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Studying the mechanism of action of new derivatives of quinoxalin-1,4-dioxide on the model organism *Mycobacterium smegmatis*

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Abstract. According to the World Health Organization (WHO), antibiotic resistance is currently one of the most serious threats to human health, food security, and development. Tuberculosis (TB) remains one of the deadliest bacterial diseases. The primary challenge in treating tuberculosis infection is the emergence of strains with multidrug resistance (MDR) to 4–9 drugs. The emergence of bacterial strains with MDR is a consequence of patients' insufficient adherence to treatment, interrupted therapy, improperly prescribed courses of chemotherapy, and, according to recent data, the accumulation of antibiotics in the environment, which can activate the natural drug resistance system in bacteria. The consequences of MDR to antibiotics include prolonged hospitalizations, increased medical expenses, and mortality. Therefore, the task is to develop new effective antibacterial agents with novel mechanisms to reduce the emergence of bacterial resistance. In this study, we investigated the mechanisms of action of new promising antimycobacterial derivatives of quinoxalin-1,4-dioxide on the model organism *Mycobacterium smegmatis*.

Keywords: antibiotics, bacterial resistance, mycobacteria, tuberculosis

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Изучение механизма действия новых производных хиноксалин 1,4-диоксида на модельном объекте *Mycobacterium smegmatis*

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Аннотация. По данным Всемирной организации здравоохранения (ВОЗ) устойчивость к антибиотикам является сегодня одной из наиболее серьезных угроз для здоровья человечества, продовольственной безопасности и развития, при этом одним из наиболее смертоносных бактериальных заболеваний остается туберкулез (ТБ). Основной проблемой лечения туберкулезной инфекции является возникновение штаммов с лекарственной устойчивостью (ЛУ) к 4–9 препаратам. Возникновение бактериальных штаммов с ЛУ является следствием недостаточной приверженности лечению пациентов, прерванного лечения, неправильно подобранного курса химиотерапии, а также, по последним данным, накопления антибиотиков в окружающей среде, которые могут приводить к активации природной системы лекарственной устойчивости у бактерий. Следствием ЛУ к антибиотикам являются продолжительные госпитализации, рост медицинских расходов и смертности в связи с чем стоит задача разрабатывать новые эффективные антибактериальные препараты, которые бы обладали новыми механизмами для снижения возникновения бактериальной устойчивости. В данной работе нами были изучены механизмы действия новых перспективных антимикобактериальных производных хиноксалин 1,4-диоксида на модельном объекте *Mycobacterium smegmatis*.

Ключевые слова: антибиотики, бактериальная устойчивость, микобактерии, туберкулез.

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Introduction

Multidrug-resistant (MDR) tuberculosis remains a major threat to modern medicine, with 649 000 new cases of rifampicin-resistant tuberculosis (RR-TB) – the most effective first-line drug – occurring between 2018 and 2021, of which 78% were MDR (rifampicin- and isoniazid-resistant) [1]. The emergence of MDR strains may result from, among other things, insufficient adherence, interrupted therapy or inappropriate chemotherapy. Russia, along with India and China, is among the countries with the highest prevalence of MDR-tuberculosis (WHO Global Tuberculosis Report 2022). Extensively drug-resistant (XDR) TB strains, resistant to 4-9 drugs, pose the greatest threat among MDR strains [2]. Increasing levels of resistance have also been attributed to the accumulation of antibiotics in nature and the activation of natural drug resistance systems in bacteria. According to recent data, one of the factors accelerating the emergence of DR is the presence of minimal selective concentrations (MSC) of antibiotics in the environment, which can lead to an increase in resistant strains in the population and activation of cell defence mechanisms against antibiotics (release or inactivation of antibiotics) [3–5]. Thus, one of the main current challenges is the search for new antituberculosis drugs (ATTs) that will have fundamentally new mechanisms of action, which will make it possible to overcome the phenomenon of drug resistance.

