MmpS5-MmpL5 Transporters Provide Mycobacterium smegmatis Resistance to imidazo[1,2-b][1,2,4,5]tetrazines

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Abstract: The emergence and spread of drug-resistant Mycobacterium tuberculosis strains (including MDR, XDR, and TDR) force scientists worldwide to search for new anti-tuberculosis drugs. We have previously reported a number of imidazo[1,2-b][1,2,4,5]tetrazines – putative inhibitors of mycobacterial eukaryotic-type serine-threonine protein-kinases, active against M. tuberculosis. Whole genomic sequences of spontaneous drug-resistant M. smegmatis mutants revealed four genes possibly involved in imidazo[1,2-b][1,2,4,5]tetrazines resistance; however, the exact mechanism of resistance remain unknown. We used different approaches (construction of targeted mutants, overexpression of the wild-type (w.t.) and mutant genes, and gene-expression studies) to assess the role of the previously identified mutations. We show that mutations in MSMEG_1380 gene lead to overexpression of the mmpS5-mmpL5 operon in M. smegmatis, thus providing resistance to imidazo[1,2-b][1,2,4,5]tetrazines by increased efflux through the MmpS5-MmpL5 system, similarly to the mechanisms of resistance described for M. tuberculosis and M. abscessus. Mycobacterial MmpS5-MmpL5 transporters should be considered as an MDR-efflux system and they should be taken into account at early stages of anti-tuberculosis drug development.

Keywords: Mycobacterium smegmatis; imidazo[1,2-b][1,2,4,5]tetrazine; MmpS5-MmpL5; efflux; drug discovery; drug resistance; tuberculosis

1. Introduction

Tuberculosis (TB), caused by Mycobacterium tuberculosis, is currently the leading killer among the infectious diseases caused by one infectious agent, responsible for an estimate of 1.2 million deaths in 2018 [1]. According to WHO, 1.7 billion people globally are infected with M. tuberculosis, and thus are at risk of developing the disease [1]. The emergence and spread of multidrug resistant TB (MDR-TB, defined as TB resistant to rifampicin and isoniazid), extensively drug-resistant TB (defined as MDR-TB with resistance to the fluoroquinolones and second-line injectables) and totally drug-resistant TB (TDR-TB) is a global threat to world-wide TB control [2–5]. Long treatment times (six months for drug-susceptible TB and up to two years for DR-TB) often lead to bad patient compliance, which is one of the causes of drug resistance development and results in worse treatment outcomes. Thus, researchers are forced to search for novel anti-TB drugs and shorter regimens [6].

Eukaryotic-type serine-threonine protein-kinases (ESTPKs) play a key role in M. tuberculosis life cycle regulation, controlling some of its vital aspects such as cell division and survival within host macrophages, and, therefore, they represent attractive targets for drug development [7,8]. We have previously described a number of imidazo[1,2-b][1,2,4,5]tetrazines with a promising antibacterial
activity on \textit{M. tuberculosis} and \textit{M. smegmatis} [9]. Most of these compounds showed activity as potential ESTPK inhibitors in the original \textit{M. smegmatis aphVIII+} test-system [10,11], and two of them were able to bind to the \textit{M. tuberculosis} PknB adenine-binding pocket according to docking studies [10]. Despite the predicted activity as ESTPK-inhibitors, both the exact mechanism of action and the mechanism of resistance to these compounds are still unknown.

We were able to obtain spontaneous \textit{M. smegmatis} mutants resistant to four imidazo[1,2-b][1,2,4,5]tetrazines (3a, 3c, 3h and 3n, Figure 1), which had cross-resistance among them, suggesting a common mechanism of drug-resistance [9]. Whole-genomic sequencing and comparative genomic analysis revealed mutations in \textit{MSMEG} \textit{0641} (binding-protein-dependent transporters inner membrane component) in 1 mutant, in \textit{MSMEG} \textit{1601} (hypothetical protein) in seven mutants, in \textit{MSMEG} \textit{2087} (transporter small conductance mechanosensitive ion channel (MscS) family protein) in one mutant [12], while all the mutants carried different mutations in \textit{MSMEG} \textit{1380} (AcrR/TetR\textsubscript{N} transcriptional regulator) – 1 nonsynonymous SNP, 2 insertions leading to a frameshift, 2 duplications (6 and 501 base pairs-long) and one deletion [13].

