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RESEARCH

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Deciphering the possible role of MmpL7 efflux pump in SQ109 resistance in *Mycobacterium tuberculosis*

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Abstract

Background SQ109 is a promising candidate drug for the treatment of patients with drug-resistant tuberculosis (DR-TB). The purpose of this study was to investigate the activity of SQ109 against clinical isolates of *Mycobacterium tuberculosis* (MTB) from patients with multidrug-resistant TB (MDR-TB) and pre-extensively drug-resistant TB (pre-XDR-TB), and to explore new drug-resistant mechanisms of SQ109.

Methods We evaluated the in vitro activity of SQ109 against clinical isolates from patients with MDR-TB and pre-XDR-TB using minimal inhibitory concentration (MIC) assay. The drug-resistant gene, *mmpL3* of SQ109-resistant strains was sequenced, and a quantitative real-time PCR assay was used to analyze 28 efflux pump genes in SQ109-resistant strains without *mmpL3* mutations. The role of candidate efflux pumps *mmpL5* and *mmpL7* on the MIC of SQ109 was evaluated using recombinantly cloned MmpL5 and MmpL7 expressed in *Mycobacterium smegmatis*.

Results The MIC_{90}, MIC_{95} and MIC_{99} values of SQ109 for 225 clinical isolates of MTB were 0.25 mg/L, 0.5 mg/L and 1.0 mg/L, respectively. Among the pre-XDR strains, six showed resistance to SQ109 despite the absence of gene mutations in *mmpL3*. In six resistant pre-XDR strains, the MIC of SQ109 decreased with the use of an efflux pump inhibitor, and there was significant upregulation of *mmpL5* and *mmpL7* in two strains after exposure to SQ109. The presence of MmpL7 in *Mycobacterium smegmatis* resulted in decreased susceptibility to SQ109, with the MIC increasing from 16 mg/L to 32 mg/L.

Conclusions Our data demonstrated that SQ109 exhibited excellent levels of in vitro activity against MTB. MmpL7 may be a potential gene for MTB resistance to SQ109, providing a useful target for detecting SQ109 resistance in MTB.

Keywords *Mycobacterium tuberculosis*, SQ109, Susceptibility, MmpL7

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Introduction

Tuberculosis (TB) remains a major cause of morbidity and mortality worldwide. The World Health Organization estimated that 10.6 million people contracted the disease and 1.3 million died from it in 2022 [1]. Although the incidence of TB is diminishing due to the implementations of global TB control strategy over the past decades, the emergence of multidrug-resistant TB (MDR-TB, defined as resistant at least to rifampin and isoniazid), especially pre-extensively drug-resistant TB (pre-XDR-TB, defined as MDR plus resistance to at least one fluoroquinolone and one second-line injectable drug) and extensively drug-resistant TB (XDR-TB, defined as MDR plus resistance to any fluoroquinolone and at least one drug from Group A) [2], is posing a major threat to the achievements of worldwide efforts in fighting TB. Due to the acquisition of drug resistance to the two most potent anti-TB drugs, only half of MDR/XDR-TB patients had a successful treatment outcome [3]. The unsatisfactory response to anti-TB regimens undoubtedly increases the risk for transmission of MDR/pre-XDR/XDR-TB within the community. Therefore, there is an urgent need to improve treatment of these particular forms of tuberculosis by introducing new drugs and regimens.

During the past few years, several promising new drugs have been developed to combat multidrug-resistant bacteria, as well as to shorten the duration of chemotherapy [4]. Examples of new drugs and regimens that have shown potential in treating MDR-TB include bedaquiline, delamanid, pretomanid, and the use of shorter treatment regimens such as the 9–12 months Bangladesh regimen [5]. Of these new antimicrobial agents, SQ109, an ethambutol (EMB) analog with a 1,2-diamine pharmacophore, was shown to be superior to EMB in MTB. The major mode of action for SQ109 is to inhibit the assembly of mycolic acid into the MTB cell wall by binding to mycolic acid transporter MmpL3 [6]. Previous experimental data demonstrated that this new agent has excellent in vitro and in vivo activity against both drug-susceptible and drug-resistant MTB isolates. In addition, SQ109 shows synergistic activity with rifampin, isoniazid and bedaquiline, which highlights its further application as an important component of new anti-TB regimens [7, 8]. Despite limited evidence from clinical trials, SQ109 has been considered as one of the most potent agents against MDR-TB. The use of this drug in treatment of MDR-TB relies on the accurate in vitro drug susceptibility testing (DST) results, whereas the breakpoint for the use of SQ109 has not been established due to a lack of in vitro DST results.

