Cl100; 5/7 | | 10[b] | 10[b] | | (15) | (15) | | | 30 |
| T100/P20/Cl100; 2/7 | | 10[b] | 10[b] | | (15) | (15) | | | 30 |
| Total (172) | 5 | 60[a], 5 | 55[a] | (16) | (46) | (60) | (15) | (15) | 172 |

*n*/7 means drugs were given *n* days/week.
Dosage (mg/kg, as indicated). R, rifampin; S, streptomycin; T, TB47; Cl, clarithromycin; Cf, clofazimine. P, rifapentine.
CFU, colony-forming units; RLU, relatively light unit; RLU-L, RLUs detected from foot pads of live mice.
Mice were infected on Day −14 and treatment was initiated on Day 0.
[a]The same batch of 5 live mice were detected for the RLU-Ls and then sacrificed.
[b]The same batch of 10 live mice were detected for the RLU-Ls and then included in the relapse evaluation.

Technology (Texas, USA). Streptomycin, clarithromycin and clofazimine were purchased from Tokyo Chemical Industry (Tokyo, Japan).

### 2.7. *Rifampin and streptomycin resistance analysis*

Ten single colonies, randomly selected from different recurrent mice treated with rifampin−streptomycin in the animal experiment testing 3-drug regimens (Table 4) were used for PCR using the newly designed primers in this study, MU_rpoB-F1/MU_rpoB-R1 and MU_ropB-F2/Mu_rpoB-R2 (Table 2; Sangon Biotech), to amplify *rpoB* gene which is associated with rifampin resistance. In addition, to amplify *rpsL* gene which is related to streptomycin resistance, the specific primers, Mu_rpsL_F and Mu_rpsL_R (Table 2; Sangon Biotech), were used in this study. Colonies from untreated control groups were used as negative controls. The PCR products were sequenced by Sangon Biotech. The resulting sequences were compared with that of the infection strains.

### 2.8. *Statistical analysis*

Relative light units (RLUs) and CFU counts were lg transformed before analysis and presented as mean ± standard deviation (SD). Group means were compared by unpaired Student's *t*-test. The significance level was set at $P < 0.05$ ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). Time-to-swelling curves were calculated using the Log-rank (Mantel−Cox) test. The relapse rates were analyzed using Fisher's exact test. All statistical tests were performed with GraphPad Prism 7 software (CA, USA).

**Table 5** Original experimental scheme to test activities of TB47 against the wild-type *M. ulcerans* or TB47-resistant mutants in BALB/c mice.

| Bacterial strain | Drug regimen (mg/kg) | Number of mice to be sacrificed for CFU counts at the following time points | | | |
|---|---|---|---|---|---|
| | | Day −7 | Day 0 | Day 14 | Total |
| $Mu^{wt}$ | Untreated | 5 | 5 | 5 | 15 |
| | R20/Cl100 | | | 5 | 5 |
| | T12.5 | | | 5 | 5 |
| $Mu^{Sm1}$ | Untreated | 5 | 5 | 5 | 15 |
| | R20/Cl100 | | | 5 | 5 |
| | T12.5 | | | 5 | 5 |
| | T25 | | | 5 | 5 |
| | T50 | | | 5 | 5 |
| | T100 | | | 5 | 5 |
| $Mu^{Sm2}$ | Untreated | 5 | 5 | 5 | 15 |
| | R20/Cl100 | | | 5 | 5 |
| | T12.5 | | | 5 | 5 |
| | T25 | | | 5 | 5 |
| | T50 | | | 5 | 5 |
| | T100 | | | 5 | 5 |
| $Mu^{Dm1}$ | Untreated | 5 | 5 | 5 | 15 |
| | R20/Cl100 | | | 5 | 5 |
| | T200 | | | 5 | 5 |
| | R20/T100/Cl100 | | | 5 | 5 |
| $Mu^{Dm10}$ | Untreated | 5 | 5 | 5 | 15 |
| | R20/Cl100 | | | 5 | 5 |
| | T200 | | | 5 | 5 |
| | R20/T100/Cl100 | | | 5 | 5 |
| Total (165) | | 25 | 25 | 115 | 165 |

$Mu^{wt}$: wild type autoluminescent *M. ulcerans* 1059 (AlMu) and other mutants with QcrB mutations were all selected based on this strain; $Mu^{Sm1}$: $QcrB^{T323A}$; $Mu^{Sm2}$: $QcrB^{T323I}$; $Mu^{Dm1}$: $QcrB^{T323A,I183T}$; $Mu^{Dm10}$: $QcrB^{T323I,G325S}$. Sm, single mutation; Dm, double mutations. CFU, colony-forming unit.
The drugs were all given 5 days/week.
Mice were infected on Day −8 and treatment was initiated on Day 0.
Dosage (mg/kg): R, rifampin (20); Cl, clarithromycin (100); T: TB47 (as indicated).

### 2.9. *Data availability*

Sequence data that support the findings of this study have been deposited in GenBank with accession codes QcrBs (*Mycobacterium tuberculosis*: AJF03548.1 [https://www.ncbi.nlm.nih.gov/protein/AJF03548.1]; *Mycobacterium smegmatis*: AFP40620.1 [https://www.ncbi.nlm.nih.gov/protein/AFP40620.1]; *Mycobacterium marinum*: ACC41667.1 [https://www.ncbi.nlm.nih.gov/protein/ACC41667.1]; *M. ulcerans*: ABL05699.1 [https://www.ncbi.nlm.nih.gov/protein/ABL05699.1]; *Mycobacterium abscessus*: SLK50456.1 [https://www.ncbi.nlm.nih.gov/protein/SLK50456.1]; *Mycobacterium leprae*: CAC31260.1 [https://www.ncbi.nlm.nih.gov/protein/CAC31260.1]). The authors declare that all other relevant data supporting the findings of this study are available within the article and its supplementary information files and from the corresponding author upon reasonable request.

