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DMNP, a Synthetic Diterpene Analogue, Reduces Mycobacterial Persistence and Biofilm Formation by Targeting (p)ppGpp Synthetases

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Summary

Bacterial persistence coupled with biofilm formation is directly associated with failure of antibiotic treatment of TB. We have now identified 4-(4,7-DiMethyl-1,2,3,4-tetrahydroNaphthalene-1-yl)Pentanoic acid (DMNP), a synthetic diterpene analogue, as a lead compound that was capable of suppressing persistence and eradicating biofilms in *Mycobacterium smegmatis*. By using two reciprocal experimental approaches – Δrel_{Msm} and $\Delta relZ$ gene knockout mutations versus rel_{Msm} and $relZ$ overexpression technique – we showed that both Rel_{Msm} and RelZ (p)ppGpp synthetases are plausible candidates for serving as targets for DMNP. *In vitro*, DMNP inhibited (p)ppGpp synthesizing activity of purified Rel_{Msm} in a concentration-dependent manner. These findings, supplemented by molecular docking simulation, suggest that DMNP targets the structural sites shared by Rel_{Msm}, RelZ and presumably by a few others as yet unidentified (p)ppGpp producers, thereby inhibiting persister cell formation and eradicating biofilms. Therefore, DMNP may serve as a promising lead for development of antimycobacterial drugs.

Introduction

The rise in antibiotic persistence coupled with biofilm formation is a major health concern directly associated with failure of antibiotic treatment of TB (Esteban and Garcia-Coca, 2017; Sharma et al., 2019). The majority of naturally occurring bacterial cells are thought to be in a dormant or a slow-growing state comparable to stationary phase (Gefen et al., 2014; Kolter et al., 1993) and exhibit high dependence of persister cell formation on (p)ppGpp (Prusa et al., 2018). The latter is a product of stringent factors Rel_{Mtb} and Rel_{Msm} for *Mycobacterium tuberculosis* (Mtb) or *Mycobacterium smegmatis* respectively (Prossliner et al., 2018; Prusa et al., 2018) that are essential for cell survival during the chronic phase of *in vivo* infection or stress impacts inducing stringent response (Primm et al., 2000). The absence of (p)ppGpp synthetases in mammalian cells (Mittenhuber, 2001) renders Rel protein an attractive target for developing new antimicrobials to combat bacterial persistence, including tuberculosis. Attempts to chemically synthesize antibiotics structurally similar to (p)ppGpp such as its analog relacin (Wexselblatt et al., 2012) had limited success (Prusa et al., 2018). Therefore, the identification of new inhibitors of (p)ppGpp synthesis is important for developing effective antitubercular medication.

We synthesized a variety of derivatives of erogorgiaene (Incerti-Pradillos et al., 2016), of which 4-(4,7- DiMethyl-1,2,3,4- tetrahydroNaphthalene-1-yl)Pentanoic acid (DMNP) emerged as the most active lead compound targeting mycobacterial persister cell formation. We now endeavored to identify the DMNP target(s) in mycobacterial cells and to elucidate its mechanism of action. Fast growing *M. smegmatis* that shares phenotypic and genetic similarities with Mtb was employed as a model organism (T et al., 2020). Batch cultures of this organism were used as a convenient tool for modeling persister cell formation during transition to stationary phase.

The findings of this study strongly support our hypothesis that DMNP targets the structural sites shared by mycobacterial Rel_{Msm}, RelZ and possibly by some other as yet unidentified (p)ppGpp producers, thereby inhibiting persister cell formation and eradicating biofilms. Therefore,

the data obtained might be used for developing novel antitubercular drugs to eradicate recalcitrant infections.

Keywords: diterpene, synthetic analogue, mycobacteria, persistence, biofilm formation, target, Rel_{Msm}, RelZ, (p)ppGpp synthetases

Results

Synthesis

The stereoselective synthesis of (–)-4-(4,7-dimethyl-1,2,3,4-tetrahydronaphthalene-1-yl)pentanoic acid (DMNP, **2**), an analogue of natural marine diterpene erogorgiaene **1**, is shown in Figure 1.

Cell Killing Activity of DMNP Is Potentiated in Standing Cultures of *M. smegmatis*

Activities of antibiotics currently used in clinical practice against *M. smegmatis* cells were compared at different growth phases (Figure 2A) with the activity of DMNP (**2**, MW = 260.38), a derivative of erogorgiane (**1**, MW = 270.50) (Figure 1, Figure 2B). Importantly, when *M. smegmatis* cells entered the stationary phase, there was an increase in the number of cells tolerant to the conventional clinical antibiotics tested, whereas DMNP decreased the number of survivor cells by more than 4-5 orders of magnitude (Figure 2C). Thus, it is reasonable to hypothesize that some target(s) specific for DMNP emerged in the cells in this period. Reduced energy state observed in these cells (Figure 2D) appears to be among the main reasons behind the deceleration of the growth-associated processes targeted by most of the clinical antibiotics, thus resulting in quiescent state and persister formation (Brauner et al., 2016; Keren et al., 2004; Torrey et al., 2016).

The stationary phase stress response is known to trigger the synthesis of Rel_{Msm}, – (p)ppGpp synthetase – that is known as a key player in bacterial stringent response and persistence (Gupta et al., 2016; Prusa et al., 2018). Furthermore, energy-consuming (p)ppGpp synthesis is one of the causes of the cell ATP hydrolysis raising the ADP/ATP ratio that also contributes to persister formation (Krasny and Gourse, 2004; Prossliner et al., 2018; Shan et al., 2017).

Using our constructed *M. smegmatis* mc²155 rel_{Msm}p::lux strain harboring rel_{Msm} promoter and luxAB reporter gene fusion, we showed that induction of rel_{Msm} gene expression occurred during potentiation of the DMNP killing effect (Figure 2C). These data prompted us to speculate that DMNP could target Rel_{Msm}.

The effect of chirality on the anti-persister activity of DMNP was assessed by comparing pure enantiomers with a racemic mixture (Figure 2E). (+)-DMNP did not show any noticeable activity against persister cell formation. In contrast, (–)-DMNP proved to be slightly more active than the racemic mixture. Taking into account only a partial advantage of (–)-DMNP over the racemic sample, the further experiments were continued with the racemate.

The reduction in the cell tolerance to DMNP was followed by its raise after the batch culture was cultivated for 24 h before being tested for tolerance to DMNP (Figure 2), which is apparently due to the increase over time in the proportion of the cells within the whole stationary-phase population that have already completed formation of persister state and whose survival no longer depended on the activity of persister forming factors, such as Rel_{Msm} protein, a suggested target for DMNP.