To address this concern, the main goal of our study was to establish a proposed epidemiological cut-off (ECOFF) value to determine in vitro susceptibility of MTB against SQ109. In addition, we firstly identified the MmpL7 as an efflux pump conferring SQ109 resistance on the basis of our proposed ECOFF value.

Materials and methods

Bacterial strains

A total of 225 *Mycobacterium tuberculosis* strains, including 110 MDR-TB and 115 pre-XDR strains, used in this study were collected from Beijing Chest Hospital, Capital Medical University, between January 2016 and December 2018. Each isolate was obtained from an individual patient and identified at the species level through multilocus sequence analysis, as previously described [9].

Minimal inhibitory concentration determinations

The MICs of MTB isolates against SQ109 were determined using the 2-fold broth dilution method as previously reported [10]. Briefly, the turbidity of 4-week MTB cultures on L-J medium was adjusted 1.0 using a McFarland standard. Followed by 1:20 dilution with sterile Middlebrook 7H9 broth containing 10% oleic acid, albumin, dextrose, and catalase (OADC), a total volume of 100 µL of this inoculum was added into the wells of a 96-well plate containing serial two-fold dilutions of SQ109 (16–0.016 mg/L) in 100 µl of 7H9 Middlebrook medium. After incubation at 37 °C for 7 days, 70 µL of alamarBlue solution was added and assessment of color development was performed whereby a change from blue to pink indicated bacterial growth. The MIC of each strain against SQ109 was defined as the minimal drug concentration at which no growth was observed. Additionally, MIC90, MIC95 and MIC99 were designated as the minimum concentration of the drugs that inhibited growth by 90%, 95% or 99% of the isolates tested, respectively. For quality control, a control MTB strain H37Rv (ATCC 27249) was included with every MIC experiment. Each isolate was tested in triplicate to assess reproducibility. The synthesis and purification of pure SQ109 powder were carried out by HanXiang Biotech Co., Ltd (Shanghai, China). The ECOFFs were determined on the basis of the distribution profile of MIC values. For unimodal MIC distributions, ECOFFs were defined as concentrations representing ≥ 99.9% of the bacterial population; For bimodal MIC distributions, ECOFFs were set between the two populations [9]. For the drug sensitivity test involving inhibitor treatment, three efflux pump inhibitors, namely verapamil (VP), carbonyl cyanide m-chlorophenylhydrazone (CCCP), and reserpine (RP), were selected. Each inhibitor was tested at three different concentrations: VP at 40 mg/L, 80 mg/L, and 160 mg/L, CCCP at 0.5 mg/L, 2 mg/L, and 8 mg/L, and RP at 6 mg/L, 12 mg/L, and 24 mg/L. In vitro MIC tests were conducted on six pre-XDR isolates with no mutations in the *mmpL3* gene. The drug sensitivity test of *Mycobacterium smegmatis*, which carries the pMV261-MmpL5 and

pMV261-MmpL7 plasmids, was conducted for SQ109 as previously reported [11]. All the experiments were performed in triplicate.

DNA amplification and sequencing

The crude genomic DNA was extracted from freshly culture mycobacteria as previously reported [12]. The entire fragment of *mmpL3* gene conferring SQ109 resistance was analyzed by Sanger sequencing. The DNA fragments were amplified with primers listed in Table S1. The 50 μL PCR mixture was prepared as follows: 25 μL 2×PCR Mixture (CWBio, Beijing, China), 5 μL of DNA template, and 0.2 μM of each primer pair. PCR cycling conditions consisted of preincubation at 94 °C for 5 min, and then 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min followed by a final cycle of 72 °C for 10 min. PCR product was purified using a Mag-Bind PCR Purification Kit (CWBio, Beijing, China) and amplicons were sent to Qingke Company (Beijing, China) for DNA sequencing. DNA sequences were aligned with the homologous sequences of the reference MTB strain H37Rv (ATCC 27249) using BioEdit Sequence Alignment Editor Version 7.1.3 (www.mbio.ncsu.edu/bioedit.bioedit.html).

