DMNP, a Synthetic Analog of Erogorgiaene, Inhibits the ppGpp Synthetase Activity of the Small Alarmone Synthetase RelZ

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Abstract. Suppression of the stringent response is a promising strategy for the treatment of persistent bacterial infections. A novel class of compounds having a mechanism of action based on alarmone synthetase inhibition and suppressing the synthesis of (p)ppGpp alarmones in bacteria may provide a more effective treatment for latent infections and resolve problems associated with bacterial persistence. Conventional antibiotics primarily act on actively growing bacteria, but they are inactive against persister cells with a slowed metabolism. Alarmone synthetase inhibitors have antipersister properties that may enhance conventional antibiotics' antibacterial action. Two groups of RSH proteins are responsible for the synthesis of alarmones: long RelA/SpoT homologs and small alarmone synthetases. Many species of bacteria possess both types of enzymes. Despite the fact that a number of inhibitors of bifunctional long synthetases/hydrolases have been described to date, their properties with respect to monofunctional small alarmone synthetases have been studied poorly. This study investigated the effect of the alarmone synthetase inhibitor DMNP on the purified RelZ small alarmone synthetase protein from *Mycolicibacterium smegmatis*.

1 Introduction

One of the fundamental problems in research aimed at improving public health is reducing the incidence rate, ineffective treatment and mortality linked to recurrent bacterial infections [1], of which tuberculosis occupies the leading place in terms of these indicators [2, 3]. This is largely due to the specific properties of the causative agent of tuberculosis *Mycobacterium tuberculosis*, which has a pronounced ability for persistence [4, 5].

The main difficulties in the treatment of tuberculosis arise from the high number of cases of multidrug-resistant TB and the high incidence of infection relapse. These manifestations of infection based on the mechanisms of bacterial insensitivity to antibiotics: genetically determined resistance, as well as phenotypically determined persistence [6]. Persistence allows mycobacteria to survive in the presence of antibiotics due to a drop in

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metabolic rate, thus increasing the chances of relapse and lengthening treatment duration [7, 4]. It is critical to note that persistence and resistance are complementary adaptations [8], so survival due to persistence might contribute to the emergence of antimicrobial resistance.

Thus, to resolve the problem of bacterial persistence, novel and more effective treatment strategies are required. One of the possible ways to solve it may be the development of new compounds that compromise bacterial adaptations to stress, such as the stringent response [1, 9], the synthesis of (p)ppGpp alarmones signaling molecules, which act as positive regulators of bacterial persistence [10].

The key enzymes responsible for the formation of the stringent response are the RelA/SpoT (RSH) homologs, which catalyze the (p)ppGpp alarmones synthesis from the guanine nucleotides GTP, GDP, and GMP, and also catalyze alarmone hydrolysis [11]. RSH superfamily proteins fall into three groups: long RelA/SpoT homologs, most of which can both synthesize and hydrolyze alarmones; small alarmone synthetases, that can only synthesize (p)ppGpp; and small alarmone hydrolases, that can only degrade alarmones [12].

There is strong evidence supporting the role of the stringent response in tuberculosis treatment failure. The *M. tuberculosis* $\Delta rel_{\mathrm{Mtb}}$ knockout strain that lacks (p)ppGpp due to a gene deletion in the only functional alarmone synthetase characterized by growth defect, deficiency in biofilm formation, impaired TB chronic infection in mice model [13], as well as inability to undergo metabolic arrest in response to nutrient starvation, and decreased starvation-induced tolerance to first-line antitubercular medication isoniazid [14].

Alarmone synthetase inhibitors present a new strategy for the treatment of tuberculosis [1, 9]. Research on the drug class having this mode of action began with relacin and its analogs, which are modified variants of (p)ppGpp that block wide range of long RSH proteins [15, 16]. Recently, a structurally distinct inhibitor GSK-X9 has been discovered for $\mathrm{Rel}_{\mathrm{Mtb}}$ protein [14]. However, relacin was unable to inhibit the activity of the small alarmone synthetase RelQ from *Enterococcus faecalis* [17].

In this work, we continue the studies of DMNP (4-(4,7-dimethyl-1,2,3,4-tetrahydronaphthalen-1-yl)pentanoic acid), a synthetic analogue of the natural compound erogorgiaene. Our previous studies demonstrated that DMNP inhibits alarmone synthetases from *Mycolicibacterium smegmatis* and reduces its persistence and biofilm formation [18]. The purpose of this study is to determine the effect of DMNP on the small alarmone synthetase RelZ from *M. smegmatis* (MSMEG_5849) in vitro.

2 Materials and methods

2.1 DMNP synthesis

The synthesis of the DMNP compound and the structural model of its interaction with the alarmone synthetase binding pocket were described previously [18].