To shed further light on the anti-mycobacterial activity of DMNP, we compared time-killing curves obtained for both exponential and stationary-phase *M. smegmatis* cells exposed to various antibiotics (Figure 3). All of these curves exhibited the typical biphasic character indicating that the fast killing of regular, susceptible cells was followed by survival of persisters, keeping their number at a relatively stable level (Balaban et al., 2019; Brauner et al., 2016; Lewis, 2010). The exponentially growing cells were killed by clinical antibiotics faster than the stationary-phase cells predominantly presented by persisters tolerant to high antibiotic concentrations (20-200 MIC) (Figure 3, see also Table S3). In contrast, DMNP was highly efficient when cells entered the stationary phase. These data suggest that when *M. smegmatis* cells enter the stationary phase they activate synthesis of cell component(s) that are targeted by DMNP.

DMNP Targets Both Rel_{Msm} and RelZ Proteins

Considering literature data (Arai et al., 2014; Incerti-Pradillos et al., 2016; Rodríguez and Ramírez, 2001) and the results presented in the previous section, we proposed that DMNP is likely to affect the Rel_{Msm} activity, thus making the cells vulnerable to stationary phase stresses.

To validate this assumption, we primarily employed a widely accepted concept that high-affinity binding of an antimicrobial agent to the abundant specific target, amplified by gene overexpression, reduces the number of the agent's free molecules to the level insufficient to bind the remaining active receptor units, thereby notably decreasing antibacterial effect (Arai et al., 2015; Sato et al., 2010) (Figure 4A). For this, we have primarily used the wild-type *M. smegmatis* mc²155 strain transformed by pMind-*rel*_{Msm} plasmid, whose availability to overexpress *rel*_{Msm} was confirmed by preliminary experiments (Figure S1). We showed that induced overexpression of *rel*_{Msm} in cells exposed to DMNP, in contrast to the cells exposed to clinical antibiotics, produced at least two orders of magnitude higher number of tolerant cells relative to the uninduced counterpart (Figure 4B). Furthermore, a similar inactivation of DMNP was also achieved when Rel_{Msm}/DMNP interaction was performed with supplementation of *M. smegmatis* cultural medium with an abundant quantity of purified recombinant Rel_{Msm} protein before addition of DMNP (Figure 4C). In contrast, unspecific BSA protein added in the equivalent concentration failed to cause a similar inactivation of DMNP (Figure S2G).

Rel_{Msm} protein is known to be responsible for the persister cell formation (Steinchen and Bange, 2016). However, now at least one more (p)ppGpp synthetase, RelZ, is known for *M. smegmatis* that constitutively make, but not hydrolyze, (p)ppGpp (Petchiappan et al., 2020). This protein is referred to as a short alarmone synthetases (SASs), whose active centers are known to bear similarity to those of a single catalytic domain integrated in the long multidomain Rel proteins (Kushwaha et al., 2019). Using *M. smegmatis* mc²155 pMind-*relZ* strain, we have now shown that *relZ* overexpression, like that of *rel*_{Msm}, led to a significant decrease in the antibacterial effect of DMNP on *M. smegmatis*, thereby demonstrating the RelZ ability to complement Rel_{Msm} in its

interaction with DMNP (Figure 4A, D). Therefore, both Rel_{Msm} and RelZ are of great interest as the proteins promoting persister cell formation that can be viewed as probable targets for DMNP. It should be noted, however, that in addition to the main (p)ppGpp synthetases (Rel_{Msm} and RelZ) there are some other (p)ppGpp producers in *M. smegmatis* cells (Petchiappan et al., 2020). These so far unidentified (p)ppGpp producer(s) may be hypothetically referred to as protein(s), i.e. SASs, that have evolved from the synthetic (SYN) domain integrated in the long multi-domain Rel proteins (Kushwaha et al., 2019). Bacterial cells may carry multiple (p)ppGpp-synthesizing SASs in the same genome that are known to contain similar catalytic domains and are widely spread among different species (Jimmy et al., 2020). These hypothetic producer(s) are responsible for contributing to the total cell pool of (p)ppGpp and persistence, and therefore could be potentially considered as additional target(s) for DMNP.

Indeed, DMNP at a sufficiently high concentration completely suppressed persistence within 12-24 h, and therefore inactivated all (p)ppGpp producers in the parental and the knockout strains, including those so far unidentified (Figure 4H). Alternatively, gradual addition of Δrel_{Msm} followed by $\Delta rel_{Msm}\Delta relZ$ knockout mutations to the parental strain exposed to relatively low DMNP concentrations resulted in a proportional decrease in cell potential to form persister cells (parent $>\Delta rel_{Msm}>\Delta rel_{Msm}\Delta relZ$) (Figures 4E, 4F, 4G). The remaining level of persister formation by knockout strains $\Delta rel_{Msm}\Delta relZ$ observed at low concentration of DMNP was apparently maintained due to the residual activity of the extant (p)ppGpp synthetases, other than Rel_{Msm} and RelZ. Therefore, this suggests that yet unidentified (p)ppGpp producer(s) should be also viewed as DMNP targets, in addition to Rel_{Msm} and RelZ.

It should be noted that the samples withdrawn from the original batch culture at later stages (48-72 h) showed an incremental growth of subpopulation of tolerant cells that have already completed persister formation by these time points and hence were less dependent on the (p)ppGpp producers that are inhibited by DMNP.

The number of persisters tolerant to DMNP in *M. smegmatis* Δrel_{Msm} or $\Delta rel_{Msm}\Delta relZ$ transformed with pMind-*rel*_{Msm} returned roughly to that of the parental strain when the plasmid was overexpressed (Figures 4I, 4J). Similar results were observed in both single and double knockout mutants transformed with pMind-*relZ* (Figures 3L, 3M), thereby supporting the idea that complementation by RelZ rescued the phenotypes of Δrel_{Msm} strain to a certain extent and therefore both Rel_{Msm} and RelZ are targeted by DMNP (Figure 4A).

Exponentially growing *M. smegmatis* cultures, irrespective of whether *rel*_{Msm} was overexpressed or not, did not show any measurable differences in the number of cells surviving after exposure to DMNP (Figure S2). However, when the cells entered the stationary phase, a clear increase in the negative effect of DMNP on the cell survival became apparent. This provides further support to our notion that killing effect of DMNP is eventually mediated through stationary phase stresses, whose impacts are controlled by the Rel_{Msm} activity.