Quantitative real-time PCR assay

The mRNA levels of the efflux pump gene in the isolates were measured using a qPCR assay. The isolates in the logarithmic phase were first placed at -80 °C for 10 min, followed by thawing in boiling water and subjecting to 240 W ultrasound treatment for 3 cycles, each lasting 3 min with intervals of 15 s, and subsequently underwent RNA extraction. The total RNA was extracted using the Trizol method following the instructions of the manufacturers [13]. After DNaseI treatment (TaKaRa, Dalian, China), cDNAs were synthesized in reverse transcription from 5 μg of total RNA using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Quantitative real-time PCR (qPCR) was performed in triplicates for each sample using TB Green® Premix Ex Taq™ II (TaKaRa, Dalian, China) on the Real-Time PCR System (Bio-Rad). The Real-Time PCR System was programmed as follows: 94 °C for 2 min to denature template; 94 °C for 45 s for denaturation; at 60 °C for 45 s for annealing; and 68 °C for 1 min for extension. Primers for transcriptional level analysis were listed in Table S1 and 16 S rRNA was used as the internal control of respective qPCR assay. The fold change values in the qPCR experiments were calculated using the $2^{(-\text{Delta Delta C(T)})}$ method [14] and were further used to create a heatmap with log2 fold changes (log2 FC), with Table S2 reporting the values from the heatmap.

Construction of Msm/pMV261-MmpL5 and Msm/pMV261-MmpL7

The DNA of H37Rv and the empty plasmid pMV261 were sent to Beijing TSINGKE biotechnology co., Ltb for plasmid construction and attachment of a 3*Flag sequence at the C terminal. In brief, the *mmpL5* and *mmpL7* genes were separately cloned into the empty plasmid pMV261 through homologous recombination and then transformed into E. coli DH5α competent cells. Confirmation was done by sequencing. The constructed plasmid was introduced into *Mycobacterium smegmatis* mc^2 155 through electroporation [11], and the presence of the vector was confirmed by PCR and western blot. The empty plasmid pMV261 was also electroporated into *Mycobacterium smegmatis* mc^2 155, but without the Flag sequence.

PCR and western blot analysis

The bacterial cultures containing *Mycobacterium smegmatis* with the pMV261-MmpL5 and pMV261-MmpL7 plasmids and empty plasmid pMV261 were centrifuged at 12,000 rpm for 2 min. The resulting pellet was then resuspended in 200 μL of sterile water and heated in a 100℃ water bath for 10 min. After centrifugation, the supernatant was collected as the template for PCR amplification. For the PCR system, A solution of 25 μL PCR reaction mixture was prepared containing 12.5 μL 2×Phanta Flash Master Mix, 5 μL of DNA template, 1 μL of each primer set and 5.5 μL ddH_2O. Amplification was conducted with an initial denaturation at 98 °C for 30 s, followed by 35 cycles of amplification (denaturation at 98 °C for 10 s, annealing at 70 °C for 5 s and extension at 72 °C for 15 s, with a final extension at 72 °C for 1 min). The PCR products were subjected to gel electrophoresis.

For western blot analysis, the protein expression of MmpL5 and MmpL7 in Msm/pMV261-MmpL5 and Msm/pMV261-MmpL7 was verified by western blotting as previously reported [11]. Msm/pMV261-MmpL5 and Msm/pMV261-MmpL7 were centrifugated at 12,000 rpm for 2 min and the pellet was resuspended in 500 μl 1×PBS buffer and 5 μl MCE protease inhibitor "Cocktail". After blending, ultrasound was performed for 2 min with low frequency, and then the cells lysates were centrifuged at 12,000 rpm for 2 min to collect the supernatant. Add 1× protein loading buffer and boil at 95 °C for 10 min. Proteins were separated by 8% SDS-PAGE at 80 V for 2 h and transferred to a PVDF membrane (Millipore). The blots were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 2 h at room temperature and then incubated for 2 h at room temperature with anti-DDDDK-tag mAb-HRP-DirecT antibody (#M185-7, MBL, 1:5000) in TBS with Tween-20 (TBST) and 5% (w/v) bovine serum albumin. Following three washes of 10 min each with TBST, the blots were developed with Immobilon Western

Chemiluminescent HRP Substrate (WBKLS0500, Millipore) and detected using an enhanced chemiluminescence detection system (iBright CL1500 imaging system; Thermo Fisher Scientific, Inc.).