## 3. Results

### 3.1. *The therapeutic efficacy of 2-drug regimens containing TB47*

We evaluated the activity of TB47 in a validated mouse model of BU[6,12,14] using AlMu[9,12]. We selected the available proved oral drugs, rifampin, clarithromycin[7] and clofazimine[6] against BU first to test their sterilizing efficacy in combination with TB47. We expected that the long half-life clofazimine could show strong synergistic activity with TB47 as clofazimine had showed very good synergistic activity with TB47 against *M. tuberculosis in vitro*[15] and against *M. abscessus* both *in vitro* and *in vivo* in our recent studies[16]. The mean lgCFU per footpad were 5.89 ± 0.11 at Day 11 (one day after infection) and 6.55 ± 0.11 at Day 0, respectively. Live RLUs of footpads from the positive control group (rifampin−streptomycin) at Day 2 were not different from that of untreated control ($P > 0.05$) and obviously higher than that from the TB47-containing groups ($P < 0.0001$), which meant TB47-containing regimens took effect quickly in only 2 days. Live RLUs of footpads from all the TB47-containing groups were even close to the background reading noise level at Day 4 (Fig. 1A) and much lower than that from the positive control group ($P < 0.0001$). Live RLUs of each mouse from rifampin−streptomycin group after 4 weeks of treatment were even still well above the ground reading noise level with mean lgRLU per footpad (2.29 ± 0.38). It should be noted that adding rifampin, clarithromycin or clofazimine to TB47 showed neither obvious better nor earlier bactericidal effect than TB47 alone in this model ($P > 0.05$).

In comparison to the standard regimen for 5 weeks, TB47 or its 2-drug combinations for 1 week all significantly extended the time-to-relapse ($P < 0.01$, Fig. 1B). In addition, swelling degrades of all mice were identified by observing them at the relapse time