Additionally, we found that pMind-*rel*_{Msm} induction in *M. smegmatis* increased the number of cells tolerant not only to DMNP, but also to Vitamin C (Figure 4K), which was recently shown to also target the Rel_{Msm} protein (Syal et al., 2017). This agrees with our data on the *rel*_{Msm} overexpression to establish the target for DMNP.

Furthermore, we exposed to DMNP the exponentially growing *M. smegmatis* cells that were preliminary treated by either streptomycin or rifampicin (Figure S3) and found significant suppression of persister formation, down to eradication (Figure S3A). Therefore, the efficiency of clinical antibiotics may be significantly enhanced through banding them together with DMNP to eradicate recalcitrant infections caused by persister forms of Mtb.

DMNP Inhibits (p)ppGpp-Synthesizing Activity

The results presented so far seem to indicate that the functional activity of Rel_{Msm} may be affected by DMNP. To test this, we purified Rel_{Msm} protein by using His-tag technology (See section METHOD DETAILS / His-tag Rel_{Msm} Protein Purification and DMNP Activity Assays). Enzyme activity of pure Rel_{Msm} protein was measured through changes in the concentrations of the

substrate (GTP) and the product ((p)ppGpp), detected by HPLC analysis, in the course of the *in vitro* reaction (Figure 5; see also Figure S7). The methanol-containing control was used in these measurements. Notably, trace amounts of GDP remained unchanged throughout the course of the reaction, thus excluding unspecific GTP hydrolysis (inset in Figure 5A). The data showed that DMNP inhibited the (p)ppGpp-synthesizing activity of Rel_{Msm} protein in terms of substrate (GTP) consumption and (p)ppGpp production in a concentration-dependent manner (Figure 5B-C).

Next, for gaining preliminary insight, we performed molecular docking simulations (METHOD DETAILS/Molecular Docking Assay) to pinpoint a plausible location of the DMNP binding site on Rel_{Msm} and RelZ proteins surfaces (Figure 6A) and to suggest a potential mechanism of inhibition of their (p)ppGpp-synthesizing activity by DMNP.

For *M. smegmatis*, crystal structure of Rel protein is not available yet; a PDB search for *M. tuberculosis* (PDB ID 5XNX) identified a structure of the Rel_{Mtb} protein (Singal et al., 2017) that is closely related to *M. smegmatis* and share large identities (92%) in the Rel proteins amino acid sequences. Since the substrate was not present in the protein structure, the calculations of the GTP interaction with amino acids (Y300, Y309, H313, Q327) (Figure 6E) of the active site were based on literature data.

To localize a plausible DMNP binding site, we analyzed surface of the N-terminal domain of the Rel_{Mtb} protein in the crystal structure and considered cluster #1 as a promising candidate due to the highest binding energy (-7.00 kcal/mol) and the location adjacent to GDP/GTP binding site (Figure 6B). In this arrangement, at least one of the amino acids (HIS177) would interact with both the DMNP inhibitor and the GTP substrate. We also considered TYR306 as an important amino acid due to its proximity to the amino acids of the active site (Y300, Y309, H313, Q327). Therefore, it seems likely that exposure of Rel protein to DMNP would interfere with its binding to GTP, thereby decreasing the (p)ppGpp-synthesizing activity.

Further, it is also reasonable to assume that RelZ, the only SAS identified thus far in *M. smegmatis*, could be targeted by DMNP in a similar fashion because, like other SASs, RelZ is

analogous to the SYN domain of the multidomain Rel protein (Kushwaha et al., 2019) (Figure 6A,E). *M. smegmatis* RelZ crystal structure also is not available yet, but there are structural data for several other small alarmone synthetases belonging to different species (Kushwaha et al., 2019). For docking, we chose *Staphylococcus aureus* RelP (YwaC, SAS2) protein (PDB ID 6EWZ) (Manav et al., 2018) because its crystal structure shows interactions with substrates in the pre-catalytic state. The results of these calculations are more difficult to extrapolate to mycobacteria due to the greater evolutionary distance between the genera *Mycobacterium* and *Staphylococcus*, however, these proteins have 29% identity at their active sites.

Searching for the structural similarity of the DMNP binding pockets specific for Rel_{Msm} and RelZ proteins (Figure 6A), the entire surface of RelP (SAS2) protein from *Staphylococcus aureus* was analyzed with Achilles Blind Docking Server and was compared with the discussed above Rel_{Mtb} docking data. The screen revealed a cluster (-5.40 kcal/mol) (Figure 6D) showing some common features with Rel_{Mtb} - DMNP binding pocket. Similar to the previous calculations, DMNP could affect the amino acids involved in the GTP interaction with SAS protein (LYS147 and ASN149).

To predict potential interaction of DMNP with both long RSH and SAS proteins in structurally similar binding pockets, we also carried out BLASTp alignment of Rel_{Mtb} and RelP (SAS2) active sites sequences (Figure 6C). The results indicate that amino acids capable of interacting with DMNP are aligned in the adjacent locations (TYR306 from Rel_{Mtb} and LYS147/ASN149 from RelP) suggesting that in both instances DMNP dockings could occur in similar binding pockets.

The presented data indicate that DMNP could target both Rel_{Msm} (long RSH) and RelZ (SAS) proteins, thereby suppressing persister cell formation through the concentration-dependent inhibition of (p)ppGpp synthetizing activities. Molecular docking simulation data supports the idea that amino acid residues involved in the GTP binding or located near the active sites of both Rel_{Msm}

and RelZ could be involved in the interactions of DMNP with these proteins leading to inhibition of their activities.

DMNP Eradicates Biofilms and Affects Cell Sliding Motility

Sliding motility, biofilm formation and related persister cell formation in mycobacteria referred to as virulence factors contribute to their expansion and resistance to the immune response of a host organism (Dong et al., 2012; Rao et al., 2009).

Based on the reported data (Petchiappan et al., 2020), we hypothesized that DMNP could interfere with the biofilm formation by targeting both (p)ppGpp synthesizing proteins Rel_{Msm} and RelZ. *M. smegmatis* forms architecturally complex biofilms (pellicles) at the air/liquid interface of standing cultures that were used as a convenient model for studying the changes in the structural and pellicle mass (dry weight) characteristics of biofilms, depending on the gene structure of *M. smegmatis* cells and their exposure to DMNP. For this purpose, parental strain of *M. smegmatis* mc²155 (WT) and two of its derivatives harboring single (Δrel_{Msm}) and double ($\Delta rel_{Msm} \Delta relZ$) knockout mutations were used, as well as their pMind-*rel*_{Msm} and pMind-*relZ* overexpressing transformants (Figure 7A – D; see also Figure S4).