In this article we describe the investigation of these mutations’ role in mycobacterial drug resistance to imidazo[1,2-b][1,2,4,5]tetrazines by different approaches: construction of targeted mutants, overexpression of the wild-type (w.t.) and mutant genes, and gene-expression studies.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure1}
\caption{Chemical structures of imidazo[1,2-b][1,2,4,5]tetrazines [9].}
\end{figure}

2. Results

2.1. Mutations in \textit{MSMEG} \textit{1380} Gene Lead to imidazo[1,2-b][1,2,4,5]tetrazines Resistance in \textit{M. smegmatis}

The list of nonsynonymous mutations found in spontaneous drug-resistant \textit{M. smegmatis} mutants used in this study is presented in Table 1. We were able to construct targeted \textit{M. smegmatis} mutants harboring each mutation in genes \textit{MSMEG} \textit{0641}, \textit{MSMEG} \textit{1601}, and \textit{MSMEG} \textit{2087}, as well as five mutations found in \textit{MSMEG} \textit{1380} gene using the p2NIL/pGOAL19 suicide system [14] for homologous recombination (Table 1).

\begin{table}[h]
\centering
\caption{Bacterial strains used in the study.}
\begin{tabular}{lll}
\hline
\textbf{Bacterial strains} & \textbf{Name} & \textbf{Comment} & \textbf{Origin} \\
\hline
\textit{M. smegmatis} \textit{mc2} 155 & Wild-type (w.t.) strain & & \\
\textit{M. smegmatis} \textit{atR1} & Spontaneous mutant of \textit{mc2} 155. Mutations: Y52H (TAC>CAC) in \textit{MSMEG} \textit{1601}; del LLA41-43 (del GCTGCTCGC480-488) in \textit{MSMEG} \textit{1380}. & [9] & \\
\textit{M. smegmatis} \textit{atR2} & Spontaneous mutant of \textit{mc2} 155. Mutations: Y52H (TAC>CAC) in \textit{MSMEG} \textit{1601}; ins GC425-426 (frameshift) in \textit{MSMEG} \textit{1380}. & [9] & \\
\end{tabular}
\end{table}
We examined the minimal inhibitory concentrations (MICs) of the four compounds on the recombinant *M. smegmatis* strains and found that mutations in *MSMEG_1380* gene lead to elevated MICs as compared to the w.t. strain (4×MIC for the compound 3a, at least 2×MIC for the compound 3c, at least 2×MIC for the compound 3h, and 4×MIC for the compound 3n), while recombinants harboring mutations in genes *MSMEG_0641*, *MSMEG_1601*, and *MSMEG_2087* had the same MICs as the w.t. strain (Table 2).

Thus we have shown that only the mutations in *MSMEG_1380* are responsible for imidazo[1,2-b][1,2,4,5]tetrazines resistance in *M. smegmatis*.

Table 2. Imidazo[1,2-b][1,2,4,5]tetrazines MICs on *M. smegmatis* strains in liquid medium.

| Compound | M. smegmatis strains MICs, µg/mL |
|----------|----------------------------------|
|          | mc2 155 | atR1c | atR2c | atR9c | atR14c | atR33c | 0641c | 1601c | 2087c |
| 3a       | 128     | 512    | 512    | 512    | 512    | 128    | 128    | 128    | 128    |
| 3c       | 64      | >128*  | >128*  | >128*  | >128*  | >128*  | 64     | 64     | 64     |
| 3h       | 128     | >256*  | >256*  | >256*  | >256*  | >256*  | 128    | 128    | 128    |
| 3n       | 64      | 256    | 256    | 256    | 256    | 64     | 64     | 64     |

*The compounds were not soluble at higher concentrations; bacterial growth was observed at the stated concentrations.*
2.2. W.t. MSMEG_1380 Overexpression Increases M. smegmatis Susceptibility to imidazo[1,2-b][1,2,4,5]tetrazines

In order to investigate further the role of MSMEG_1380 in M. smegmatis resistance to imidazo[1,2-b][1,2,4,5]tetrazines, we cloned the w.t. MSMEG_1380 gene and two of its mutant variants in the tetracycline inducible plasmid pMINDKm [15]. We used the paper-disc assay to assess the drug susceptibility of M. smegmatis strains to imidazo[1,2-b][1,2,4,5]tetrazines and found that the overexpression of the w.t. MSMEG_1380 gene increases M. smegmatis susceptibility to the tested compounds, while the overexpression of its mutant variants had no effect on the phenotype (Table 3), thus suggesting that the disruption of MSMEG_1380 protein’s function leads to the drug-resistant phenotype.