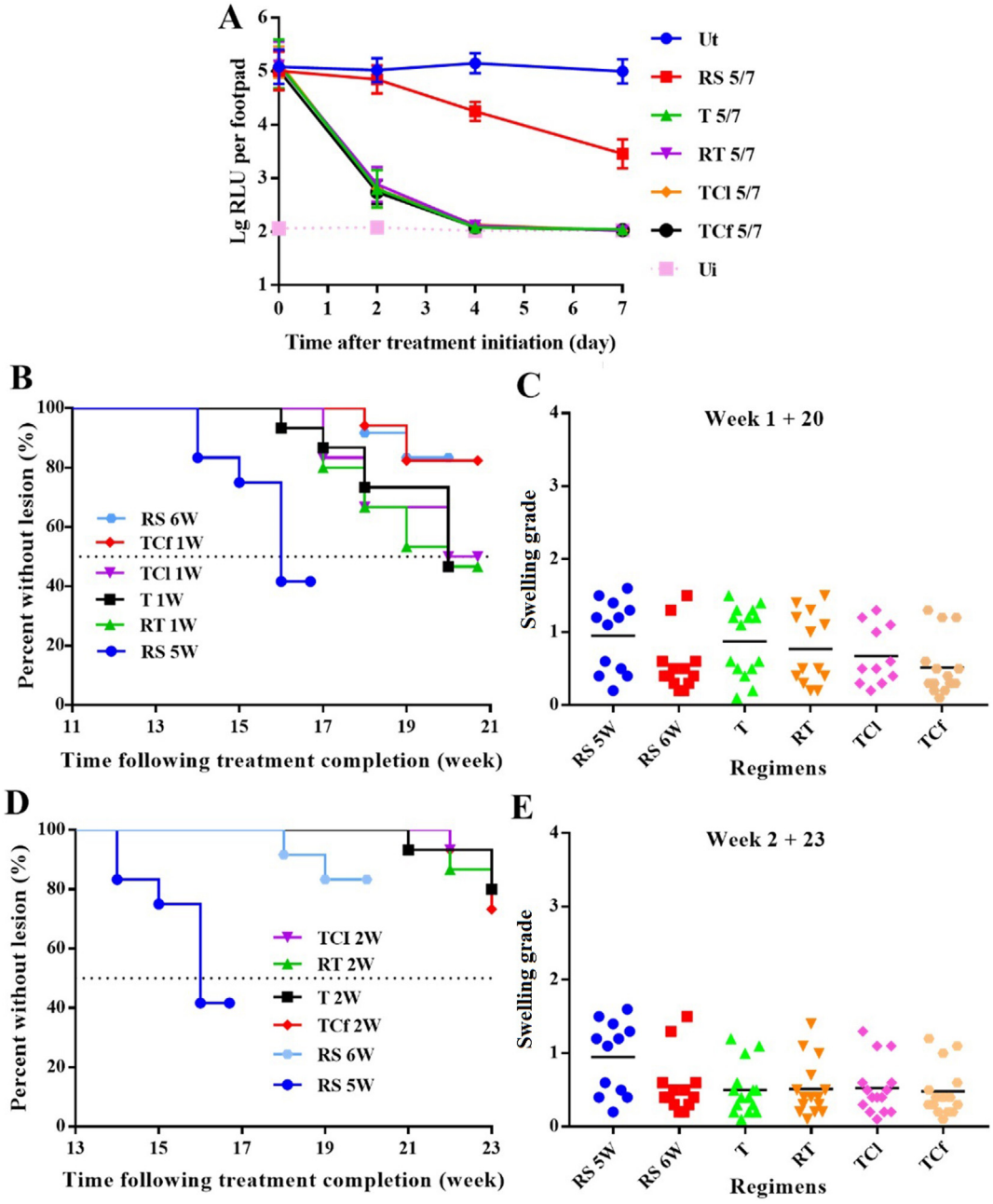


**Figure 1** Activity of TB47 (T) alone or in a 2-drug regimen against BU. (A) Kinetic curves of RLUs from the footpads of the same batch of live mice treated with different regimens. Data are expressed as mean ± SD of five samples from Ui or Ut group and ten samples from each T-containing group. Statistical analysis was performed using unpaired Student's *t*-test. The dotted pink line indicated the base line (the limit of detection). (B) and (D) Time to footpad swelling after completion of antibiotic treatment. Time to footpad swelling in mice treated for 1 week (B) or 2 weeks (D). 13 mice held for relapse in RS 5W and 12 mice held for relapse in RS 6W, 15 mice held for relapse in other groups. (C) Swelling after 1 week of treatment with TB47-containing regimens and 20 weeks without treatment. (E) Swelling after 2 weeks of treatment with TB47-containing regimens and 23 weeks without treatment. (B)−(D) Swelling after 5 and 6 weeks of treatment with RS and 17 and 20 weeks without treatment, respectively. Bars represent median swelling grade. Dosage (mg/kg) given daily for 5 days/week: R, rifampin (10); S, streptomycin (150); T, TB47 (25); Cl, clarithromycin (100); Cf, clofazimine (25). Ut: untreated; Ui: uninfected (for RLU detection, Ui is the base line). W: week.

points when mice had been treated for 1 week and then left without treatment for the next 20 weeks for TB47-containing groups or treated in the positive control groups for 5 or 6 weeks and then left without treatment for the next 17 and 20 weeks, respectively (Fig. 1C), which showed a certain proportion of mice in each group had swollen footpads. Longer duration of treatment of TB47 or TB47-containing regimens further prolonged time-to-relapse and much longer than the standard regimen for 6 weeks ($P < 0.01$, Fig. 1D). However, no TB47-containing regimens could completely cure BU in two weeks with relapse rates 20%,

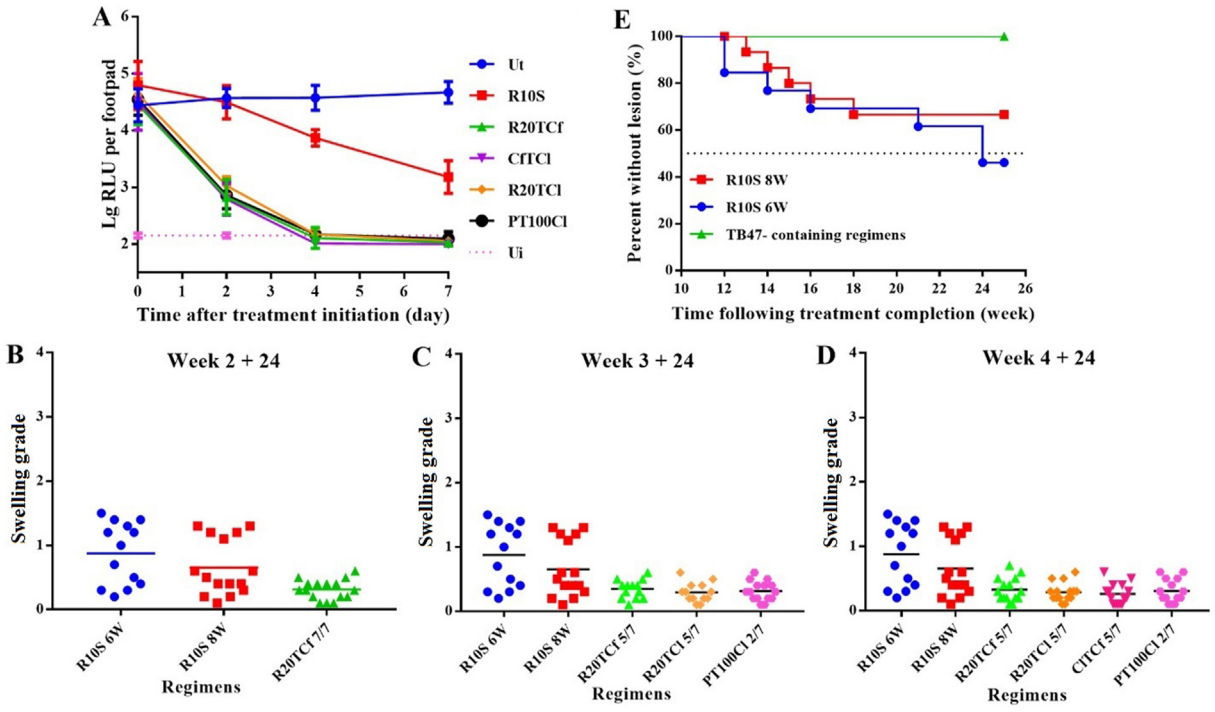


**Figure 2** Activity of 3-drug regimens containing TB47 (T) against BU. (A) RLUs detected from footpads of the same batch of live mice treated. Data are expressed as mean ± SD of ten samples. Statistical analysis was performed using unpaired Student's *t*-test. The dotted pink line indicated the base line (the limit of detection). *n*/7, drugs given *n* days/week. PT100Cl were given at Day 0 and Day 3. (B)−(D) Swelling after treatment and 24 weeks without treatment. Mice were treated with TB47-containing regimens for 2 weeks (B), 3 weeks (C) or 4 weeks (E). Bars represent median swelling grade. (E) Time to footpad swelling of mice from different groups after treatment cessation. Statistical differences were determined by Lg-rank (Mantel−Cox) test in each group (Table 6). Dosage (mg/kg): R, rifampin (as indicated); S, streptomycin (150); T, TB47 (as indicated); P, rifapentine (20); Cl, clarithromycin (100); Cf, clofazimine (25). Un: untreated; Ui: uninfected (for RLU detection, Ui is the base line); W, week. *n*/7: the frequency of administration is *n* days/week.