The results showed that single (Δrel_{Msm}) and double ($\Delta rel_{Msm} \Delta relZ$) knockout mutants formed atypical biofilms: cells clumped together to form floating islands rather than a single continuous layer across the surface. However, overexpression of *rel*_{Msm} or *relZ* in either Δrel_{Msm} or $\Delta rel_{Msm} \Delta relZ$ knockout strains of *M. smegmatis* recovered the biofilm surface, similar to that of the parental strain (Figure 7A).

DMNP inhibited biofilm formation in a concentration-dependent manner (Figure 7B; see also Figure S4), thereby producing the pellicle disassembly in WT, similar to that formed in single (Δrel_{Msm}) or double ($\Delta rel_{Msm} \Delta relZ$) knockout strains, even when they were not exposed to DMNP (Figure 7A).

M. smegmatis cells exposed to different DMNP concentrations showed decreased ability to form pellicles proportionally to the gradual addition of single Δrel_{Msm} and double $\Delta rel_{Msm}\Delta relZ$ knockout mutations in a concerted manner, similar to that observed for persister cell formation: WT> Δrel_{Msm} > $\Delta rel_{Msm}\Delta relZ$ (Figure 7B, see also Table S4). The fact that even double knockout mutation ($\Delta rel_{Msm}\Delta relZ$) by itself still fails to completely suppress the formation of biofilms, akin to persister cell formation, supports the notion that *M. smegmatis* cells contain some other (p)ppGpp producer(s), yet unidentified, in agreement with the report by Petchiappan et al (Petchiappan et al., 2020). Furthermore, the activity of these producer(s) can be completely suppressed by sufficiently high concentration of DMNP (Figure 7B; see also Figure S4) and therefore, as already discussed, they can be considered as likely additional targets(s) for DMNP.

Alternatively, overexpression of *rel_{Msm}*, or to a lesser extent *relZ*, has partially rescued biofilm formation by either shifting the minimal biofilm destructive concentrations (MBDC, $\mu\text{g/mL}$) to higher values in WT exposed to DMNP or increasing p.m. values in WT and knockout strains (Figure 7B, see also Figure S4 and Tables S4, S5). However, planktonic growth was still detectable in the range from minimal biofilm destructive concentrations to minimal bactericidal concentrations (MBC) (Figure 7C; see also Table S6). Both MBDC and MBEC (minimal biofilm eradication concentration) actually have the same nature. The latter is commonly used to characterize biofilm eradication activity of antibacterials through MBC/MBEC ratios that for the biofilm-eradicating agents typically have values in the range of 1 to 3 (Garrison and Huigens, 2017). Considering the high levels of the MBC/MBDC ratios observed here (about 2 to 3), DMNP can be viewed as a biofilm-eradicating agent (Figure 7C). Furthermore, this DMNP property was dependent on the *rel_{Msm}* and *relZ* genes: the MBC/MBDC ratio increased from 2.25 for WT to 3.25 for knockout strains (Δrel_{Msm} and $\Delta rel_{Msm}\Delta relZ$) and returned to the original values in response to *rel_{Msm}*-overexpression (Figure 7C). We compared the results of the MBC/MBDC measurements with those for the MBC/MBEC ratios for WT that were conducted with the MBEC™ Biofilm Inoculator (Innovotech, Canada) for DMNP and the conventional antibiotics (Table S3). Although DMNP

exhibited lower MBC/MBEC value (1.25) for WT strain as compared to MBC/MBDC (2.25), it still falls into the category of biofilm-eradicating agents, unlike the conventional antibiotics we tested (Garrison and Huigens, 2017).

These results were validated by experiments showing that DMNP could selectively inhibit biofilm formation in a concentration-dependent manner over a range of concentrations (0.07 - 0.57 mM) that had no discernible inhibitory effect on the growing planktonic cells (Figure 7D). However, unlike DMNP, all of the conventional antibiotics tested in this work, as exemplified by rifampicin (Figure 7E), were equally effective against both planktonic and biofilm *M. smegmatis* cells. This confirms our findings that DMNP preferentially inhibits biofilm formation versus planktonic growth and therefore is consistent with the idea that it targets Rel_{Msm} and RelZ proteins. Such alterations form “macroscopic surface phenotype” displaying a limited cell sliding motility and rough colony morphology (Gupta et al., 2016; Gupta et al., 2015).

Measurement of Zeta potential in *M. smegmatis* cells exposed to DMNP displayed a concentration-dependent reduction in the value of the cell surface negative charge (Figure S5A). Together with the increase in the cell wall hydrophobicity, this indicates that DMNP, akin to Δrel_{Msm} mutation, is able to affect the cell surface properties. Furthermore, DMNP contributed to the formation of rough colony morphology in both WT and knockout derivatives (Figure 7F), thereby emulating macroscopic cell surface phenotype that was observed specifically for Δrel_{Msm} *M. smegmatis* cells (Gupta et al., 2015). In contrast, conventional antibiotics do not produce similar alterations (Figure S6).

We have now demonstrated that DMNP reduced the sliding motility of *M. smegmatis* cells owing to its ability to impair cell surface (Figure 7G). The area of sliding colonies (mm²) (See section METHOD DETAILS/ Cell Sliding Motility) decreased while their specific optical density (SOD) proportionally increased over a range of subinhibitory DMNP concentrations (0.02 – 0.05 mM) as compared to the control (Figure S5B). The data indicate that the limitation of sliding motility at these concentrations occurred with no discernible inhibition of cell growth as suggested

by an increase in SOD that was reversely proportional to the area of sliding colony. However, a further increase in DMNP concentration led to decrease in SOD, thus exhibiting partial inhibition of cell growth occurring in addition to inhibition of sliding motility (Figure S5B).

The results show that DMNP, by interfering with functions of the Rel_{Msm}, RelZ and presumably of some other unidentified (p)ppGpp producer(s), inhibits them in a manner similar to phenotype of Δrel_{Msm} or/and $\Delta relZ$ knockout mutations. This adds to the body of evidence that both Rel_{Msm} and RelZ could be indeed targeted by DMNP.