Table 3. Growth inhibition halos, produced by imidazo[1,2-b][1,2,4,5]tetrazines on M. smegmatis strains.

| Compound | Concentration, nmole/disc | Growth inhibition halo, mm |
|----------|---------------------------|---------------------------|
|          |                           | M. smegmatis transformants |
|          |                           | pMINDKm | pMINDKm:msmeg_1380 | pMINDKm:msmeg_1380-19 | pMINDKm:msmeg_1380-33 |
| 3a       | 300                       | 9.8 ± 1.5 | 17.0 ± 3.6 | 8.8 ± 0.8 | 8.2 ± 0.6 |
| 3c       | 300                       | 7.0 ± 0.8 | 15.0 ± 2.9 | 6.3 ± 0.5 | 6.3 ± 0.5 |
| 3h       | 40                        | 6.7 ± 0.5 | 11.5 ± 0.4 | 6.3 ± 0.5 | 6.5 ± 0.4 |
| 3n       | 100                       | 9.7 ± 2.4 | 16.0 ± 0.8 | 9.2 ± 1.3 | 9.3 ± 1.2 |

2.3. MSMEG_1380 Represses the Expression of the mmpS5-mmpL5 Operon in M. smegmatis

MSMEG_1380 gene lies 179 b.p. upstream the mmpS5-mmpL5 operon (genes MSMEG_1381-MSMEG_1382) in the M. smegmatis genome and is transcribed in the opposite direction (Figure 2). The structure of this operon is conserved in different mycobacterial species: the mmpS5-mmpL5 genes are controlled by a TetR-family transcriptional repressor, encoded by a gene located upstream the operon. Mutations in genes encoding the TetR-repressor, which lead to the upregulation of the mmpS5-mmpL5 genes, are involved in M. abscessus resistance to the derivatives of thiacetazone [16], as well as in cross-resistance of M. tuberculosis to bedaquiline and clofazimine [17]. We have also identified a possible operator sequence in the 5′-untranslated region of MSMEG_1381, similar to the one described in [16]: 5′-AAGCGGATTGACCTTATCCACTT-3′.

To test the hypothesis that resistance to imidazo[1,2-b][1,2,4,5]tetrazines in M. smegmatis has a similar origin to the ones described for M. tuberculosis and M. abscessus [16,17], we analyzed the expression of MSMEG_1380 gene and mmpL5 (MSMEG_1382) genes in different conditions. All the spontaneous M. smegmatis mutants had increased mmpL5 expression (54.16–80.45 times) as compared to the w.t. M. smegmatis mc2 155 strain (Figure 3A). The overexpression of the w.t. MSMEG_1380 gene, cloned into the pMINDKm plasmid led to a 7.90-fold repression of the mmpL5 gene expression (P < 0.001, Figure 3B), confirming that MSMEG_1380 encodes the repressor of the
mmpS5-mmpL5 operon, and explaining the drug-susceptible phenotype, observed in the MSMEG_1380 overexpressing strain. On the contrary, the expression of MSMEG_1380 was upregulated in the mutant strains (Figure 3A), indicating that this transcriptional repressor is self-regulatory and that mutations lead to the loss of its function.

We also observed that the addition of subinhibitory concentrations of the compound 3a upregulated the expression of mmpL5 in a dose-dependent manner (Figure 3C).

Figure 3. Relative expression levels of mmpS5-mmpL5 operon genes in different conditions: expression levels of MSMEG_1380 and mmpL5 genes in spontaneous M. smegmatis imidazo[1,2-b][1,2,4,5]tetrazine-resistant mutants (A); expression levels of MSMEG_1380 and mmpL5 genes in M. smegmatis pMINDKm:msmeg_1380 (B); expression levels of the mmpL5 gene after the addition of different concentrations of the compound 3a (shown on the X-axis)(C). Error bars represent standard deviations from triplicates.

3. Discussion

Deorphaning phenotypic screening hits, that is determining their mechanism of action and/or resistance, is a key part in the early-stage anti-TB drug development [18]. Here we determine the mechanism of M. smegmatis imidazo[1,2-b][1,2,4,5]tetrazines resistance based on the previously obtained whole-genome sequencing data for 12 spontaneous mutants with cross-resistance to four compounds [9,13].