2.2 Cloning of the *relZ* gene sequence into an expression vector

To obtain an insert of the small alarmone synthetase *relZ* gene from *M. smegmatis*, relZ (pET) NdeI 5'-TGGAcAtATGCACCACCCCCCGT-3' и relZ (pET) HindIII 5'-aGCAagCTtGGCCTGCAGCTTCTCGA-3' primers were used for PCR with Phusion polymerase (Thermo Fisher Scientific) using *M. smegmatis* MC^2155 genomic DNA template. After ethanol precipitation, adenyl ends were added to the PCR fragment using Taq polymerase. TA cloning was used to insert the linear fragment into the plasmid pAL2 (Evrogen). The presence of the insert was confirmed by PCR with M13/pUC primers.

Then, restriction by NdeI and HindIII (Thermo Fisher Scientific) was carried out with the pET23b vector (Novagen) and the resulting pAL2-relZ intermediate vector. The corresponding sticky-end fragments were isolated by electrophoresis and gel purification. The purified fragments were mixed with T4 DNA ligase and used to transform the *Escherichia coli* BMH strain. As a result of selection and subsequent analysis by PCR, the successful construction of the pET23b-*relZ* plasmid was confirmed. The insert sequence in the purified pET plasmid was fully sequenced from primers T7 Promoter, T7 Terminator, and SAS seq 5'-CGCCTCGACCAAAAGTGAGCT-3'. No nucleotide substitutions were found.

2.3 Protein expression and purification

For protein production, the *E. coli* BL21(DE3) culture with the pET23b-*relZ* plasmid was used: 2 ml of the culture with OD600 0.6–1.0 was centrifuged and the medium was removed, resuspended, and transferred into a flask with 50 ml LB with ampicillin 100 mg/ml. After 2 hours of induction, the culture was centrifuged and the medium was removed. The cells were resuspended in 1 ml extraction buffer (20 mM Tris-HCl pH 7.4, 500 mM NaCl) and lysed with sonication on ice for 30 s at 35% amplitude 3 times with 30 s intervals. Then the total protein was centrifuged for 5 minutes at 15000×g at 4°C to separate the soluble and insoluble fractions. Protein electrophoresis was used to assess the expression efficiency of both fractions.

RelZ protein was purified using HisPur Ni-NTA Spin Purification Kit (Thermo Fisher Scientific). The soluble total protein fraction with 5 mM imidazole was applied to a column pre-equilibrated with a buffer (20 mM Tris-HCl pH 7.4, 500 mM NaCl, 5 mM imidazole). After that, columns were incubated with slow rotation on ice for 0.5-1 h. Unbound proteins were removed by centrifugation (2 min 700×g 4°C here and below). The bound protein was then washed with 2 ml of washing solution (20 mM Tris-HCl pH 7.4, 500 mM NaCl, 25 mM imidazole). The remaining protein fraction was eluted from the column with 200 µl of wash solution (20 mM Tris-HCl pH 7.4, 500 mM NaCl, 250 mM imidazole) in 3 steps. We then dialyzed the protein solution overnight with a buffer (20 mM Tris-HCl pH 7.4 and 500 mM NaCl) to remove imidazole. The resulting protein solutions were analyzed by protein electrophoresis. A NanoDrop 2000C spectrophotometer was used to determine protein concentrations. Protein extinction coefficients were calculated using the Expasy ProtParam tool.

2.4 Enzymatic reaction

Enzymatic reactions with RelZ, a small alarmone synthetase from *M. smegmatis*, were performed under the conditions described previously [19] with minor modifications. The reaction mixture included 40 mM Tris-HCl (pH 7.4), 500 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mM GDP, 1 mM ATP, 0.75 µM RelZ. To analyze the effect of increasing concentrations of DMNP on the ppGpp-synthesizing activity of RelZ, DMNP in 5% methanol or 5% methanol alone (control) or reaction buffer (control 2) was added to the reaction mixture. The reaction mixture was incubated for 1 h at 37°C. In accordance with the previously described protocol [20], the reaction was stopped by addition of 4N perchloric acid (1:10), centrifuged, 4-fold diluted, and analyzed by high performance liquid chromatography.

2.5 Statistical analysis and visualization of results

Python Seaborn package was used for visualization and statistical analysis of RelZ in vitro inhibition data. The Shapiro-Wilk test was used to assess the normality of data distribution. Data are presented as mean ± 95% confidence interval. Statistical significance of differences was assessed using t-test ($p < 0.05$). The Quest Graph IC_{50} Calculator was used to determine the half-maximal inhibition concentration (IC_{50}) for DMNP using a three parameter formula.

3 Results and discussion

The ability of DMNP to suppress the activity of the small alarmone synthetase RelZ from *M. smegmatis* under in vitro assay conditions is studied. In order to obtain the purified RelZ protein, the pET23b-*relZ* plasmid was used to express the RelZ protein. RelZ is characterized by a higher affinity for GDP than GTP [19]. Therefore, GDP and ATP were used as reaction substrates, so ppGpp and AMP were the reaction products, accordingly.