20%, 20% and 26.67% in groups TB47, TB47-rifampin, TB47-clarithromycin, TB47-clofazimine, respectively, at 23 weeks after treatment completion (Fig. 1E). Swelling degrades of all mice were identified by observing them at the relapse time points when mice had been treated for 2 week and then left without treatment for the next 23 weeks with the same positive control groups as that in Fig. 1C, which showed lower proportion of relapse mice in each group containing TB47 than that treated only for 1 week. It should be noted that adding rifampin, clarithromycin or clofazimine to TB47 showed the same sterilizing activity as TB47 alone ($P > 0.05$) in this model, so TB47 could be the main driver in all the 2-drug regimens.

### *3.2. The therapeutic efficacy of 3-drug regimens containing TB47 and the potential drug resistance analysis*

As TB47 showed low toxicity and dose-dependent bactericidal activity against *M. ulcerans* both *in vitro* and *in vivo* in our previous study[9] and higher doses of TB47 above the potential drug resistance selection window[17] may avoid selection of low-level TB47-resistant spontaneous mutants *in vivo*. TB47 could not get 100% cure at 25 mg/kg even in 2-drug combinations as shown above, we tried to test higher doses of TB47 and in 3-drug combinations, which could have better sterilizing activity and be preferred in prevention of potential drug resistance *in vivo*. In

**Table 6** Results of relapse assessments of 3-drug regimens containing TB47.

| Drug regimen[a] | Percentage (proportion) with positive *M. ulcerans* cultures 6 months after completing treatment for: | | | | |
|---|---|---|---|---|---|
| | 2 weeks | 3 weeks | 4 weeks | 6 weeks | 8 weeks |
| R10/S150 (5/7)[b] | | | | 53.85 (7/13) | 26.67 (4/15) |
| T50/R20/Cf25 (7/7)[b] | 0 (0/16) | | | | |
| T50/R20/Cf25 (5/7)[b] | | 0 (0/16) | 0 (0/15) | | |
| T50/Cf25/Cl100 (5/7)[b] | | | 0 (0/15) | | |
| T50/R20/Cl100 (5/7)[b] | | 0 (0/13) | 0 (0/15) | | |
| T100/P20/Cl100 (2/7)[b] | | 0 (0/15) | 0 (0/14) | | |

[a]R, rifampin; S, streptomycin; Cf, clofazimine; T, TB47; Cl, clarithromycin; P, rifapentine. Dosage: mg/kg as indicated. S, given by subcutaneous injection; other drugs, given by oral administration.

[b]*n*/7 means the frequency of administration is *n* days/week.

addition to the three oral drugs selected above, we included rifapentine this time, a rifamycin similar to rifampin with longer half life[18,19]. We also tried higher rifampin dose because it showed better *in vivo* activity at higher doses which were well tolerated in human beings[5,19]. The mean lgCFU counts per footpad were 6.01 ± 0.37 at Day −14 (one day after infection) and 6.36 ± 0.35 at Day 0, respectively. Similar to the above animal experiment at Day 4, RLUs from footpads of live mice treated with the standard regimen were still very high with 3.87 ± 0.146 lgRLU per footpad while the RLUs from footpads of all TB47-containing groups reached the background reading noise level (Fig. 2A). We noted that all TB47-containing regimens showed almost the same bactericidal effect ($P > 0.05$) demonstrated by the kinetic curves of RLUs from the footpads of the same batch of live mice treated (Fig. 2A).

Furthermore, all mice treated with 3-drug regimens containing TB47 showed no relapse analyzed by using footpad swelling index (Fig. 2B−D) and by microbiological confirmation of the presence of *M. ulcerans* in footpad of each mouse held till 24 weeks after treatment completion (Table 6 and Fig. 2E). While in the positive control group, mice treated with rifampin−streptomycin for 6 weeks started to display swollen footpads (swelling grade ≥1) from 12 weeks after treatment completion and the relapse rate was 53.85% (7/13; Table 6 and Fig. 2E), and those treated with rifampin−streptomycin for 8 weeks started to display swollen footpads from 13 weeks after treatment completion and the relapse rate was 26.67% (4/15; Table 6 and Fig. 2E). The rifampin−streptomycin therapy for 8 weeks was not significantly better than the same therapy for 6 weeks for both time-to-swelling ($P > 0.05$) and relapse rates ($P > 0.05$), which was similar to a previous report in which some mice treated with rifampin−streptomycin for 6 weeks relapsed even later than those treated with rifampin−streptomycin for 8 weeks[6].

The number of colonies in each relapse footpad exceeded $10^5$ CFUs. To ascertain whether the obtained colonies from relapse mice were due to emergency of drug resistance or not, randomly selected 10 colonies from each footpad of 11 relapse mice from both positive control groups were used to amplify the *M. ulcerans rpoB* and *rpsL* genes by PCR. In all cases, the colonies selected showed no mutation in *rpoB* and *rpsL* genes by DNA sequencing. So the results inferred that the relapse mice in the control groups could be due to inadequate treatment, which is similar to the previous report[5], in which the authors sequenced the 400-bp rifampin resistance determining region and found no mutation.