The construction of targeted mutants showed that only mutations in MSMEG_1380 are responsible for drug resistance. In M. smegmatis, MSMEG_1380 encodes a TetR-family transcriptional repressor, which controls the mmpS5-mmpL5 operon, encoding transmembrane transporters, conserved throughout mycobacterial species. Mutations occurring in MSMEG_1380 led to the upregulation of the mmpS5-mmpL5 operon and increased efflux of the drug-candidates from the cells, similarly to the mechanisms described for M. tuberculosis and M. abscessus [16,17]. Overexpression of the w.t. MSMEG_1380 led to the repression of the mmpS5-mmpL5 operon and expectedly to an increased drug susceptibility phenotype. Interestingly, we observed a dose-dependent upregulation of the mmpS5-mmpL5 operon upon the addition of one of the compounds, which may indicate the ability of this compound to bind to the MSMEG_1380 protein, inhibiting its affinity to the operator sequence; however, this needs to be examined in vitro in future studies.

The tested compounds showed activity as ESTPK inhibitors [9,11]; however, we have not observed any mutations in ESTPK genes. One or more ESTPKs might still be the biotargets of imidazo[1,2-b][1,2,4,5]tetrazines but determining them by spontaneous mutagenesis might be difficult: some of the ESTPKs may fulfill the functions of others in the situation when they might be inhibited [8], and there is a possibility that more than one mutation might be required.

The primary biological role of the MmpS5-MmpL5 system consists in siderophore transport, which is crucial for M. tuberculosis survival under low-iron conditions within macrophages [19]. Yet, this efflux system has also shown itself to be an important factor of drug resistance: besides the mentioned efflux-mediated resistance to thiacetazone derivatives, bedaquiline and clofazimine [16,17], it has also been reported to provide M. tuberculosis resistance to azoles [20]. We can expect that M. tuberculosis strains resistant to bedaquiline and clofazimine might also be resistant to imidazo[1,2-b][1,2,4,5]tetrazines; however, a 36% mismatch in the amino acid sequences of the
MmpL5 proteins in *M. smegmatis* and *M. tuberculosis* may affect the drug-specificity of the transporter, and this should be additionally examined in future studies. Still, the MmpS5-MmpL5-mediated resistance mechanism needs to be considered during early stages of anti-TB drug development, and convenient in silico and in vitro test-systems for rapid analysis should be developed.

4. Materials and Methods

4.1. Bacterial Strains and Growth Conditions

*M. smegmatis* strains described in this study are presented in Table 1. Middlebrook 7H9 medium (Himedia, India) supplemented with OADC (Himedia, India), 0.1% Tween-80 (v/v), and 0.4% glycerol (v/v) was used as liquid medium, while the M290 Soyabean Casein Digest Agar (Himedia, India) was used as solid medium. *Escherichia coli* DH5α was used for plasmids propagation. Cultures in liquid medium were incubated in the Multitron incubator shaker (Infors HT, Switzerland) at 37 °C and 250 rpm.

4.2. Targeted *M. smegmatis* mutants’ Construction

Targeted *M. smegmatis mc2 155* mutants were constructed by homologous recombination using the p2NIL/pGOAL19 suicide vector system [14]. Briefly, genes *MSMEG_0641, MSMEG_1380, MSMEG_1601,* and *MSMEG_2087* with adjacent 1-1.5 kb fragments were amplified from genomic DNA, isolated from respective mutants by phenol-chloroform/isoamyl alcohol extraction after enzymatic cell lysis [21], with Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA) using the following primers, picked with primer-BLAST [22]: pN_0641_f 5′-TTTTCTGCAGCCAACAACGATCCAGATGTCCGT-3′ and pN_0641_r 5′-TTTTAGCTTCAATGGCGGCGTCCTTCATCTCG-3′ for *MSMEG_0641*; pN_1380_f 5′-TTTTAAGCTTGTACTACTCGCTGGTGGCGTC-3′ and pN_1380_r 5′-TTTTGGATCCTGCTGCACGTGTTCGGTGTC-3′ for *MSMEG_1380*; pN_1601_f 5′-CCCATGACGGGCATCATCAACC-3′ and pN_1601_r 5′-TTTTTATAAAGCGGATCCAGCCATCCACCACC-3′ for *MSMEG_1601*; pN_2087_f 5′-TTTTAAGCTTCCAGAAGGTCACCAGCGATCTG-3′ and pN_2087_r. The amplified products were digested with respective restriction enzymes (Thermo Scientific, USA) and ligated in the p2NIL plasmid. The cassette from pGOAL19 was subsequently cloned in the obtained plasmids at the *PacI* restriction site. The plasmids were electroporated in *M. smegmatis mc2 155* cells as described in [23] and plated on M290 plates supplemented with kanamycin (50 μg/mL), hygromycin (50 μg/mL), and X-Gal (50 μg/mL); blue single-crossover colonies were selected. Blue colonies were grown overnight in liquid 7H9 medium with ADC, and serial 10-fold dilutions were plated on M290 plates supplemented with X-Gal (50 μg/mL) and sucrose (2% w/v); white double-crossover colonies were selected and tested for Km susceptibility. Target genes were then Sanger-sequenced for a final confirmation of the mutation.