The aim of this study was to investigate the mechanism of action of promising PTP candidates – a new quinoxaline 1,4-dioxide derivative synthesised by us earlier 4 [6]. These compounds were selected due to their high activity against mycobacteria – compounds of this class have been shown to introduce single- and double-strand breaks in DNA, leading to cell death, which makes them promising for further study and modification [7]. Using reverse genetics methods, we have shown that mutations in the *MSMEG_4646, MSMEG_5122,* and *MSMEG_1380* genes confer resistance to compound 4 [6]. In this work, we studied the mechanisms of cross-resistance to quinoxaline 1,4-dioxide derivatives on the model object *Mycobacterium smegmatis* using genetic constructs with an increased level of gene expression.

Materials and methods

Bacterial strains and incubation conditions

Cells of *Mycolicibacterium (Mycobacterium) smegmatis* strains (Table 1). were grown in Middlebrook 7H9 liquid medium (Himedia) supplemented with OADC (oleic acid, albumin, dextrose, catalase), 0.05% Tween 80, 0.4% glycerol and in Lemco-Tween liquid medium. Composition of Lemco-Tween medium (per 1 litre): 5 g peptone (Oxoid), 5 g Lab Lemco (Oxoid), 5 g NaCl, 0.05% Tween 80. M290 medium (HiMedia Laboratories Pvt. Ltd) was used to grow *M. smegmatis* on agarised medium. *M. smegmatis* was incubated at $t = 37°C$.

Table 1. **Bacterial strains used in the study**

Bacterial strains		
Title	**Description**	**Reference**
M.smegmatis mc^2 155	Wild-type strain (*w.t.*)	(8)
M. smegmatis qdr	Spontaneous mutants of *M. smegmatis* qdR1, qdR4 and qdR5 resistant to the compound **1**	(9)
M. smegmatis	*M. smegmatis* strains carrying pMind containing mutant genes: pM4646w, pM4646q314, pM4648w, pM4648q1, pM5122.	Present paper

Source: compiled by the authors.

Cloning of genes containing mutations in the MSMEG_4646, MSMEG_4648, MSMEG_5122 genes into the pMind plasmid vector

MSMEG_4646, MSMEG_4648, MSMEG_5122 genes of *M. smegmatis* were aplified from genomic DNA of mutant strains resistant to quinoxaline 1,4-dioxide-4 derivative and WT strain *M. smegmatis* mc^2 155 using primers selected using NCBI BLAST primer (see Table 2). The optimal annealing temperature of the primers was selected using gradient PCR on a Bio-Rad T100 instrument (USA). Tersus Plus PCR kit (Eurogen) was used for amlification. The amlified fragment was cloned into the shuttle replicative vector pMind... by restriction sites *NdeI* and *SpeI* (Fast digest, Thermo Scientific, USA). T4-DNA ligase (Thermo Scientific, USA) was used for ligation. The obtained constructs were transfected into competent *E. coli* cells according to the standard procedure [10]. The presence of the target insert of the desired length was assessed by PCR screening of colonies. Constructs with target gene inserts were isolated from *E. coli* cells using a plasmid DNA isolation kit (Eurogen, Russia). The obtained constructs were transformed into competent *M. smegmatis* mc^2 155 cells by electroporation according to the method [11].

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

Primer's title	Primers for cloning in pMind
pM_4646_f	NdeI 5′ ttttCATATGggaggaaatgttATGGGTGACAACGGCAACGG 3′
pM_4646_r	SpeI 5′ ttttACTAGTTCATGCGTTCGCTCCCACAG 3′
pM_4648_f	NdeI 5′ ttttCATATGggaggaaatgttATGGCGCACCGGTACAAGG 3′
pM_4648_r	SpeI 5′ ttttACTAGTTCACATCGGCAGGTTGTAGGG 3′
pM_5122_f	NdeI 5′ ttttCATATGggaggaaatgttATGACGTACGTCATTGCCGAAC 3′
pM_5122_r	SpeI 5′ ttttACTAGTTCAGTCCTCACCCTGAGGC 3′

Source: compiled by the authors.