The activity of the RelZ enzyme was assessed by measuring two parameters: GDP substrate and ppGpp product concentrations. A linearly dependent function of the GDP solution concentration related to the area of the chromatographic peak was used to determine the molar concentration of GDP in samples, while the concentration of ppGpp is expressed in peak area units. Hence, the activity is calculated as the difference between the starting and ending concentrations of substrate or product per µM of protein per hour.

According to the results of the experiments, when DMNP concentration (0–1200 µM) increased in the reaction mixture, the RelZ enzyme ppGpp synthesizing activity decreased when the activity was measured by either the decrease of GDP substrate or the increase of ppGpp product concentrations (Fig. 1). This is consistent with previous experimental data produced in vivo indicating that RelZ protein from *M. smegmatis* is a DMNP target [18].

The relative concentration of half-maximal inhibition IC_{50} of RelZ activity by DMNP was determined using a nonlinear regression model equals to 303 µM when measured by the change in ppGpp concentration and 260 µM when measured by GDP. IC_{50} analysis for previously obtained data on the suppression of the activity of Rel_{Msm} from *M. smegmatis* in vitro [18] showed a value of 195 µM DMNP (activity was estimated by change of GTP substrate).

RelZ activity is increased by 10–18% when methanol is added at a concentration of 5% to the reaction mixture. The effect of increasing the (p)ppGpp synthetase activity with the addition of methanol was previously observed for Rel_{Msm} from *M. smegmatis* [18], for RelA from *E. coli* [21], and for PNPase/GPSI from *Streptomyces antibioticus* [22]. Since this effect is observed for alarmone synthetases of various groups (RelA, actRel, PNPase) [12], it can be caused by the effect of 5–10% methanol on the reaction rate as a solvent.

Taken together, the results we obtained demonstrate the ability of DMNP to inhibit not only the long alarmone synthetase Rel_{Msm} [18], but also the small alarmone synthetase RelZ from *M. smegmatis* in vitro, which has not been observed yet for other alarmone synthetase inhibitors.

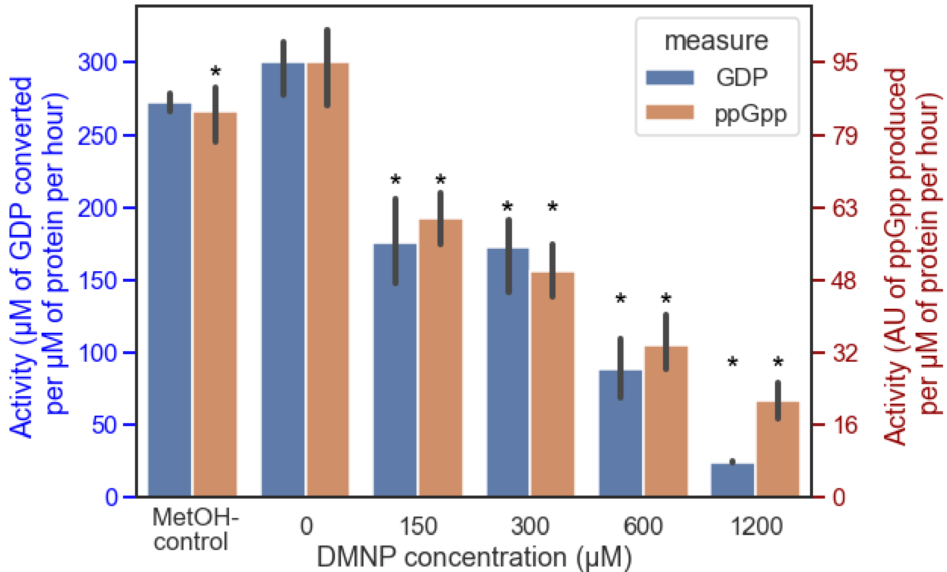

Fig. 1. The effect of DMNP inhibitor concentration on the in vitro activity of the small alarmone synthetase RelZ from *M. smegmatis*. The reaction without the addition of methanol and DMNP is designated MetOH- control. Enzyme activity values measured by concentration change of GDP substrate or ppGpp product in the reaction mixture are scaled to display the results in one figure for uniform comparison.

*statistically significant difference with 0 μM DMNP control (t-test, $p<0.05$).

4 Conclusion

According to the obtained experimental data, the DMNP compound is able to inhibit small alarmone synthetases. It is shown by using RelZ protein from *M. smegmatis*. Taking into account the IC_{50} values that we obtained for RelZ in comparison to the data on the activity of the long RSH protein Rel_{Msm}, DMNP concentrations of the same order of magnitude are required to achieve a similar inhibition effect on RelZ activity.

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