4.3. MIC Determination

MICs of the studied compounds on *M. smegmatis* were determined in liquid medium. *M. smegmatis* strains were cultured overnight in 7H9 medium, then diluted in the proportion of 1:200 in fresh medium (to approximately OD600 = 0.05). 196 μl of the diluted culture was poured in sterile nontreated 96-well flat-bottom culture plates (Eppendorf, Germany) and 4 μL of serial two-fold dilutions of the tested compounds in DMSO were added to the wells. The plates were incubated at 37°C and 250 rpm for 48 h. The MIC was determined as the lowest concentration of the compound with no visible bacterial growth.

4.4. MSMEG_1380 Cloning, Expression and Drug-Susceptibility Testing

MSMEG_1380 genes from respective strains were amplified by Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA) using primers pM_1380_f 5′-
GACACATATGGGAGGAAATGTTGTAGTGCCCCCGAGACG-3' and pM_1380_r 5'-TTTTACTAGTTCAGGTGGCGCAGGGCG-3' picked with primer-BLAST [22] and cloned in the pMINDKm: plasmid [15], a modification of pMIND [24] lacking the kanamycin resistance gene, at the NdeI and SpeI restriction sites, to obtain the following plasmids: pMINDKm:msmeg_1380, pMINDKm:msmeg_1380-19, and pMINDKm:msmeg_1380-33, containing, respectively, the w.t. msmeg_1380 gene as well as its mutant variants from strains atR19 and atR33. The resulting plasmids were electroporated in M. smegmatis mc2 155 cells as described in [23].

M. smegmatis transformants were grown in Middlebrook 7H9 broth supplemented with hygromycin (50 μg/mL) and tetracycline (10 ng/mL) to midexponential phase (OD₆₀₀ = 1.2). Afterwards the cultures were diluted in the proportion of 1:9:10 (culture:water:M290 medium) and 5 mL were poured as the top layer on Petri dishes with agarized M290 medium. Both top- and bottom-layers were supplemented with hygromycin (50 μg/mL) and tetracycline (10 ng/mL). The plates were allowed to dry for at least 30 min, afterwards sterile paper discs with impregnated imidazo[1,2-b][1,2,4,5]tetrazines were plated. The plates were incubated for 2–3 days at 37°C, until the bacterial lawn was fully grown. Growth inhibition halos were measured to the nearest 1 mm. The experiments were carried out as triplicates; the average diameter and standard deviation (SD) were calculated.