Sensitivity test of M. smegmatis to quinoxaline 1,4-dioxide derivatives

Cultures were incubated at 200 rpm and 37 °C overnight until OD600 = 1.2. Sensitivity to quinoxaline 1,4-dioxide derivatives was then determined using the paper disc method (diffusion-disc method): *M. smegmatis* culture was diluted 1 : 9 : 10 (culture : water : M290 medium (HiMedia Laboratories Pvt. Ltd.)) and seeded on top of the agar base layer on Petri dishes. Petri dishes have been incubated for 2 days at 37 °C until complete growth of the bacterial lawn. Growth inhibition halos were measured to the nearest 1 mm. Experiments were performed in triplicate; mean diameter and standard deviation (SD) were calculated. The criterion for selecting positive results was the presence of a significant difference in the diameter of the growth inhibition zone and the absence of the intersection of standard deviations of the diameters of the growth inhibition zones of the experimental and control samples of *M. smegmatis* [12].

Results

A cross-resistance study of M. smegmatis qdR mutants.

To investigate cross-resistance to quinoxaline 1,4-dioxide derivatives, we used spontaneous mutants of *M. smegmatis* mc^2 155 resistant to 4-fold MIC of compound 4 (Table 3), obtained and partially characterized previously [6]. Mutations in four different genes, MSMEG_1380, MSMEG_4646, MSMEG_4648, and in the gene and its promoter region MSMEG_5122, were identified by comparative genomic analysis. Using reverse genetics methods, it was shown that mutations in the MSMEG_1380, MSMEG_4648, and MSMEG_5122 genes lead to resistance to the previously described quinoxaline 1,4-dioxide derivatives [6].

Spontaneous mutants of *M. smegmatis* resistant to compound 4: *M. smegmatis* qdR1, *M. smegmatis* qdR4 and *M. smegmatis* qdR5 (Table 2) were used to investigate cross-resistance to quinoxaline 1,4-dioxide derivatives, and therefore to understand whether there is a common mechanism of action or resistance of *M. smegmatis* to different quinoxaline 1,4-dioxides. Possible cross-resistance

was tested to the following compounds: 4, 13c, 12c, 13a, 16c, 14a, 15c (compounds shown in Table 3).

Table 3. **Chemical compounds used in the study**

No.	Title	link
4	2-carboethoxy-3-methyl-6-(piperazin-1-yl)-7-chloroquinoxaline 1,4-dioxide hydrochloride	(6)
13c	2-acetyl-7-(piperazin-1-yl)-3-trifluoromethyl-6-chloroquinoxaline 1,4-dioxide hydrochloride	(9)
12c	7-(piperazin-1-yl)-3-trifluoromethyl-6-chloro-2-ethoxycarbonylquinoxaline 1,4-dioxide hydrochloride	
13a	2-acetyl-7-(piperazin-1-yl)-3-trifluoromethylquinoxaline 1,4-dioxide hydrochloride	
16c	7-(piperazin-1-yl)-3-trifluoromethyl-2-furanoyl-6-chloroquinoxaline 1,4-dioxide hydrochloride	
14a	7-(piperazin-1-yl)-2-propanoyl-3-trifluoromethylquinoxaline 1,4-dioxide hydrochloride	
15c	2-benzoyl-7-(piperazin-1-yl)-3-trifluoromethyl-6-chloroquinoxaline 1,4-dioxide hydrochloride	

Source: compiled by the authors.

The study revealed cross-resistance to all tested compounds in almost all cases. The qdR series mutants were resistant to most of the quinoxaline 1,4-dioxide derivatives analysed (Figure 1). The *M. smegmatis* qdR1 strain was generally more sensitive than the other mutants, although significantly more resistant than the wild-type strain to all compounds.