### 3.3. *High-level resistance to TB47 due to double mutations in QcrB*

In the previous study, we obtained nine TB47-resistant spontaneous *M. ulcerans* mutants by screening using only very low concentrations (≤0.02 μg/mL) of TB47 but none using ≥0.05 μg/mL with many repeats and identified single-nucleotide polymorphisms (SNPs) at the same codon in all TB47-resistant isolates ACC → GCC or ATC resulting in Thr323Ala or Thr323Ile substitution in QcrB[9] (Table 1). The spontaneous resistance mutation rates of low-level TB47-resistant *M. ulcerans* mutants against TB47 were $0.83 \times 10^{-9}$ at 0.02 μg/mL[9]. MICs of TB47 were 0.2−0.4 μg/mL to all nine low-level TB47-resistant *M. ulcerans* mutants we obtained previously which all showed single mutation at Thr323 in QcrB. Here we obtained ten new types of high-level TB47-resistant spontaneous mutants using low-level TB47-resistant mutants, $Mu^{Sm1}$ and $Mu^{Sm2}$ (Table 1) we had by screening at higher concentrations of TB47 (≥0.8 μg/mL). All the 10 types of high-level TB47-resistant mutants harbored novel double mutations in their QcrBs (Fig. 3) and MICs of TB47 were >50 μg/mL to all of them (Table 1) while the MICs of TB47 to TB47-sensitive *M. ulcerans* strains were only 0.0016 μg/mL[9]. Among them, it seems that Met352 and Leu185 are mutant hot sites, since we got 6 and 5 mutants, respectively (Table 1).

All the QcrB mutations in *M. ulcerans* occurred at 6 sites indicated by one red dot and five blue dots in Fig. 3, spanning from 158 to 352 in a fragment containing 195 amino acids. Only two amino acid residues at position Thr323 and Gly325 are highly conservative in QcrBs from all the mycobacteria selected here and the Thr323 is the only amino acid mutation site causing resistance

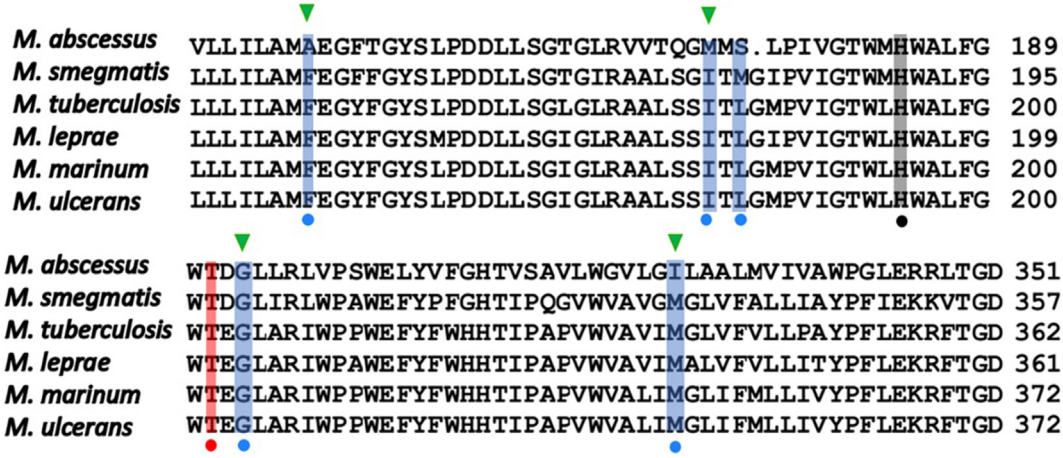


**Figure 3** The amino acid sequences alignment of QcrB fragments. The only one mutation site from the first step of screening using *M. ulcerans* is indicated in the red background and spot. The five mutation sites from the second step of screening are indicated in blue backgrounds and spots. The four green triangles indicate the mutation sites from the second step of screening and amino acid residues at these positions in *M. tuberculosis* may interact with Q203 when Q203 binds to QcrB from a report[31]. The mutation site in the QcrB of TB47-resistant *M. smegmatis* is indicated in the black background and spot[30].