Result

In vitro susceptibility of SQ109 against mycobacterial isolates

MIC distributions for SQ109 are shown in Fig. 1. Overall, the MIC_{90}, MIC_{95}, MIC_{99} values of SQ109 for MTB isolates were 0.25 mg/L, 0.5 mg/L and 1.0 mg/L, respectively. Against MDR isolates, the MIC99 of SQ109 (MIC_{99}, 1.0 mg/L) was lower than that against pre-XDR isolates (MIC_{99}, 2.0 mg/L). Based on the distribution of SQ109 minimum inhibitory concentrations (MICs) among MTB isolates, we suggest an epidemiological cutoff (ECOFF) value of 0.5 mg/L for identifying SQ109-resistant tubercle bacilli. A strain with MIC≤0.500 mg/L was considered sensitive, while a strain with MIC>0.500 mg/L was classified as drug-resistant. Therefore, all 110 (100%) MDR strains and 109 (94.8%) pre-XDR strains were found to be sensitive to SQ109. Among the pre-XDR strains, six were resistant to SQ109, with MIC values of 1.000 mg/L in four strains, 2.000 mg/L in one strain, and 4.000 mg/L in one strain.

We further analyzed the *mmpL3* gene, target gene of SQ109 in six resistant pre-XDR strains for SQ109. The sequencing results revealed that the *mmpL3* gene sequence of the 6 strains was identical to that of the MTB standard strain H37Rv, with no gene mutations detected, suggesting that the SQ109 resistance was not caused by mutations in the target gene.

In vitro susceptibility of SQ109 against six resistant pre-XDR strains in combined with efflux pump inhibitors

To investigate the impact of efflux pumps on SQ109 resistance, multiple efflux pump inhibitors were employed to determine whether efflux pump mechanism confers the elevated SQ109 MICs of clinical MTB isolates. The decrease in MIC of SQ109 were observed in six resistant pre-XDR strains with efflux pump inhibitor treatment. As depicted in Table 1, the MIC values of four strains were significantly decreased when treated with VP and RP, suggesting that these strains may exhibit resistance to SQ109 due to increased efflux pump activity. Conversely, the MIC values of the other two isolates remained unchanged in the presence of efflux pump inhibitors, indicating that alternative mechanisms contributed SQ109 resistance rather than efflux pump in these isolates.

Identification of candidate efflux pump genes conferring SQ109 resistance

The expression levels of 28 efflux pump genes were examined in four pre-XDR strains exhibiting decreased SQ109 MICs after exposure to efflux pump inhibitors. As illustrated in Fig. 2, the expression levels of the mmpL5 and mmpL7 genes in two strains (Strain 2 and Strain 3) were significantly upregulated. After 24 h of SQ109 exposure, the expression of mmpL5 and mmpL7 in Strain 2