4.5. Mycobacterial RNA Isolation and Real-Time qPCR

M. smegmatis strains were grown overnight in Middlebrook 7H9 broth to midexponential phase (OD₆₀₀ = 1.0-1.2); cells from 10 mL culture were harvested by centrifugation for 10 min at 3000g and washed by 1 mL of RNAprotect Bacteria Reagent (Qiagen, USA). Total RNA was extracted by homogenization in Trizol solution (Invitrogen, USA) [25], followed by phenol (pH = 4.5)-chloroform/isoamyl alcohol (25:24:1) purification and precipitation in high salt solution (0.8 M Na citrate, 1.2 M NaCl) with isopropanol. Remaining genomic DNA was removed by DNAse I, Amplification grade (Invitrogen, USA). 50 ng of total RNA was used for cDNA synthesis by iScript Select cDNA Synthesis Kit (Bio-Rad, USA). 1 ng of cDNA was used for real-time qPCR with the qPCRmix-HS SYBR kit (Evrogen, Russia) on a CFX96 Touch machine (Bio-Rad, USA). CFX Manager V 3.1 (Bio-Rad, USA) was used to analyze the qPCR results: relative normalized expression of three biological replicates was calculated as ΔΔCq and genes sigA and ftsZ were used as reference. The following primers were picked by primer-BLAST [22] for qPCR: q1380-f 5'-CTGCTCGACGAACCATGCGAAAC-3' and q1380-r 5'-AAGGGTCTTGAGCCGAATCTCAACG-3' (MSMEG_1380), q1382-f 5'-ACCACGCAGATCATGAACAACGACT-3' and q1382-r 5'-GAAATCTCGAAGTCCGCCAGATGA-3' (MSMEG_1382), qsigAs-sm-f 5'-CGACCTTTGTATCACCCTGAGCAT-3' and qsigAs-sm-r 5'-CTCGACCTCATCCAGGAAGGCAAC-3' (sigA), qftsZs-sm-f 5'-AGCAGCTCTCGATGTGTCGCTT-3' and qftsZs-sm-r 5'-GCCTGAAGGGCGTCGAGTTT-3' (ftsZ).