Discussion

Bacterial persistence coupled with biofilm formation are associated with the failure of antibiotic treatment of TB that poses a unique challenge for developing new therapies. Preliminary screening of a variety of synthetic derivatives of marine diterpene erogorgiaene identified 4-(4,7-DiMethyl-1,2,3,4- tetrahydroNaphthalene-1yl)Pentanoic acid (DMNP) as the most active antipersister lead compound (Figure 2B), which was used in this study.

Presently, one of the most important regulators of bacterial persistence involved in cell adaptation to stationary-phase stresses is a hyperphosphorylated guanosine analog (p)ppGpp (Brown et al., 2016). In mycobacteria, (p)ppGpp is synthesized and is broken down by the dual-function enzyme Rel that is referred to as RSH (RelA/SpoT homolog) proteins (Atkinson et al., 2011; Gupta et al., 2016).

Recently, the main Rel_{Msm} (p)ppGpp synthetase/hydrolase in *M. smegmatis* was complimented by RelZ protein that is referred to as short alarmone synthetases (SASs). RelZ, in addition to ability of maintaining basal (p)ppGpp level, contains a RNase HII domain that is implicated in R-loop induced stress response (Petchiappan et al., 2020). Furthermore, Petchiappan et al found that the $\Delta rel_{Msm}\Delta relZ$ *M. smegmatis* strain is still capable of synthesizing (p)ppGpp, thereby indicating the presence of some other (p)ppGpp producer(s) in this organism (Petchiappan et al., 2020). Although they have not been so far identified and characterized in *M. smegmatis*, these

(p)ppGpp producer(s) may be putatively referred to as SAS(s) because a large number of bacterial species are known to carry multiple (p)ppGpp-synthesising SASs in the same genome (Jimmy et al., 2020). All currently known short alarmone synthetases have evolved from the synthetic (SYN) domain integrated in the long multi-domain Rel proteins reflecting their similarity to its single catalytic domain (Kushwaha et al., 2019).

The literature data and our initial results indicated that, in addition to Rel_{Msm} and RelZ, some other, so far unidentified (p)ppGpp producer(s) are capable to contribute to the formation of persister cells and can be targeted by DMNP. Recently, formation of biofilms containing persister cells responsible for the recalcitrance of mycobacterial infections (Sharma et al., 2014) was also shown to be a (p)ppGpp-dependent process (Pacios et al., 2020). Furthermore Δrel_{Msm} and $\Delta rel_{Msm}\Delta relZ$, single and double gene knockout mutants, are defective in biofilm formation and have altered cell surface properties (Petchiappan et al., 2020). Therefore, our hypothesis that (p)ppGpp synthetases (Rel, SASs) represent the multiple targets for DMNP seems to be also relevant for the biofilm formation.

We verified this hypothesis by using two reciprocal experimental approaches, Δrel_{Msm} and $\Delta relZ$ gene knockout mutations versus rel_{Msm} and $relZ$ overexpression technique. Accordingly, at sufficiently high concentration, DMNP exhibited complete suppression of persistence and biofilm formation mediated through the inhibition of all (p)ppGpp producers functioning in the parental strain, including Rel_{Msm}, RelZ and so far unidentified species (Figure 4H; Figure 7; see also Figure S4). At the same time, sequential addition of Δrel_{Msm} and $\Delta rel_{Msm}\Delta relZ$ knockout mutations to the cells that were then exposed to DMNP in the range of lower concentrations resulted in a proportional decrease in the cell potential to form persister cells and biofilms (parent > Δrel_{Msm} > $\Delta rel_{Msm}\Delta relZ$). It should be noted, however, that in this case the remaining level of persister and biofilm formation was maintained at the expense of the residual activity of the extant (p)ppGpp synthetase(s), other than Rel_{Msm} and RelZ (Figure 4E,F,G; Figure 7; see also Figure S4; Table S4).

Conversely, overexpression of *rel*_{Msm} and *relZ* genes in Δ *rel*_{Msm} or Δ *rel*_{Msm} Δ *relZ* knockout strains transformed with either pMind-*rel*_{Msm} or pMind-*relZ* plasmids reverted formation of persisters and biofilms close to their natural levels (Figure 4 I,J,L,M; Figure 7; see also Figure S4; Table S5). Similar data were obtained when the protocol was reproduced with Vitamin C (Figure 4K), another agent capable to competitively inhibit (p)ppGpp-synthesizing activity of Rel_{Msm} (Syal et al., 2017).

These data indicate that both Rel_{Msm} and RelZ can be viewed as the targets for DMNP, which are able to at least partially complement each other in the formation of persister cells and biofilms. Therefore, both approaches realized in this work support our hypothesis that DMNP can target not only Rel_{Msm} and RelZ proteins but also other yet unidentified (p)ppGpp synthetase(s).

Finally, we purified recombinant Rel_{Msm} protein and measured its (p)ppGpp-synthesizing activity in the presence of DMNP at different concentrations with reference to the control with no antibiotic (Figure 5). The data revealed that the activity of Rel_{Msm} is inhibited by DMNP in a concentration-dependent manner, thus supporting the conclusion that Rel_{Msm} is indeed targeted by DMNP.

The experimental findings and preliminary computational data obtained from molecular docking modeling (Figure 6) strongly suggest that DMNP can bind to the structurally similar active sites in the proteins, collectively referred to as Rel and SAS, through a common mechanism, thereby inhibiting their (p)ppGpp-synthesizing activities.

We showed here that DMNP used in combination with either streptomycin or rifampicin significantly enhanced their killing effect on *M. smegmatis* cells by interfering with persister cell formation (Figure S3). Furthermore, DMNP, unlike the conventional antibiotics, can be viewed as a biofilm-eradicating agent characterized by a favorable MBC/MBDC ratio (about 2 to 3) (Figure 7C) or MBC/MBEC ratio of 1.25 (Table S3) (Garrison and Huigens, 2017). Therefore, antipersistent substances elaborated on the basis of DMNP could be used potentially in combination with conventional antibiotics to eradicate chronic infections including tuberculosis.

Collectively, both direct and indirect observations reported here for *M. smegmatis* cells contribute to a body of evidence that the mechanism of the antimycobacterial effect of DMNP involves its binding to (p)ppGpp synthetases (primarily Rel_{Msm} and RelZ proteins) leading to inhibition of the processes relevant to persistence and biofilm formation. Therefore, DMNP may serve as a promising lead compound for developing antitubercular therapies relying on suppression of persister cell formation and thus effective against chronic and recalcitrant tuberculosis infections.