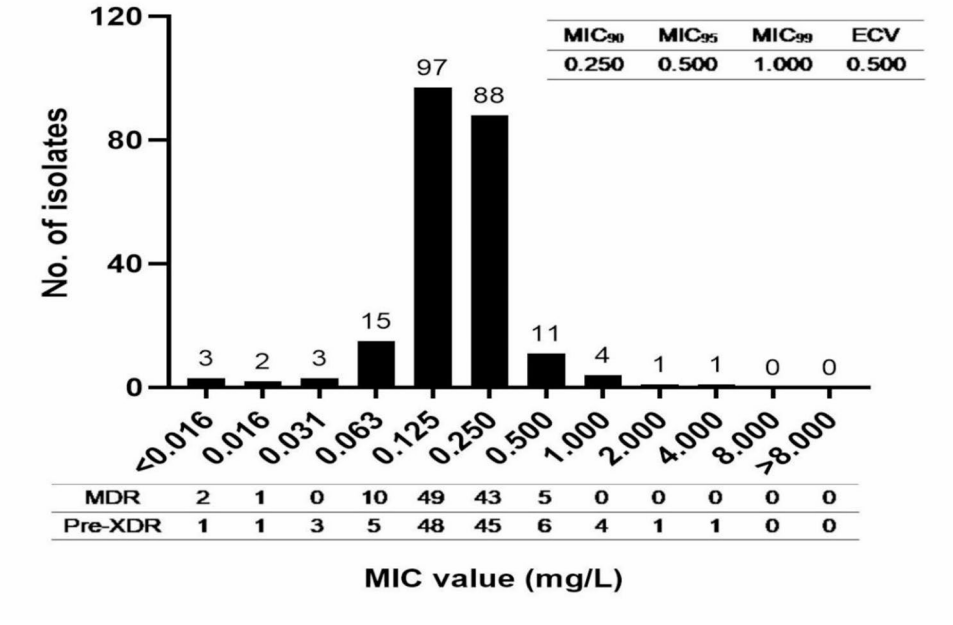


Fig. 1 MIC distributions for MDR and pre-XDR species against SQ109. *Abbreviations*: ECV, epidemiological cutoff value; MIC, minimal inhibitory concentration

Table 1 MIC values of SQ109 resistant clinical strains treated with different efflux pump inhibitors

Strains	SQ109	SQ109 +VP40	SQ109 +VP80	SQ109 +VP160	SQ109 +RP6	SQ109 +RP12	SQ109 +RP24	SQ109 +CCCP0.5	SQ109 +CCCP2	SQ109 +CCCP8
1	1.000	≤ 0.016	≤ 0.016	≤ 0.016	0.125	0.125	0.125	0.500	0.500	0.500
2	1.000	≤ 0.016	≤ 0.016	≤ 0.016	0.125	≤ 0.016	≤ 0.016	0.250	0.250	0.031
3	1.000	≤ 0.016	≤ 0.016	≤ 0.016	0.063	≤ 0.016	≤ 0.016	0.250	0.250	0.063
4	1.000	0.063	≤ 0.016	≤ 0.016	0.250	0.125	0.125	1.000	1.000	1.000
5	2.000	2.000	4.000	2.000	4.000	4.000	2.000	2.000	2.000	2.000
6	4.000	4.000	4.000	4.000	8.000	4.000	4.000	4.000	4.000	4.000

Note Concentration unit of MIC value: mg/L; MIC, minimal inhibitory concentration

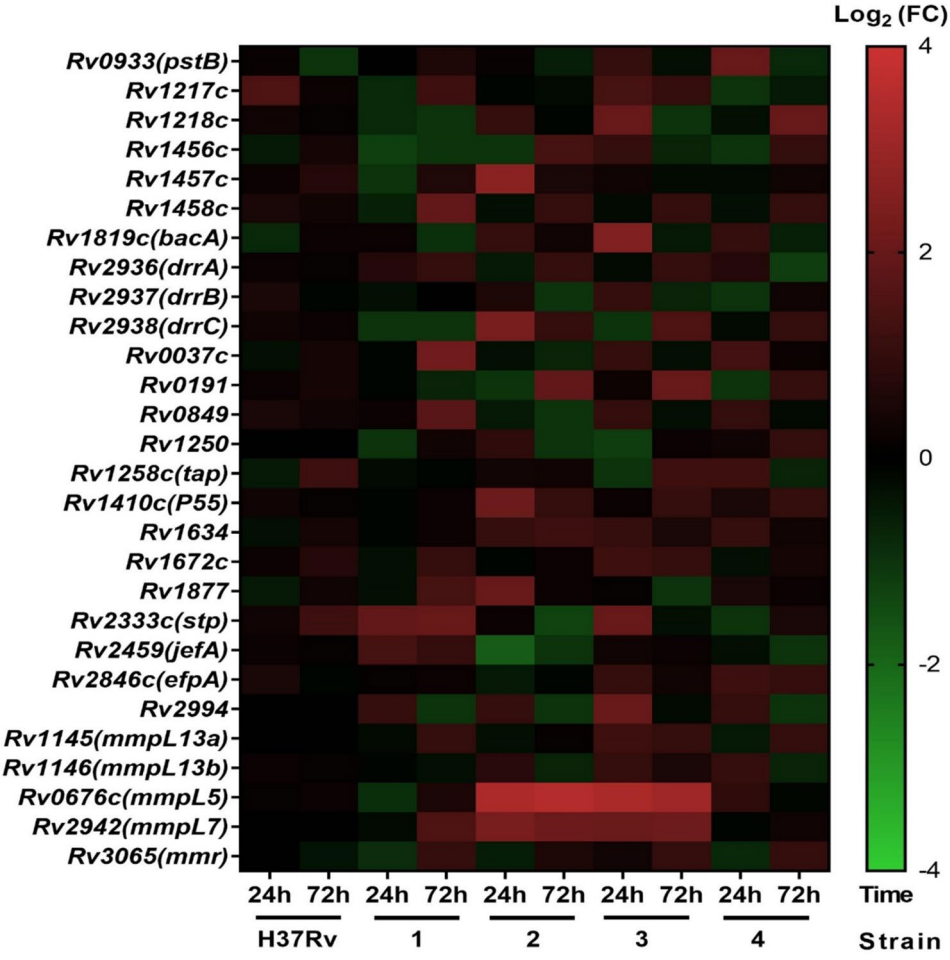
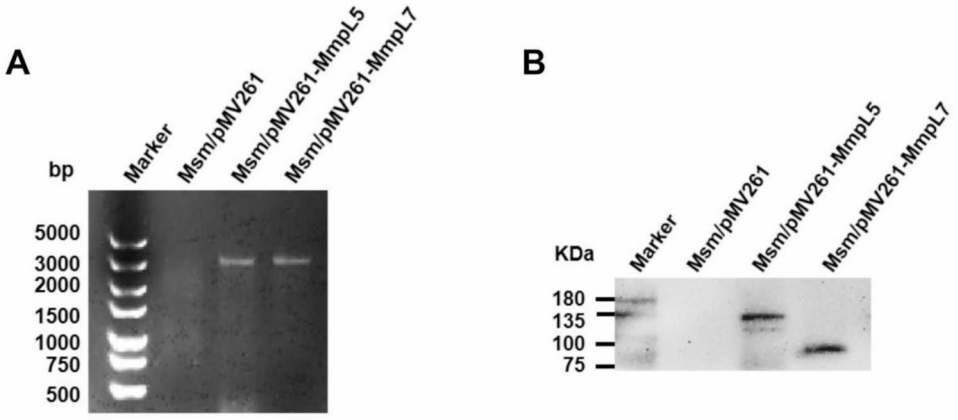