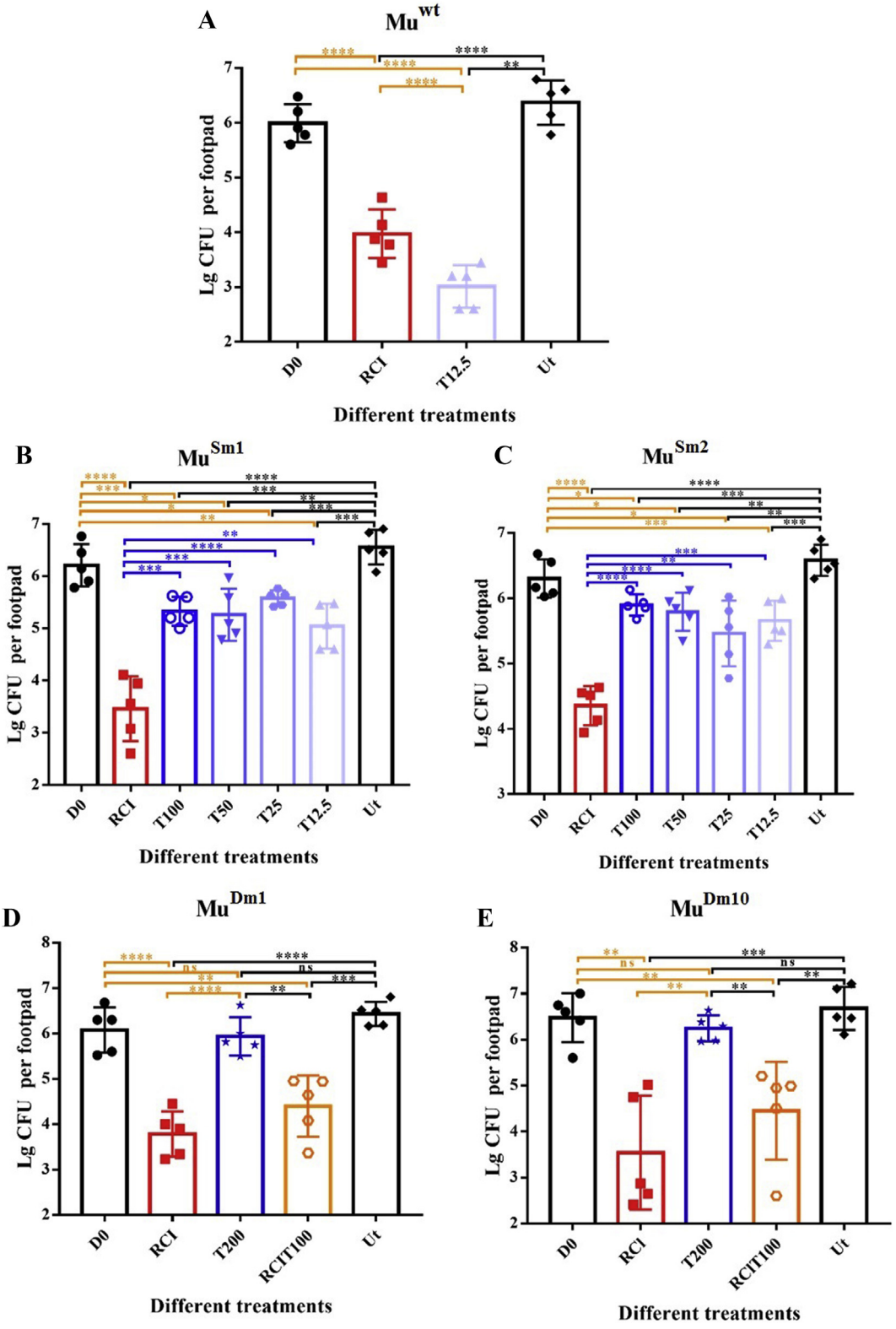


**Figure 4** Activities of TB47 (T) against TB47-sensitive and TB47-resistant *M. ulcerans* strains *in vivo*. CFUs of footpad tissue suspensions from different treatment groups infected with (A) the wild-type autoluminescent *M. ulcerans* 1059 ($Mu^{wt}$), (B) the low-level TB47-resistant strains $Mu^{Sm1}$ and (C) $Mu^{Sm2}$, and the high-level TB47-resistant strains (D) $Mu^{Dm1}$ and (E) $Mu^{Dm10}$. Features of the mutant strains are shown in Table 1. Sm, single mutation; Dm, double mutations. Data are expressed as mean ± SD of five samples. D, day; Ut, untreated. Dosage (mg/kg) given daily 5 days/week: R, rifampin (20); T, TB47 (as indicated); Cl, clarithromycin (100). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

for the first step, indicated by a red dot in Fig. 3. Amino acid residues at the other 4 sites of QcrB from *M. abscessus* are all different from that of the other mycobacteria selected, which may explain why TB47 alone has no detectable activity against it. Based on >6 independent mutant screening attempts using $Mu^{Sm1}$ and $Mu^{Sm2}$, we determined spontaneous resistance mutation rates against TB47 at high level (for the 2nd step only) to be $4.76 \times 10^{-8}$–$7.79 \times 10^{-8}$ for both strains at all the concentrations used.

### 3.4. Activity of TB47 alone and in combination against TB47-resistant mutants

As WHO recommended replacing streptomycin with oral clarithromycin in combination with rifampin recently[20], we used the combination of rifampin (20 mg/kg which was doubled as that in the standard recommendation)−clarithromycin (100 mg/kg) as a positive treatment control for this experiment in the same BU mouse model with different *M. ulcerans* mutants (Table 1) and their TB47-sensitive parent strain $Mu^{wt}$. For mice infected with $Mu^{wt}$, TB47 at 12.5 mg/kg alone was much more effective that rifampin−clarithromycin combination by footpad CFUs ($P < 0.01$, Fig. 4A), even though the dose of rifampin doubled (20 mg/kg) in combination with clarithromycin. For mice infected with $Mu^{Sm1}$ or $Mu^{Sm2}$, footpad CFUs from mice treated with TB47 at different doses all showed anti-BU activity compared with that from the untreated control groups ($P < 0.05$) or that from the Day 0 groups ($P < 0.05$) for $Mu^{Sm1}$ and $Mu^{Sm2}$ experiments, respectively (Fig. 4B and C). In addition, footpad CFUs from mice treated with rifampin−clarithromycin combination were significantly lower than that from mice treated with any dose of TB47 for both $Mu^{Sm1}$ and $Mu^{Sm2}$ experiments (all $P < 0.01$ and some even <0.0001, Fig. 3B and C). This indicate that in case of treating infection with low-level TB47-resistant *M. ulcerans* strains, TB47 showed activity at all doses (12.5−100 mg/kg) used but not as strong as rifampin−clarithromycin combination. In case of $Mu^{Dm1}$ and $Mu^{Dm10}$ strains, footpad CFUs from mice treated with TB47 at very high dose (200 mg/kg) were much higher than that from the corresponding groups treated with regimens containing rifampin 20−clarithromycin 100 combination ($P < 0.01$ for both $Mu^{Dm1}$ and $Mu^{Dm10}$ experiments, respectively, Fig. 4D and E) and showed no difference from that from the corresponding untreated control groups ($P > 0.05$ for both $Mu^{Dm1}$ and $Mu^{Dm10}$ experiments, respectively, Fig. 4D and E). Nevertheless, the bacterial burden of mice treated with TB47−rifampin−clarithromycin combination was not significantly different from that of corresponding mice treated with rifampin−clarithromycin ($P > 0.05$ for both $Mu^{Dm1}$ and $Mu^{Dm10}$ experiments, respectively) and lower than that of corresponding mice in the untreated control groups ($P < 0.001$ and $P < 0.0127$ for $Mu^{Dm1}$ and $Mu^{Dm10}$ experiments, respectively, Fig. 4D and E). This indicates that in case of treating infection with high-level TB47-resistant *M. ulcerans* strains, TB47 at very high dose (200 mg/kg) showed no effect and adding TB47 (100 mg/kg) to rifampin−clarithromycin combination could not improve the effect.

