In Vitro Susceptibility Testing of GSK656 against *Mycobacterium* Species

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ABSTRACT In this study, we aimed to assess the *in vitro* susceptibility to GSK656 among multiple mycobacterial species and to investigate the correlation between leucyl-tRNA synthetase (LeuRS) sequence variations and *in vitro* susceptibility to GSK656 among mycobacterial species. A total of 187 mycobacterial isolates, comprising 105 *Mycobacterium tuberculosis* isolates and 82 nontuberculous mycobacteria (NTM) isolates, were randomly selected for the determination of *in vitro* susceptibility. For *M. tuberculosis*, 102 of 105 isolates had MICs of ≤ 0.5 mg/liter, demonstrating a MIC₅₀ of 0.063 mg/liter and a MIC₉₀ of 0.25 mg/liter. An epidemiological cutoff value of 0.5 mg/liter was proposed for identification of GSK656-resistant *M. tuberculosis* strains. For NTM, the MIC₅₀ and MIC₉₀ values were >8.0 mg/liter for both *Mycobacterium intracellulare* and *Mycobacterium avium*. In contrast, all *Mycobacterium abscessus* isolates had MICs of ≤ 0.25 mg/liter, yielding a MIC₉₀ of 0.063 mg/liter. LeuRS from *M. abscessus* showed greater sequence similarity to *M. tuberculosis* LeuRS than to LeuRSs from *M. avium* and *M. intracellulare*. Sequence alignment revealed 28 residues differing between LeuRSs from *M. avium* and *M. intracellulare* and LeuRSs from *M. tuberculosis* and *M. abscessus*; among them, 15 residues were in the drug binding domain. Structure modeling revealed that several different residues were close to the tRNA-LeuRS interface or the entrance of the drug-tRNA binding pocket. In conclusion, our data demonstrate significant species diversity in *in vitro* susceptibility to GSK656 among various mycobacterial species. GSK656 has potent efficacy against *M. tuberculosis* and *M. abscessus*, whereas inherent resistance was noted for *M. intracellulare* and *M. avium*.

KEYWORDS GSK656, LeuRS, susceptibility, mycobacteria

Tuberculosis (TB), which is caused by *Mycobacterium tuberculosis* complex, remains a major cause of morbidity and death worldwide, with an estimated 10.0 million new incident cases and 1.6 million deaths in 2017 (1). The emergence of multidrug-resistant TB (MDR-TB), i.e., bacteria resistant to isoniazid and rifampin, hampers progress toward elimination of tuberculosis at a global level (2, 3). Nearly 0.6 million new cases of MDR-TB occur annually (1). Due to its intrinsic resistance to the most potent drugs, the treatment of MDR-TB requires up to 2 years of therapy with second-line medications (which are less effective and more toxic), and only 50% of MDR-TB cases receiving second-line drugs achieve favorable outcomes, as reported by the World Health Organization (1, 4). The situation is much worse for patients infected with extensively drug-resistant TB (XDR-TB), which is defined as MDR-TB with additional resistance to

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TABLE 1 Distribution of GSK656 MICs among *M. tuberculosis* isolates

Classification	No. of strains with MIC (mg/liter) of:													Total no.	MIC ₅₀ (mg/liter) ^a	MIC ₉₀ (mg/liter)
	<0.008	0.008	0.016	0.031	0.063	0.125	0.25	0.5	1.0	2.0	4.0	8.0	>8.0			
Non-MDR	1	3	9	6	16	4	1	0	0	0	0	0	0	40	0.063	0.125
MDR	0	2	4	6	14	8	4	1	0	1	0	0	0	40	0.063	0.25
XDR	0	0	3	4	9	5	1	1	0	1	0	1	0	25	0.063	0.5
Total	1	5	16	16	39	17	6	2	0	2	0	1	0	105	0.063	0.25

^aMIC₅₀, concentration required to inhibit the growth of 50% of the isolates tested; MIC₉₀, concentration required to inhibit the growth of 90% of the isolates tested.

any fluoroquinolone and at least one of the three injectable second-line drugs (2). The extensively resistant form beyond MDR-TB dramatically reflects treatment option exhaustion, resulting in even worse clinical outcomes (5). Therefore, there is an urgent need for novel drugs that assist in the management of drug-resistant TB, especially MDR-TB and XDR-TB (3).