Fig. 2 Increased expression of *mmpL5* mRNA and *mmpL7* mRNA in SQ109 resistant clinical strains (strain 2 and strain 3) treated with SQ109 for 24 h and 72 h

increased by 3.36 and 2.36, respectively, while in Strain 3, the expression of mmpL5 and mmpL7 increased by 3.31 and 2.03, respectively. Following 72 h of SQ109 exposure, the expression of mmpL5 and mmpL7 in Strain 2 increased by 3.50 and 2.10, respectively, and in Strain 3, the expression of mmpL5 and mmpL7 increased by 3.11 and 2.11, respectively. The $\log_2$ FC were all greater than 2. This suggests that MmpL5 and MmpL7 may be associated with SQ109 resistance through the efflux of antimicrobial agents from tubercle bacillus cells. In addition, the $\log_2$ FC of the 28 efflux pump genes did not exceed 2 at both 24 and 72 h in the other two strains.

Decreased SQ109 susceptibility of mycobacterium smegmatis via overexpression of MmpL7

We constructed the recombinant plasmids by cloning the MTB mmpL5 or mmpL7 genes into the pMV261 vector, and confirmed their presence in *Mycobacterium smegmatis* by PCR (Fig. 3A) and expression by Western blot assays (Fig. 3B). Next, the antibacterial effectiveness



C

Species	MIC (mg/L)
Msm/pMV261	16
Msm/pMV261-MmpL5	16
Msm/pMV261-MmpL7	32

Fig. 3 Increased MIC value in the expression of MmpL7 in Msm strain against SQ109. **A**: Verification of the *mmpL5* and *mmpL7* expression Msm strain by PCR amplification; **B**: Verification of the MmpL5 and MmpL7 expression Msm strain by Western blotting; **C**: MIC value in MmpL5 and MmpL7 expression Msm strain against SQ109. *Abbreviations*: Msm, *Mycobacterium smegmatis*

of SQ109 against *Mycobacterium smegmatis* strains expressing recombinant MmpL5 or MmpL7 proteins from MTB was assessed and presented in Fig. 3C. The results showed that the presence of MmpL7 led to a decrease in SQ109 susceptibility, with the MIC increasing from 16 mg/L to 32 mg/L. However, the overexpression of MmpL5 did not alter the susceptibility of *Mycobacterium smegmatis* to SQ109.