Significance

Synthetic analogue of marine diterpene erogorgiaene, 4-(4,7-Dimethyl-1,2,3,4-tetrahydronaphthalene-1-yl)pentanoic acid (DMNP), emerged as a promising lead compound targeting mycobacterial persister cell formation. To identify the DMNP target(s), fast growing *Mycobacterium smegmatis* was used as a model organism that shares phenotypic and genetic similarities with *Mtb*. Hyperphosphorylated guanosine analogue (p)ppGpp, the product of Rel_{Msm} and RelZ alarmone synthetases is known to be one of the most important regulators of bacterial persistence. Cells treated with DMNP exhibited a phenotype similar to that caused by the loss of functions by alarmone synthetases, including persistence and biofilm formation. *In vitro* experiments demonstrated that (p)ppGpp-synthesizing activity of purified recombinant Rel_{Msm} protein is inhibited by DMNP in a concentration-dependent manner. Finally, molecular docking simulations suggest that DMNP could be binding to Rel_{Msm} and RelZ proteins in close proximity to their highly similar GTP binding sites, thereby inhibiting their (p)ppGpp-synthesizing activity. Therefore, the data obtained might be used for developing antitubercular drugs to eradicate recalcitrant infections.

Acknowledgements

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Author Contributions

A.E.R., A.G.T. and A.V.M. initiated the study and directed the project. A.E.R., V.Yu.V., S.A.Sh. and A.V.M. carried out the stereoselective synthesis and characterized the diterpenoids used in this study. N.M.K. evaluated killing potency of DMNP and persister cell formation of *M. smegmatis* throughout the experimental work and assessed *relMsm* and *relZ* gene expression. R.Yu.S. prepared

all genetic constructs used in this work, including pMind-*rel*_{Msm} and pMind-*relZ* overexpressing plasmid, *rel*_{Msm}::*luxAB* gene fusion, *rel*_{Msm} and *relZ* knockout strains and pET23b-*rel*_{Msm} (*His*) plasmid for His-Tag protein purification, and performed molecular docking simulation to predict the probable binding mode between DMNP and Rel_{Msm} and RelZ proteins. Biofilm eradicating assays, antibiotic susceptibility, as well as Zeta potential and hydrophobicity assays were performed by L.Yu.N. Rel_{Msm} protein His-Tag purification and *in vitro* (p)ppGpp synthesis assay, as well as HPLC analysis of nucleotides were carried out by A.V.A. Effect of DMNP on *M. smegmatis* pellicle formation, sliding motility and colony morphology assays, were performed by I.V.T. A.G.T. composed the paper with input from A.V.M. and A.E.R. All authors read and approved the final manuscript.

Main figure titles and legends

Figure 1. Stereoselective synthesis of (–)-DMNP 2

(See METHOD DETAILS/Chemical Synthesis)

Figure 2. Negative Effect of DMNP on Cell Survival Is Potentiated during Transition to Stationary Phase

(A) Experimental design (See METHOD DETAILS /Cell Viability and Persister Cell Number; Overexpression Assays).

(B) Structures of (–)-DMNP (1) and (+)-DMNP (2).

(C) Dynamics of cells exposed to conventional antibiotics versus those treated by racemic DMNP in relation to the expression level of *rel*_{Msm}.

Culturable cells – the control cells untreated with antibiotics or DMNP; bioluminescence – the expression level of *rel*_{Msm}; antibiotics were added to final concentrations (mM): 1.06 DMNP, 1.17 rifampicin (Rif), 0.094 tetracycline (Tetr), 0.0034 streptomycin (Str)

(D) Decline in cell energy state as an indicator of persister cell formation.

The EC (adenylate energy charge) and ADP/ATP values are represented vs the growth curve.

(E) Comparative dynamics of tolerant cells in *M. smegmatis* mc²155 batch culture survived after exposure to (±)-DMNP, (–)-DMNP- and (+)-DMNP, all in concentration of 1.06 mM. Experimental data are presented as mean values of at least three independent experiments, and the error bars represent the standard error of the mean of the independent measurements.

Figure 3. DMNP Cell Killing Activity, Unlike Conventional Antibiotics, Is Maximal in Standing Cultures

(A) Experimental design. For the cultivation conditions, see METHOD DETAILS /Cell Viability and Persister Cell Number.

(B, C) Rifampicin 1.17 mM.

(D, E) Tetracycline 0.094 mM.

(F, G) Streptomycin 0.0034 mM.

(H, I) DMNP 1.06 mM.

Experimental data are presented as mean values of at least three independent experiments, and the error bars represent the standard error of the mean of the independent measurements.

Figure 4. DMNP Targets both Rel_{Msm} and RelZ proteins of *M. smegmatis*

(A) Experimental design. For details see METHOD DETAILS / Cell Viability and Persister Cell Number.

(B) Overexpression of *rel*_{Msm} in *M. smegmatis* cells results in the decrease in DMNP antibacterial effect unlike that of conventional antibiotics. Following concentrations of antibiotics were used (mM): 0.96 DMNP, 0.0034 streptomycin, 0.094 tetracycline, 1.17 rifampicin. For details see METHOD DETAILS / Overexpression Assays.

(C) Mitigation effect of exogenously added recombinant Rel_{Msm} protein on DMNP activity against *M. smegmatis*. For details see METHOD DETAILS / His-tag Rel_{Msm} Protein Purification and DMNP Activity Assays.

(D) Overexpression of *relZ* in *M. smegmatis* cells results in the decrease in DMNP antibacterial effect. For details see METHOD DETAILS / Overexpression Assays.

(E, F, G, H) Concentration-dependent effect of DMNP against *M. smegmatis* is potentiated in parallel with addition of chromosome knockout mutations in *rel*_{Msm} and *relZ* alarmone synthetase genes.

(I, J) Overexpression of *rel*_{Msm} in *M. smegmatis* Δ *rel*_{Msm} pMind-*rel*_{Msm} or Δ *rel*_{Msm} Δ *relZ* pMind-*rel*_{Msm} strains mitigates DMNP effect.

(K) Growth phase-dependent effect of *rel*_{Msm} overexpression on survival of *M. smegmatis* mc²155 pMind-*rel*_{Msm} cells exposed to Vitamin C. The strain and growth conditions were used as in METHOD DETAILS/ Overexpression Assays, except the cells were taken from both induced and uninduced batch cultures at the following time points: 0-4 h (exponential phase), 6 h (the onset of transition to stationary phase), 24 h (early stationary phase), and 48 h (mid-stationary phase). Vitamin C was added to the cells in the volume of 20 μL to the final 75 mM concentration.