Infections with nontuberculous mycobacteria (NTM) have become increasingly common in past years (6, 7). Unfortunately, low public health priority has been placed on this neglected aspect, due to a lack of solid evidence for person-to-person transmission, especially in countries with poor settings in which TB is endemic (8). Treatment of NTM disease remains difficult, in part because of resistance to the first-line antituberculous agents (9). As an alternative, multiple new anti-TB drugs have been used in salvage therapy to achieve optimal outcomes for patients with NTM disease (10). In view of their heterogeneity, significant differences in *in vitro* drug susceptibility are noted among NTM species (11, 12). Hence, the evaluation of susceptibility to the promising novel agents among NTM species is essential to determine correct therapeutic applications of these agents for the treatment of NTM disease.

GSK656 is a novel member of the 3-aminomethyl 4-halogen benzoxaboroles that targets *M. tuberculosis* leucyl-tRNA synthetase (LeuRS) (13). Oxaborole drugs can trap the 3' end of tRNA in the editing site of LeuRS by forming a nonproductive covalent adduct between the drug and tRNA, thereby inhibiting leucylation and protein synthesis (14). Due to this new mechanism of action, GSK656 could be used as a promising drug candidate against drug-resistant TB, especially MDR-TB and XDR-TB. Primary data demonstrated that GSK656 had remarkable efficacy in eliminating pathogenic tubercle bacilli both *in vitro* and *in vivo* (13). The question of whether this novel agent is efficacious for the management of NTM disease also arises. To address these concerns, we carried out this study to assess the *in vitro* susceptibility of multiple mycobacterial species to GSK656. The second objective of this study was to investigate the correlation between sequence variations within LeuRS and *in vitro* susceptibility to GSK656 among mycobacterial species.

RESULTS

***In vitro* susceptibility to GSK656 among *M. tuberculosis* isolates.** A total of 105 clinical *M. tuberculosis* isolates, comprising 40 non-MDR-TB, 40 MDR-TB, and 25 XDR-TB isolates, were randomly selected for the determination of *in vitro* susceptibility. The majority of the isolates tested (95.2%) had GSK656 MICs of ≤ 0.25 mg/liter (Table 1). For non-MDR-TB, all isolates had MICs of ≤ 0.25 mg/liter, demonstrating a MIC₅₀ of 0.063 mg/liter and a MIC₉₀ of 0.125 mg/liter. MDR-TB and XDR-TB isolates showed slightly higher MICs; 95.0% and 88.0% of MDR-TB and XDR-TB isolates, respectively, had MICs of ≤ 0.25 mg/liter. The MIC₅₀s of the MDR-TB and XDR-TB isolates were 0.25 and 0.5 mg/liter, respectively. According to the distribution of GSK656 MICs for *M. tuberculosis* isolates, we proposed an epidemiological cutoff (ECOFF) value of 0.5 mg/liter for identification of GSK656-resistant tubercle bacilli. As shown in Table 1, 1 MDR-TB isolate and 2 XDR-TB isolates were resistant to GSK656.

The candidate gene conferring GSK656 resistance was analyzed for the 3 GSK656-resistant *M. tuberculosis* isolates. All 3 isolates were found to contain the Ser403Pro allele in the LeuRS gene, whereas this mutant type was identified in 18 of 20 GSK656-

TABLE 2 Distribution of GSK656 MICs among NTM isolates

Species	No. of strains with MIC (mg/liter) of:													Total no.	MIC ₅₀ (mg/liter) ^a	MIC ₉₀ (mg/liter)
	<0.008	0.008	0.016	0.031	0.063	0.125	0.25	0.5	1.0	2.0	4.0	8.0	>8.0			
<i>M. intracellulare</i>	0	0	0	0	0	0	0	0	1	0	0	1	28	30	>8.0	>8.0
<i>M. avium</i>	0	0	0	0	0	0	0	0	0	0	0	0	16	16	>8.0	>8.0
<i>M. abscessus</i>	0	0	1	18	14	2	1	0	0	0	0	0	0	36	0.031	0.063

^aMIC₅₀, concentration required to inhibit the growth of 50% of the isolates tested; MIC₉₀, concentration required to inhibit the growth of 90% of the isolates tested.

susceptible *M. tuberculosis* isolates, indicating that this mutation was not associated with GSK656 resistance; however, there may be other potential resistance mechanisms within this region.