## 4. Discussion

Despite the relative good efficacy of the regimen of daily rifampin−streptomycin or rifampin−clarithromycin for 2 months recommended by WHO for treatment of BU, they have significant drawbacks including daily parenteral administration for streptomycin and potential side effects, such as ototoxicity for streptomycin[6,12], gastrointestinal reactions for clarithromycin[21,22] and the drug−drug interactions of rifampin with many drugs, including drastically reducing clarithromycin exposures[23,24], and affecting metabolism of anti-retroviral agents. In addition, surgery is still needed for serious cases of BU and rifampin-resistant *M. ulcerans* isolates from patients and infected animals have been reported[14,25]. Therefore, new powerful drugs with new mechanisms of action which can form fully oral, less toxic, shorter-course or intermittent treatment regimens are highly desirable and sought after by WHO.

Here, in the first attempt, all TB47-containing 2-drug regimens showed similar bactericidal and sterilizing activities as TB47 alone, and could not obtain 100% cure (without relapse). It has been shown previously that higher doses of rifampin and rifapentine together with clofazimine[5] or clarithromycin[18] were able to reduce treatment time by half in the mouse footpad model of BU. The high doses of rifapentine have been tested for the treatment of tuberculosis in humans and were found well tolerated when administrated weekly[19,20,26,27]. Then we tried to test 3-drug combinations containing TB47 and to i) double the dose of rifampin as it showed better activities and well tolerated[19] ; ii) increase the dose of TB47 as it was dose-dependent and had low toxicity in our previous *in vivo* study[9]; iii) include the long half-life rifapentine at high dose in the intermittent regimen; and iv) treat mice in longer duration. All TB47-containing 3-drug regimens showed 100% cure while 4 out of 15 mice (26.67%, Table 6) relapsed in the positive control group which is consistent with clinical practice that a few BU patients could not be cured by the current antibiotics alone and need surgery. The main driver could be TB47 in both 2-drug and 3-drug combinations. Whether TB47 used alone or combined with less drugs at 50 mg/kg for daily use or at 100 mg/kg for intermittent use could cure BU or not in the same design (Table 4) is a question. Another question is if any of the 3-drug regimens containing TB47 can cure BU in a shorter course. The questions need to be illustrated further in the future studies. However, from the current treatment results (Figs. 1 and 2 and Table 6), we could draw the following conclusions clearly: i) all the 3-drug regimens tested were more powerful than the rifampin−streptomycin combination for 8 weeks ($P < 0.05$, Fig. 2D and Table 6). ii) TB47−rifampin−clofazimine combination given daily could cure BU in ≤2 weeks (Fig. 2E and Table 6) but lower doses of TB47 in 2-drug regimens may be not (Fig. 1D and E). iii) TB47−rifampin combined either with clofazimine or clarithromycin could cure BU in ≤3 weeks. iv) TB47−clofazimine−clarithromycin without rifamycins could cure BU in ≤4 weeks. This can be useful for BU patients infected with rifampin-resistant *M. ulcerans* or co-infected with HIV for avoiding drug−drug interaction.

To the best of our knowledge, this is the first report discovering double mutations in the QcrB of mycobacteria. It is interesting that total eight novel mutations in five novel mutation sites were found indicated by the blue dots in Fig. 3 (Table 1). To date, all TB47-resistant *M. ulcerans* mutants we obtained have mutations in QcrB and all high-level TB47-resistant mutants harbor double mutations, which indicates again that mycobacterial QcrB is the target of TB47. All the mutations of QcrB found in *M. ulcerans* and other mycobacteria spread within the region from 158 to 352 amino acid residues corresponding to a 585-bp DNA fragment of

*qcrB* gene until now, which could be a QcrB inhibitor resistance determining region, similar to the concept of rifampin or quinolones resistance determining regions in *M. tuberculosis*[28], and could possibly be a molecular diagnosis marker for this types of drugs.