(L, M) Overexpression of *relZ* in *M. smegmatis* Δ*rel*_{Msm} pMind-*relZ* or Δ*rel*_{Msm}Δ*relZ* pMind-*relZ* strains mitigates DMNP effect.

Data are presented as mean ± SEM values of at least three independent experiments.

Figure 5. DMNP Inhibits (p)ppGpp-Synthesizing Activity of Rel_{Msm} Protein in a Concentration-Dependent Manner

(A) Rel_{Msm} activity is quantified by decrease in the concentration of the substrate (GTP). Data are presented as mean ± SEM values of at least three independent experiments each performed in triplicate. Asterisks indicate significant differences (*t*-test, *p* < 0.05) compared to the control with added methanol (met); cross indicates significant difference compared to the control with no added methanol (contr).

The box in the top right corner shows HPLC analysis of guanosine nucleotides in the samples taken from the enzymatic system at different time points. The data are shown as overlapping HPLC traces taken at the specified time points (UV detection at 254 nM, See also Figure S7).

(B) Dynamics of the substrate (GTP) concentration in the process of the (p)ppGpp synthesis catalyzed by purified Rel_{Msm} protein depending on the DMNP concentration.

(C) Dynamics of the product ((p)ppGpp) concentration synthesized by purified Rel_{Msm} protein depending on the DMNP concentration.

For details see METHOD DETAILS / His-tag Rel_{Msm} Protein Purification and DMNP Activity Assays

Figure 6. Molecular Docking Modelling of DMNP Targeting Long RSH and SAS (p)ppGpp Synthetases

(A) DMNP binds Rel_{Msm} and RelZ from *M. smegmatis* in proximity to the active sites due to the structural similarities in synthetase domains (Scheme). HD – hydrolase domain; SYN – synthetase domain; REG – regulatory lobe, which includes TGS, AH, ZFD and ACT domains; RHII – RNase HII domain. Images were computationally generated and may not be consistent with actual Rel_{Msm} and RelZ proteins structures, which are not available at the time (See METHOD DETAILS/ Molecular Docking Assay).

(B) Molecular docking simulation shows a probable binding mode between DMNP and *M. tuberculosis* Rel_{Mtb} protein. Amino acids (AAs) interacting with DMNP only are colored in orange, AAs interacting with GTP only are colored in purple, AAs interacting with both GTP and DMNP are colored in red. HIS177 can interact with both DMNP (inhibitor) and GTP (substrate). TYR306 might be crucial to DMNP activity due to a close location to the AAs of the active site. Both Rel_{Mtb}-DMNP and Rel_{Mtb}-GTP interactions are calculated using molecular docking.

(C) BLASTp alignment of Rel_{Mtb} and SAS2 amino acid sequences shows that amino acids binding with DMNP close to the active site belong to similar binding pockets.

(D) Molecular docking simulation predicts a probable binding mode between DMNP and *S. aureus* SAS2 protein. AAs are colored as in (B). LYS147 and ASN149 can interact with both DMNP (inhibitor) and GTP (substrate). SAS2-DMNP interactions were calculated using docking, while SAS2-GTP interactions were shown in the SAS2 crystal structure.

(E) AAs that contribute to Rel_{Seq}-GDP interaction are marked by golden asterisks at the bottom of the alignment of Rel proteins from different species to identify Rel_{Mtb} residues contributing to the interaction with the substrate.

Figure 7. Effect of DMNP on Biofilm Formation and Cell Surface Phenotype of *M. smegmatis* mc²155

For detail see in METHOD DETAILS / Antibiotic Susceptibility and Biofilm-Eradication Assays.

(A) Biofilm disassembly produced by DMNP or single (Δrel_{Msm}) and double ($\Delta rel_{Msm}\Delta relZ$) knockout mutations is rescued by overexpression of either *rel_{Msm}* or *relZ* genes (Photo).

(B) Concentration-dependent inhibitory effect of DMNP on biofilm formation in WT strain is potentiated by single (Δrel_{Msm}) and double ($\Delta rel_{Msm}\Delta relZ$) knockout mutations. Enhancement of DMNP inhibitory effect on biofilm formation produced by Δrel_{Msm} mutation is rescued by *rel_{Msm}*-overexpression (hatched area). For detail see Tables S3, S4. The area of difference in p.m. values between of Δrel_{Msm} and $\Delta rel_{Msm}\Delta relZ$ knockout strains is marked in yellow, whereas that between WT and *rel_{Msm}*-overexpression area is marked in blue.

(C) Biofilm eradication activity of DMNP in terms of MBDC and MBC/MBDC interrelations is dependent on *rel_{Msm}* and *relZ* genes. For detail see Tables S5.

(D) Subinhibitory DMNP concentrations specifically inhibit biofilm formation, not planktonic growth.

(E) Rifampicin, in contrast to DMNP, has nearly equal inhibitory effect against both biofilm formation and planktonic growth.

(F) DMNP effect on colony surface morphology of the parent *M. smegmatis* mc²155 strain and its knockout derivatives (Photos).

(G) DMNP effect on sliding motility (Photos). For detail see Figure S6 and METHOD DETAILS / Sliding Motility.

Data are presented as mean $\pm$ SEM values of at least three independent experiments.

STAR★Methods

Resource Availability

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Bacterial and Virus Strains		
<i>Escherichia coli</i> BMH	Promega	K12 BMH 71-18
<i>E. coli</i> BL21(DE3)	Novagen	Cat#69450
<i>E. coli</i> RO91	Dr.R. Hengge-Aronis (Institut für Biologie, Mikrobiologie, Freie Universität Berlin) (Lange and Hengge-Aronis, 1994)	N/A
<i>Mycolicibacterium smegmatis</i> (basonym <i>Mycobacterium smegmatis</i>) mc ² 155	A.N. Bach Institute of Biochemistry	NC_008596
Other <i>E. coli</i> and <i>M. smegmatis</i> strains constructed and used in this study, see Table S1		
Biological Samples		
Chemicals, Peptides, and Recombinant Proteins		
DMNP (4-(4,7-DiMethyl-1,2,3,4-tetrahydroNaphthalene-1-yl)Pentanoic acid)	This paper	N/A
<i>N,N</i> -Diisopropylethylamine	Sigma-Aldrich (USA)	Cat#387649
Potassium hydride	Sigma-Aldrich (USA)	Cat#215813
2,4,6-Tri-tert-butylphenol	abcr GmbH (Germany)	Cat#AB142941
18-Crown-6	Sigma-Aldrich (USA)	Cat#186651
Trimethyl phosphonoacetate	AlfaAesar (UK)	Cat#A13301
Methanesulfonic acid	abcr GmbH (Germany)	Cat#AB110742
Palladium on carbon	Sigma-Aldrich(USA)	Cat#205699
Rh ₂ (R-DOSP) ₄	Strem Chemicals, Inc (USA)	Cat#45-2100
His-tagged <i>M.smegmatis</i> Rel _{Msm}	This paper	N/A
Restriction enzymes, DNA polymerases, T4 DNA Ligase, CIAP	Thermo Fisher Scientific (USA)	N/A
Kanamycin Sulfate	PanReac AppliChem (Germany)	Cat#A1493