In vitro susceptibility to GSK656 among NTM isolates. We also tested the *in vitro* susceptibility to GSK656 among 82 NTM isolates, including 30 *Mycobacterium intracellulare* isolates (36.6%), 16 *Mycobacterium avium* isolates (19.5%), and 36 *Mycobacterium abscessus* isolates (43.9%). The results are summarized in Table 2. For the *M. avium* complex, GSK656 exhibited poor activity against both *M. intracellulare* and *M. avium*. Although 1 *M. intracellulare* isolate (3.3%) showed a MIC of 1.0 mg/liter, the MICs of the remaining 29 isolates (96.7%) were ≥ 8.0 mg/liter. In addition, all of the test *M. avium* isolates had MICs of >8.0 mg/liter. The MIC₅₀ and MIC₉₀ values were >8.0 mg/liter for both *M. intracellulare* and *M. avium* isolates. In contrast, GSK656 showed excellent activity against *M. abscessus* isolates; all isolates had MICs of ≤ 0.25 mg/liter, yielding a MIC₅₀ of 0.031 mg/liter and a MIC₉₀ of 0.063 mg/liter, which were 2-fold lower than those for *M. tuberculosis* isolates.

Sequence analysis of LeuRSs from *M. tuberculosis* and NTM. According to the result of phylogenetic analysis (Fig. 1), LeuRS from *M. abscessus* showed greater sequence similarity to *M. tuberculosis* LeuRS than LeuRSs from *M. avium* and *M. intracellulare*. Based on this result and the results of *in vitro* susceptibility testing, we divided the proteins into group A (LeuRSs from *M. tuberculosis* and *M. abscessus*, with a drug-susceptible phenotype) and group B (LeuRSs from *M. avium* and *M. intracellulare*, with a drug-resistant phenotype). Sequence comparison revealed 28 residues differing between group A and group B (see Fig. S1 in the supplemental material). Among them, 15 residues were in the editing domain (residues V300 to K516 in *M. tuberculosis* LeuRS). However, none of the 15 residues were located in the active site of the editing domain, which contains three regions, namely, a threonine-rich region (residues T336 to T341 in *M. tuberculosis* LeuRS), a GTG region (residues G436 to V443 in *M. tuberculosis* LeuRS), and a catalytic region (residues H446 to D452 in *M. tuberculosis* LeuRS).

To further analyze the possible roles of 28 different residues in interactions between LeuRS and a drug-tRNA adduct, we performed structure modeling. In SWISS-MODEL analysis, the search for templates revealed that the LeuRS from *Thermus thermophilus* had the highest sequence identity (39.8%) to *M. tuberculosis* LeuRS. In order to analyze the binding status of both tRNA and GSK656, we chose PDB file 2V0G as the modeling template, because this structure file contained not only LeuRS from *T. thermophilus* but also the tRNA and a benzoxaborole (AN2690).

In Fig. 2, 28 residues that differed between group A and group B are displayed on *M. tuberculosis* LeuRS as cyan sticks. Among 15 residues in the editing domain, 3 residues (T476, P493, and R497 in *M. tuberculosis* LeuRS) were around the entrance of the binding pocket for the drug-tRNA adduct. Among the 11 residues outside the

FIG 1 Phylogenetic tree of LeuRS proteins from *M. tuberculosis*, *M. abscessus*, *M. avium*, and *M. intracellulare*.

FIG 2 Structure of the *M. tuberculosis* LeuRS complex with a benzoxaborole (AN2690) and tRNA. The LeuRS protein (yellow) and tRNA (gray) are displayed in cartoon mode. The 28 different residues (cyan) and the benzoxaborole drug (red) are shown as sticks and CPK balls.

editing domain, 1 residue (Q299 in *M. tuberculosis* LeuRS) was close to the tRNA-LeuRS interface.