Discussion

SQ109 is a new diamine antitubercular drug candidate that interferes with cell wall synthesis in MTB [15–17]. Our study demonstrated that all 110 (100%) MDR strains and 109 (94.8%) pre-XDR strains were found to be susceptible to SQ109, which was in agreement with previous studies [17, 18] that demonstrating that against H37Rv, MDR and XDR-TB, the MIC distribution of SQ109 ranges from 0.2 to 0.78 μg/ml. In vivo study, SQ109 also showed comparable potency and efficacy with INH in inhibiting the growth of *M. tuberculosis*, but superior to EMB [19]. In clinical trials conducted in Russia, SQ109 has shown promise in a Phase 2b-3 clinical study as an anti-TB agent [20]. The sputum negative conversion by the end of the 6th month of the intensive phase of chemotherapy in pulmonary MDR-TB patients receiving SQ109 was observed confidently more often versus treatment regimens containing only existing anti-tuberculosis drugs: 61.0% versus 42.9%. Also, the use of SQ109 and basic chemotherapy in the treatment of MDR-TB did not lead to higher incidence of adverse events [20]. In addition, multiple literatures reported that SQ109 exhibited synergistic effect in combination with other drugs such as INH, RIF and BDQ have been reported to be was observed [7, 8], highlighting its promising perspectives in combination therapy.

MmpL3, a member of the efflux pump RND protein superfamily, is the most probable cellular target of SQ109 [21]. In this study, all the SQ109-resistant MTB isolates had no mutations within this locus. We found that two strains exhibited significant upregulation of the *mmpL5* and *mmpL7* after exposure to SQ109. Numerous studies have shown that the overexpression of MmpL5 was associated with drug resistance to bedaquiline and clofazimine [22]. Here, in our findings suggested that MmpL5 did not confer SQ109 resistance, and the upregulation of MmpL5 may be a stress signature induced by initial drug exposure.

MmpL7, a protein of 920 amino acids with a predicted molecular mass of 95.1 kDa, is a RND family transporter [23]. Previous study demonstrated that the mmpL7 gene

from MTB confers a high level of resistance to isoniazid (INH) when overexpressed in *Mycobacterium smegmatis* [23]. The MmpL7 has been shown to be involved in the transport of the surface-exposed lipid phthiocerol dimycocerosate (PDIM) across the cell membrane [24]. Our study found that an overexpression of the mmpL7 gene in M. tuberculosis could be responsible for SQ109 resistance in those clinical isolates for which no mutation in the known gene targets has been identified.

This study has some limitations. First, this study did not obtain exact MIC, and we will further explore precise MIC changes in subsequent research to clarify the impact of efflux pump inhibitors on the bactericidal activity of SQ109. Second, we only expressed the constructed plasmid in *Mycobacterium smegmatis* and did not validate it in MTB. Next, we will analyze the structure and function of mmpL7, clarify the action sites and transmission pathways of mmpL7 in MTB. Third, although we identified the potential role of mmpL7 in SQ109 resistance in MTB, the exact mechanism conferring the upregulation of mmpL7 after exposure to SQ109 remained unclear. Fourth, the resistance mechanisms of the other four drug-resistant strains are unclear and further research is needed. Nevertheless, this study found for the first time that mmpL7 is upregulated in SQ109 resistant bacterial strains.

In conclusion, our data demonstrated SQ109 exhibited excellent levels of in vitro activity against MTB. In addition, mmpL7 upregulated after exposure to SQ109. Our work highlights that mmpL7 might be a target of SQ109 resistance, and the specific mechanism may require further research.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12941-024-00746-8.

Supplementary Material 1

Acknowledgements
Not applicable.

Author contributions
Naihui Chu, Jie Lu and Jinfeng Yuan conceived the study. Wei Jing, Fuzhen Zhang, Yuanyuan Shang, Wenhui Shi, Cong Yao, Xuxia Zhang and Jinfeng Yuan performed the experiments. Wei Jing, Fuzhen Zhang, Yuanyuan Shang, Naihui Chu, Jie Lu and Jinfeng Yuan analyzed the data. Wei Jing, Fuzhen Zhang, Jie Lu and Jinfeng Yuan wrote the paper, with critical input from all other authors; all authors read and approved the final version of the paper.

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Data availability
Data is provided within supplementary information files. If the dataset used and/or analyzed in this study is not shown in the supplementary information files, it can be obtained from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participants
Not applicable.

Competing interests
The authors declare no competing interests.

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