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References
1. World Health Organization Global Tuberculosis Report 2019; WHO: Geneva, Switzerland, 2019; pp. 1–297.
2. Gandhi, N.R.; Nunn, P.; Dheda, K.; Schaaf, H.S.; Zignol, M.; van Soolingen, D.; Jensen, P.; Bayona, J. Multidrug-resistant and extensively drug-resistant tuberculosis: A threat to global control of tuberculosis. *Lancet* **2010**, *375*, 1830–1843.
3. Caminero, J.A.; Solé, G.; Zumla, A.; Migliori, G.B. Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis. *Lancet Infect. Dis.* **2010**, *10*, 621–629.
4. Klopper, M.; Warren, R.M.; Hayes, C.; Gey van Pittius, N.C.; Streicher, E.M.; Müller, B.; Sirgel, F.A.; Chabula-Nxiweni, M.; Hoosain, E.; Coetzee, G.; et al. Emergence and Spread of Extensively and Totally Drug-Resistant Tuberculosis, South Africa. *Emerg. Infect. Dis.* **2013**, *19*, 449–455.
5. Velayati, A.A.; Farnia, P.; Farahbod, A.M. Overview of drug-resistant tuberculosis worldwide. *Int. J. Mycobacteriol.* **2016**, *5*, S161.
6. Muñoz-Torrico, M.; Duarte, R.; Dalcolmo, M.; D’Ambrosio, L.; Migliori, G.B. New drugs and perspectives for new anti-tuberculosis regimens. *Rev. Port. De Pneumol.* **2018**, *24*, 86–98.
7. Danilenko, V.N.; Osolodkin, D.I.; Lakatosh, S.A.; Preobrazhenskaya, M.N.; Shitl, A.A. Bacterial eukaryotic type serine-threonine protein kinases: from structural biology to targeted anti-infective drug design. *Curr. Top. Med. Chem.* **2011**, *11*, 1352–1369.
8. Prisić, S.; Husson, R.N. *Mycobacterium tuberculosis* Serine/Threonine Protein Kinases. *Microbiol. Spectr.* **2014**, *2*, 1–26.
9. Maslov, D.A.; Korotina, A.V.; Shur, K.V.; Vatlin, A.A.; Bekker, O.B.; Tolschina, S.G.; Ishmetova, R.I.; Ignatenko, N.K.; Rusinov, G.L.; Charushin, V.N.; et al. Synthesis and antimycobacterial activity of imidazo[1,2-b][1,2,4,5]tetrazines. *Eur. J. Med. Chem.* **2019**, *178*, 39–47.
10. Bekker, O.B.; Danilenko, V.N.; Ishmetova, R.I.; Maslov, D.A.; Rusinov, G.L.; Tolschina, S.G.; Charushin, V.N. Substituted azolo[1,2,4,5]tetrazines-inhibitors of antibacterial serine-threonine protein kinases, Patent RU 2462466, NPO SRC “BIOAN”, Moscow, Russia, 27 September 2012.
11. Bekker, O.B.; Danilenko, V.N.; Maslov, D.A. Test system of *Mycobacterium smegmatis* aphVIII+ for screening of inhibitors of serine-threonine protein kinases of eukaryotic type, Patent RU 2566998, NPO SRC “BIOAN”, Moscow, Russia, 27 October 2015.
12. Maslov, D.A.; Bekker, O.B.; Shur, K.V.; Vatlin, A.A.; Korotina, A.V.; Danilenko, V.N. Whole-genome sequencing and comparative genomic analysis of *Mycobacterium smegmatis* mutants resistant to imidazo[1,2-b][1,2,4,5]tetrazines, antituberculosis drug candidates. *BRSMU* **2018**, 19–22.
13. Vatlin, A.A.; Shur, K.V.; Danilenko, V.N.; Maslov, D.A. Draft Genome Sequences of 12 *Mycobacterium smegmatis* Strains Resistant to Imidazo[1,2-b][1,2,4,5]Tetrazines. *Microbiol. Resour. Announc.* **2019**, *8*, 263–2.
14. Parish, T.; Stoker, N.G. Use of a flexible cassette method to generate a double unmarked *Mycobacterium tuberculosis* tlyA pLCABC mutant by gene replacement. *Microbiology* **2000**, *146*, 1969–1975.
15. Maslov, D.A.; Bekker, O.B.; Alekseeva, A.G.; Kniazeva, L.M.; Mavletova, D.A.; Afansayev, I.I.; Vasilievich, N.I.; Danilenko, V.N. Aminopyridine- and aminopyrimidine-based serine/threonine protein kinase inhibitors are drug candidates for treating drug-resistant tuberculosis. *BRSMU* **2017**, 38–43.
16. Richard, M.; Gutiérrez, A.V.; Viljoen, A.J.; Ghigo, E.; Blaise, M.; Kremer, L. Mechanistic and Structural Insights Into the Unique TetR-Dependent Regulation of a Drug Efflux Pump in *Mycobacterium abscessus*. *Front. Microbio*. **2018**, *9*, 649.
17. Andries, K.; Villellas, C.; Coeck, N.; Thys, K.; Gevers, T.; Vranckx, L.; Lounis, N.; de Jong, B.C.; Koul, A. Acquired resistance of *Mycobacterium tuberculosis* to bedaquiline. *PLoS ONE* **2014**, *9*, e102135.
18. Cooper, C.B. Development of *Mycobacterium tuberculosis* Whole Cell Screening Hits as Potential Antituberculosis Agents. *J. Med. Chem.* **2013**, *56*, 7755–7760.
19. Sandhu, P.; Akhter, Y. Siderophore transport by MmpL5-MmpS5 protein complex in *Mycobacterium tuberculosis*. *J. Inorg. Biochem.* **2017**, *170*, 75–84.
20. Milano, A.; Pasca, M.R.; Provvedi, R.; Lucarelli, A.P.; Manina, G.; de Jesus Lopes Ribeiro, A.L.; Manganelli, R.; Riccardi, G. Azole resistance in *Mycobacterium tuberculosis* is mediated by the MmpS5-MmpL5 efflux system. *Tuberculosis* **2009**, *89*, 84–90.
21. Belisle, J.T.; Mahaffey, S.B.; Hill, P.J. Isolation of *Mycobacterium* Species Genomic DNA. In *Mycobacteria Protocols; Methods in Molecular Biology; Humana Press*: Totowa, NJ, USA, 2010; Volume 465, pp. 1–12.
22. Ye, J.; Coulouris, G.; Zaretskaya, I.; Cutcutache, I.; Rozen, S.; Madden, T.L. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinform.* **2012**, *13*, 134.
23. Goude, R.; Parish, T. Electroporation of Mycobacteria. In *Mycobacteria Protocols*, 2nd ed.; Methods in Molecular Biology; Humana Press: Totowa, NJ, USA, 2010; Volume 465, pp. 203–215.
24. Blokpoel, M.C.J. Tetracycline-inducible gene regulation in mycobacteria. *Nucleic Acids Res.* **2005**, *33*, e22–e22.
25. Rustad, T.R.; Roberts, D.M.; Liao, R.P.; Sherman, D.R. Isolation of Mycobacterial RNA. In *Mycobacteria Protocols*; Methods in Molecular Biology; Humana Press: Totowa, NJ, USA, 2010; Vol. 465, pp. 13–22.

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