DISCUSSION

The epidemic of MDR-TB remains an increasing public health concern worldwide (1). The development and clinical application of novel efficacious agents are urgently needed to shorten treatment and to increase cure rates for MDR-TB cases (15). In this study, we first assessed the *in vitro* susceptibility to GSK656 among multiple mycobacterial species. Our data demonstrate significant species diversity in *in vitro* susceptibility to GSK656 among various mycobacterial species, and *M. tuberculosis* isolates are susceptible to GSK656 regardless of initial drug resistance patterns. The majority of non-MDR-TB isolates tested exhibited MICs lower than 0.063 mg/liter. This observation is similar to a previous study demonstrating a MIC of 0.024 mg/liter for the H37Rv strain. In addition, we suggest a tentative ECOFF value of 0.5 mg/liter for GSK656. The pharmacokinetic data represent another important issue to be taken into consideration when determining clinical breakpoints for antimicrobial agents. A recent study by Li and colleagues revealed that GSK656 exhibited low clearance and excellent exposure, with an area under the concentration-time curve for GSK656 of 11.12 h · μ g/ml, in a murine model (13). The comparison between the ECOFF value and pharmacokinetic data indicates that the proposed ECOFF value can be used to detect low-level resistance among *M. tuberculosis* isolates, which might still be treated with increased dosage. Therefore, further clinical trials are essential to determine the correlation between plasma concentrations of GSK656 and clinical outcomes among TB patients.

Three *M. tuberculosis* isolates tested, including 1 MDR-TB isolate and 2 XDR-TB isolates, had MICs higher than the proposed ECOFF. Considering that all isolates were isolated from patients without prior exposure to GSK656, it is highly unlikely that they could have accumulated various types of genetic mutations within the target gene for GSK656, which is supported by our sequence analysis of LeuRS. Therefore, we speculate that cell surface permeability barriers and active efflux in tubercle bacilli may play important roles in the emergence of low-level resistance to GSK656 (2). In a previous experimental study, Velayati et al. found that the cell wall of drug-resistant isolates, especially MDR-TB and XDR-TB isolates, was significantly thicker than that of susceptible isolates (16). The increased thickness of the cell wall would prevent the entry of antimicrobial agents into bacterial cells. In addition, higher MIC values were observed only for 1 MDR-TB isolate and 2 XDR-TB isolates. Considering that increased thickness of the cell wall is more likely to confer low-level resistance to GSK656, potential novel

mechanisms of action for GSK656 may be responsible for isolates with high-level resistance.

Another interesting finding is that GSK656 exhibits potent efficacy against *M. abscessus* isolates. Due to natural resistance to most of the antibiotics that are currently available, *M. abscessus* infections are difficult to treat (17). Our findings suggest that GSK656 has potent antimicrobial activity against *M. abscessus* in *in vitro* susceptibility testing. Further studies are needed to evaluate the *in vivo* activity and pharmacokinetic parameters of GSK656 against *M. abscessus* strains. Notably, although *M. abscessus* is a member of the rapidly growing mycobacteria (RGM), its LeuRS has greater sequence similarity to the LeuRS of slowly growing *M. tuberculosis* than to those of *M. intracellulare* and *M. avium*, which partly explains the diversity of GSK656 effects against various groups of mycobacteria.

As previously confirmed, the editing domain of LeuRS is the binding domain of oxaboroles such as GSK656, and the editing active site is the oxaborole binding site (14, 18). Although the 15 residues that differed between protein group A and group B in the editing domain were not located in the active site, the changes of these residues might remodel the binding pocket for the drug-tRNA adduct and LeuRS protein. In addition, several residues were close to the tRNA-LeuRS interface or the entrance of the drug binding pocket, which might influence substrate binding. Therefore, these differences are proposed as possible mechanisms for differential efficacy among mycobacterial species.

There were several obvious limitations to this study. First, in view of the absence of outcome data, the ECOFF value is a salvage solution for distinguishing resistant and susceptible isolates. Our experimental findings require confirmation with larger clinical studies. Second, another major limitation is the small number of enrolled NTM isolates, which may weaken the overall significance of our study conclusion. Third, as endorsed by Clinical and Laboratory Standards Institute (CLSI) guidelines, *M. abscessus* inocula are incubated at 30°C instead of 37°C used in this study. Despite the fact that the incubation temperature would not affect the growth of this mycobacterial species (19), the deviation in culture conditions may lead to potential bias in the interpretation of our results. Fourth, only GSK656, rather than other antimicrobial agents, was tested in *in vitro* susceptibility testing. We could not provide a direct comparison of efficacy between GSK656 and standard anti-TB agents. Finally, the model for LeuRS-GSK656 binding is computerized rather than accurately depicted. Despite these limitations, this study provides a comprehensive description of *in vitro* susceptibility to GSK656 among mycobacterial species.