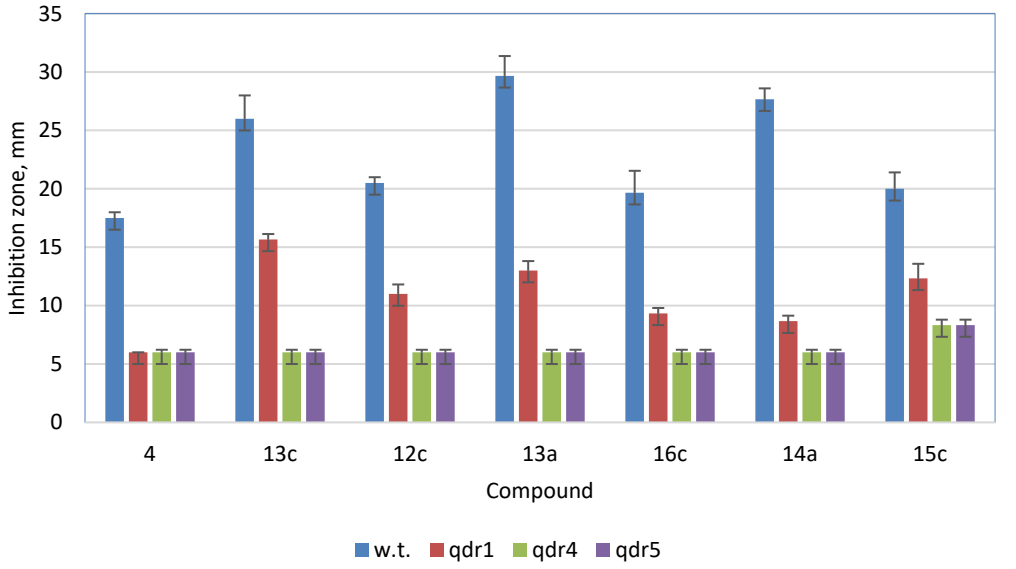

Figure 1. Diameters of growth inhibition zones around discs containing different quinoxaline 1,4-dioxides against spontaneous mutants of *M. smegmatis* resistant to compound 4. The concentration of compounds is 10 nmol/disc. Error bars reflect standard deviation.
Source: compiled by the authors.

Investigation of the role of individual genes and mutations in the formation of resistance to quinoxaline 1,4-dioxides

Previous full-genome sequencing showed that *M. smegmatis*, *qdR1, M. smegmatis, qdR4, and M. smegmatis, qdR5 mutants* have unique nonsynonymous mutations [6], confirming previous reports on the DNA-damaging properties of quinoxaline 1,4-dioxides [7].

The number of unique mutations in each strain correlated with the level of resistance, suggesting that their combination has a synergistic effect. We analysed the possible linkage of all mutant genes and identified several genes related to the oxidation of pyruvate to acetyl-CoA.

Overexpression of wild-type target genes and their mutant variants in M. smegmatis.

To investigate the role of individual genes and mutations in them in the formation of resistance to quinoxaline 1,4-dioxide, we used the approach of overexpression of target genes of wild type and their mutant variants (Table 4). For this approach, we used the pMIND vector [13], which has origins for replication in *E. coli* and mycobacterial cells, as well as an inducible tetracycline promoter.

Table 4. **Unique mutations in the genomes of *M. smegmatis qdr1, M. smegmatis qdr4 and M. smegmatis qdr5 mutants***

Protein ID	Locus tag	Annotation	Codon	SNP	A.a.	Distance to gene
M. smegmatis qdr1						
YP_888911.1	*MSMEG_4648*	DNA-binding protein	49	CAG>CCG	Q>P	–
YP_889369.1	*MSMEG_5122*	ferredoxin	–	–	–	71–72
M. smegmatis qdR4						
YP_888909.1	*MSMEG_4646*	pyruvate synthase	95	AAC>CAC	N>H	–
M. smegmatis qdR5						
YP_888909.1	*MSMEG_4646*	pyruvate synthase	274	CCG>CTG	P>L	–

Source: compiled by the authors.

We obtained the following constructs:

- pM4646wt: pMIND containing the wild-type *MSMEG_4646* gene;
- pM4646qdr4: pMIND containing the *MSMEG_4646* gene with an AAC > CAC mutation at codon 95 (N→H), corresponding to the *M. smegmatis* qdR4 mutant;
- pM4646qdr5: pMIND containing the *MSMEG_4646* gene with a CCG > CTG mutation at codon 274 (P→L), corresponding to the *M. smegmatis* qdR5 mutant;
- pM4648wt: pMIND containing the wild-type *MSMEG_4648* gene;

• pM4648qdr1: pMIND containing the MSMEG_4648 gene with a CAG > CCG mutation in codon 274 (Q→P), corresponding to the *M. smegmatis* qdR1 mutant;

• pM5122: pMIND containing the wild-type MSMEG_5122 gene.