Furthermore, we speculated that QcrB could be the only target because all the TB47-resistant *M. ulcerans* mutants had single mutation or double mutations in QcrB and the spontaneous resistant rates were very low for each step of QcrB mutation selection. Q203 has the same mechanisms of action as TB47[29]. We noted that the only reported mutation site, Thr313, in QcrB in Q203-resistant *M. tuberculosis* mutants[29] is the same as the corresponding site Thr323 in *M. ulcerans* found in the first step selection which only caused low-level resistance to TB47. However, the mutation site His190 found in QcrB from TB47-resistant *M. smegmatis* mutants reported by us[30] indicated by black dot in Fig. 3 is different from all the corresponding mutation sites found in TB47-resistant *M. ulcerans* mutants. Hence all these infer that the amino acid residues of QcrB from *M. smegmatis* interacting with TB47 or Q203 could be different from that from *M. ulcerans* and *M. tuberculosis*. Though the differences in Cyt-bds, components of the complementary pathway of the respiratory cytochrome bc1:aa3, from different mycobacteria potentially explain their differential susceptibilities to TB47[9], the differences in QcrBs from them can also play an important role for their susceptibilities. So this may explain well that the MIC of TB47 to *cydA* and *cydB* genes-deleted *M. smegmatis* was still much higher than that to *M. ulcerans* or *M. tuberculosis*[9]. Atomic structures of bd oxidases of *M. ulcerans* or *M. tuberculosis* will be more informative than that of *M. smegmatis* according to results obtained here. Though none of atomic structures of mycobacterial bd oxidases has been reported yet, an interesting study built the putative 3D structures of wild-type and T313A mutant *M. tuberculosis* QcrBs using the X-ray structures of other species' QcrBs as templates and used them for analyzing Q203-binding mode[31]. Four out of five novel QcrB mutation sites in *M. ulcerans* found in this study indicated by green triangles in Fig. 3 were predicted in the previous putative *M. tuberculosis* QcrB models[31], though TB47 adopts a different conformation[31] (cf. the Q203 model in the report[31]). So the results from this study can be considered as experimental validations of these models and will be informative to build and improve the putative 3D structures of different QcrBs from mycobacteria and could possibly be useful for designing new generations of drugs targeting QcrBs.

In the previous pharmacokinetic study of TB47, the maximum blood concentration ($C_{max}$) of TB47 in mice could reach 0.63 ± 0.28 μg/mL and its $t_{1/2}$ was 35.6 ± 2.7 h after oral administration of 10 mg/kg TB47. In addition, the concentrations in mouse foot tissue within 48 h were >4 times higher than that in the plasma[9]. For *M. ulcerans* mutants with a single mutation in QcrB, MICs of TB47 were 0.2−0.4 μg/mL which is lower than $C_{max}$, while for *M. ulcerans* mutants with double mutations in QcrB, MICs were >50 μg/mL. TB47 at from 12.5 to 100 mg/kg showed mainly bacteriostatic activities against the two low-level TB47-resistant *M. ulcerans* mutants bearing single mutation in QcrB (Fig. 3). TB47 showed no anti-BU activity in mice infected with the two high-level TB47-resistant *M. ulcerans* mutants bearing double mutations in QcrB (Fig. 3) neither at 100 mg/kg in combination with rifampin−clarithromycin nor even at 200 mg/kg for single use. So higher doses of TB47 may be useful for prevent the emergence of low-level TB47-resistance and further the high-level TB47-resistance *in vivo*, especially in combination with other drugs.

## 5. Conclusions

The treatment duration of BU could possibly be shortened from current 8 weeks using WHO recommended antibiotic regimens with surgery as the adjuvant treatment for serious cases to ≤2 weeks given daily or ≤3 weeks given twice per week (6 doses in total) using regimens containing high-dose TB47 as the cornerstone. The latter might cure serious BU completely with less surgery. These regimens should be further evaluated in clinical trials in hopes of preventing drug resistance, relieving BU patients from long-term treatment and side effects, reducing the costs and improving adherence. At the same time, novel mutations of *qcrB* gene could potentially be used not only for BU but also for tuberculosis or leprosy[9] to guide the clinical rapid molecular diagnosis of resistance to prevent abuse QcrB inhibitor drugs, and the discovery of new drugs with the same mechanism of action or that could overcome drug resistance.

## Acknowledgments

We thank Professor Eric L. Nuermberger at Johns Hopkins University (USA) for providing us *M. ulcerans* strains as kind gifts for our study. We thank Dr. Xiantao Zhang, the board chairman of Guangzhou Eggbio Co. Ltd. for providing us with TB47 for the experiments. We thank the UCAS Postdoctoral Fellowship (to Hameed HMA) and CAS-TWAS President's Ph.D. Fellowship Program (to Islam MM) for international students. Tianyu Zhang received Science and Technology Innovation Leader of Guangdong Province (2016TX03R095, China). This work was supported by the National Mega-Project of China for Innovative Drugs (2019ZX09721001-003-003), the Chinese Academy of Sciences grant (154144KYSB20190005, China), the Key-Area Research and Development Program of Guangdong Province (2019B110233003, China), the Special Funds for Economic Development of Marine Economy of Guangdong Province (GDME-2018C003, China) and partially by the Grants (SKLRD-OP-201919 and SKLRD-Z-202016) from the State Key Laboratory of Respiratory Disease, Guangzhou Institute of Respiratory Diseases, First Affiliated Hospital of Guangzhou Medical University, Guangzhou, China.

## Author contributions

Yamin Gao, Yang Liu, and Tianyu Zhang conceived and designed this study; Yamin Gao, Yang Liu, and Zhiyong Liu performed the experiment; Yamin Gao, H.M. Adnan Hameed, Cuiting Fang, Shuai Wang and Tianyu Zhang discussed the issues and initially drafted the manuscript; Yamin Gao, H.M. Adnan Hameed and Tianyu Zhang wrote the manuscript. Yamin Gao, H.M. Adnan Hameed, Lingmin Guo, Zhili Lu assisted in evaluation of the article; Yamin Gao, Yang Liu, H.M. Adnan Hameed, Md Mahmudul Islam, Xirong Tian and Tianyu Zhang provided technical support. Yamin Gao, H.M. Adnan Hameed and Tianyu Zhang

critically assessed and guided up to final version. All the authors contributed to finalize the manuscript writing as well as analyzing the data and approved the final version.

## Conflicts of interest

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. 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.

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