In conclusion, our data demonstrate significant species diversity in *in vitro* susceptibility to GSK656 among various mycobacterial species. GSK656 has potent efficacy against *M. tuberculosis* and *M. abscessus*, whereas inherent resistance is noted for *M. intracellulare* and *M. avium*. The LeuRS from *M. abscessus* shows greater sequence similarity to *M. tuberculosis* LeuRS than to the LeuRSs from *M. avium* and *M. intracellulare*. Further clinical trials are needed to validate our experimental findings.

MATERIALS AND METHODS

Bacterial strains. The mycobacterial isolates used in this study were collected from Beijing Chest Hospital, Capital Medical University, between 2011 and 2016. Each isolate was isolated from a unique patient and identified at the species level using multilocus sequence analysis, as described previously (20).

MIC testing. The *in vitro* susceptibility of *M. tuberculosis* isolates to GSK656 was determined by a microdilution MIC testing method with 7H9 broth, as reported previously (21). For NTM, isolates were tested with cation-adjusted Mueller-Hinton broth (CAMHB), according to the guidelines from the CLSI (22). CAMHB enriched with 5% oleic acid-albumin-dextrose-catalase (OADC) was used for slowly growing mycobacteria (SGM), while CAMHB without OADC was used for RGM. Briefly, a suspension of 0.5 McFarland standard was prepared with freshly grown bacteria. Then, 100 μ l of diluted inoculum was pipetted into each well of the plate, which contained 100 μ l of corresponding drug at concentrations prepared from 2-fold serial dilutions in CAMHB broth. The final concentration of organisms in each well reached 10⁵ CFU/ml. The tested concentrations of GSK656 ranged from 0.008 to 8.0 mg/liter. Normally, after 7 days of incubation for *M. tuberculosis* and SGM isolates or 3 days of incubation for RGM isolates at 37°C, 70 μ l of alamarBlue solution was added to each well. For isolates with insufficient growth in the

drug-free control by the end of the first time point, 3 additional days of incubation were used to accumulate adequate growth prior to addition of the alamarBlue solution. The plates were then reincubated at 37°C. A blue color in the well indicated no mycobacterial growth, while a pink color indicated growth occurrence. The MIC was defined as the lowest concentration of antimicrobial agent that prevented the color change from blue to pink. Each experimental batch was tested using the reference *M. tuberculosis* strain H37Rv (ATCC 27294) for quality control purposes. All experiments were performed in triplicate. For isolates giving discordant MIC results, the experiments were repeated until three identical MIC values were obtained, which were determined as the MIC values of the corresponding isolates. The ECOFF values were determined on the basis of the MIC distribution profiles. For unimodal MIC distributions, ECOFF values were defined as concentrations inhibiting $\geq 99.9\%$ of the bacterial population; for bimodal MIC distributions, ECOFF values were set between the two populations (12).

DNA sequencing. The crude genomic DNA of *M. tuberculosis* isolates was isolated with a rapid boiling method, as reported previously (23). The freshly grown bacteria were transferred into a microcentrifuge tube containing 500 μ l Tris-EDTA (TE) buffer. After centrifugation at 13,000 rpm for 2 min, the supernatant was discarded, and the sediment was resuspended in 500 μ l TE buffer. The resuspension was heated in a 95°C water bath for 1 h, followed by centrifuging at 13,000 rpm for 5 min. The supernatant was used as the template for PCR amplification. According to previous reports (13), *LeuRS* (Rv0041) is the GSK656 antibiotic target in tubercle bacilli. The entire *LeuRS* gene of *M. tuberculosis* isolates with high MIC values (MICs of >0.5 mg/liter) was amplified and sequenced.

Sequence alignment and structure modeling. Protein sequences of *LeuRS*s from *M. tuberculosis* (UniProt accession no. P9WVF1), *M. abscessus* (UniProt accession no. B1MMK6), *M. avium* (UniProt accession no. A0Q8W7), and *M. intracellulare* (UniProt accession no. H8IHP3) were obtained from UniProtKB (<http://www.uniprot.org>). Sequence alignment was performed using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo>), and the results were sent to Simple Phylogeny (http://www.ebi.ac.uk/Tools/phylogeny/simple_phylogeny) to generate a phylogenetic tree, using the neighbor-joining clustering method. The structure modeling of *M. tuberculosis* *LeuRS* (UniProt accession no. P9WVF1) was performed with SWISS-MODEL, using PDB file 2VOG as the modeling template (24). The sequence alignment was further formatted in ESPript, according to the results of Clustal Omega and structure modeling.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.4 MB.

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