These constructs were used to transform *M. smegmatis* strain mc^2 155. The drug susceptibility phenotype of the obtained *M. smegmatis* transformants carrying the constructed plasmids was assessed by the disc-diffusion method, rifampicin (rif) was used as a control compound (Figure 2).

The results (Figure 2) showed a significant increase in resistance to compounds **4, 12c, 14a and 13c** upon overexpression of *MSMEG_4646* mutant genes. Overexpression of *MSMEG_4648* mutant genes did not result in increased resistance. Overexpression of the wild-type gene *MSMEG_5122* consistently resulted in increased sensitivity to compounds 4 and 12c, probably shifting the redox reaction equilibrium due to the presence of more electron donor.

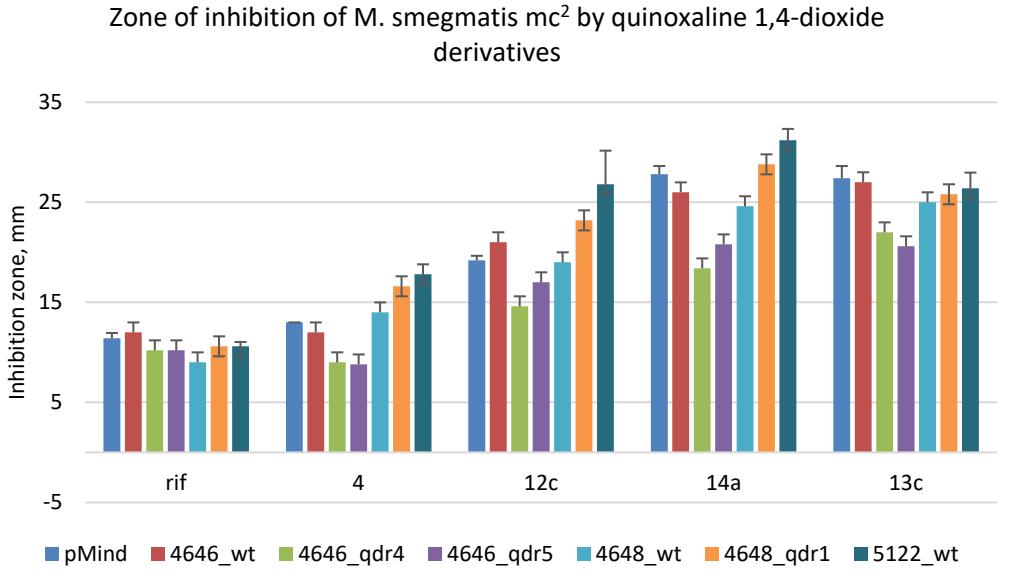

Figure 2. Diameters of growth inhibition zones around discs containing different quinoxaline 1,4-dioxides on *M. smegmatis* cultures. The concentration of compounds is 10 nmol/disc. Error bars reflect standard deviation.

Source: compiled by the authors.

Conclusion

As a result of this research, we were able to establish the putative mechanism of action of quinoxaline 1,4-dioxide derivatives on the model object *Mycobacterium smegmatis*. It can be assumed that the differences in the level of sensitivity of mutant strains are based on different sets of mutations in different strains. Thus, mutants, qdR4 and qdR5 have mutations in the *MSMEG_4646* gene (see Table 4) encoding the alpha subunit of ferredoxin oxidoreductase (pyruvate

synthase) involved in pyruvate metabolism. The qdR1 mutant has a mutation in the *MSMEG_4648* gene, annotated as a DNA-binding protein, which may act as a regulator of transcription of the adjacent operon encoding the alpha- and beta-subunits of the aforementioned pyruvate synthase and *MSMEG_5122*. In one of the previously studied mutants we could detect a mutation only in the *MSMEG_5122* gene encoding ferredoxin directly, and the qdR1 mutant also has two mutations in the putative promoter region of the *MSMEG_5122* gene (positions -71-72), and both mutant strains were resistant to the tested compound. Ferredoxin acts as an electron acceptor for pyruvate synthase, in the oxidation of pyruvate to acetyl-CoA. Probably, the mutant subunit competes with the wild-type subunit in the formation of the pyruvate synthase complex, which in total reduces the efficiency of its operation and activation of quinoxaline 1,4-dioxides, leading to reduced sensitivity of the strain to the compound under study.

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