Ampicillin Sodium Salt	PanReac AppliChem (Germany)	Cat#A0839
Hygromycin B Gold	InvivoGen (USA)	Cat#31282-04-9
Tetracycline hydrochloride	Sigma (USA)	Cat#T3383
Streptomycin sulfate	PanReac AppliChem (Germany)	Cat#A1852
Rifampicin	PanReac AppliChem (Germany)	Cat#A2220
Isoniazid	Sigma (USA)	Cat#I3377
Ethambutol dihydrochloride	Sigma (USA)	Cat#E4630
Guanosine-3',5'-bisdiphosphate	Jena Bioscience (Germany)	Cat#NU884S
Critical Commercial Assays		
Deposited Data		
Experimental Models: Cell Lines		
Experimental Models: Organisms/Strains		
Oligonucleotides		
Primers used in this study, see Table S1 for details	This paper	N/A
Recombinant DNA		
Plasmid: pET23b	Novagen	Cat#69746
Plasmid: pTZ57R	Thermo Fisher Scientific	Cat#K1214
Plasmid: pMind	Addgene (Blokpoel et al., 2005)	RRID:Addgene_24 730

Plasmid: pKW08-Lx-Int	Addgene (Williams et al., 2010)	RRID:Addgene_25015
Plasmid: p2NIL	Addgene (Parish and Stoker, 2000)	RRID:Addgene_20188
Plasmid: pGOAL19	Addgene (Parish and Stoker, 2000)	RRID:Addgene_20190
Plasmid: pET23b- <i>rel</i> _{Msm}	This paper	RRID:Addgene_166043
Plasmid: pMind- <i>rel</i> _{Msm}	This paper	RRID:Addgene_166044
Plasmid: pMind- <i>rel</i> Z	This paper	RRID:Addgene_166045
Plasmid: pKW08-rel-Lx-Int	This paper	RRID:Addgene_166046
Plasmid: p2NIL- <i>rel</i> _{Msm}	This paper	RRID:Addgene_166047
Plasmid: p2NIL- <i>rel</i> Z	This paper	RRID:Addgene_166048
Additional information about plasmids is provided in Table S1.		
Software and Algorithms		
AutoDock Vina v1.1.2	(Trott and Olson, 2010)	RRID:SCR_011958
ClustalX v2.1	(Larkin et al., 2007)	RRID:SCR_017055
Achilles Blind Docking Sever	(Sanchez-Linares et al., 2012)	N/A
BLASTp v2.10.0	(Camacho et al., 2009)	RRID:SCR_001010
PyMOL v2.3.0	Schrodinger, Inc.	RRID:SCR_000305
GalaxyTBM	(Ko et al., 2012)	N/A
Protein-Ligand Interaction Profiler v2.1.3	(Salentin et al., 2015)	GitHub c8d4c8d
Statistica for Windows 5.0	StatSoft, Inc., 1995	RRID:SCR_014213
Other		

Lead Contact

Further information and requests for responding to material, resources and reagents should be directed to and will be fulfilled by the Lead Contact, Andrei V. Malkov (a.malkov@lboro.ac.uk).

Materials Availability

All unique reagents generated in this study are available from the Lead Contact. The availability of DMNP may be limited to a small batch and might require resynthesis. Plasmid generated in this study will be deposited to Addgene (<https://www.addgene.org/>).

Data and Code Availability

The published article includes all biological data generated during this study. This study did not generate code.

Experimental Model and Subject Details

Growth Conditions

M. smegmatis mc²155 cells were grown overnight in a glass tube with 5 mL of Middlebrook 7H9 broth (Difco) containing 0.5% glycerol, 0.05% Tween 80 and 50 µg/mL ampicillin with agitation on shaker GFL1092 (GFL, Germany) (200 rpm, 37°C). The grown cells were then diluted 1:50 with fresh medium and 50 mL of culture were further cultivated in 250 mL Erlenmeyer flasks under the above conditions with the registration of growth by optical density (A₆₀₀) recorded by UV-1650PC spectrophotometer (Shimadzu, Japan). *E. coli* culture grown overnight in LB broth was brought to OD 0.1 (A₆₀₀) with the same medium and further cultivated in the volume of 50 mL in 250 mL Erlenmeyer flasks on the shaker with agitation (120 rpm, 37°C). Genetically modified strains harboring resistance genes were grown in appropriate antibiotics concentrations (ampicillin 50 µg/mL, kanamycin 50 µg/mL, hygromycin 100 µg/mL).

Protein expression conditions

To express the recombinant protein, *E. coli* BL21(DE3) pET23b-*rel*_{Msm} cells were grown in LB medium containing ampicillin 100 µg/mL until optical density OD₆₀₀ of 0.6 was reached. Then cultures were supplemented with IPTG to a final concentration of 1 mM, followed by 3 hours incubation at 30°C.

Method Details

Construction of Bacterial Strains and Plasmids

The list of the *M. smegmatis* and *E. coli* strains and plasmids used in this work is presented in **Key Resources Table** and Table S1.

*pMind-rel*_{Msm} and *pMind-relZ*

The *rel*_{Msm} gene coding sequence was amplified by PCR with *M. smegmatis* mc²155 as a template by using high-fidelity polymerase Phusion (Thermo Fisher Scientific) and primers *rel* Msm NdeI and *rel* Msm PacI listed in Table S1. Adenylate overhangs were added to amplified DNA fragment by incubation with Taq polymerase to allow cloning into intermediate vector pTZ57R via InsTAclone PCR Cloning Kit (Thermo Fisher Scientific). Insert in pTZ57R-*rel*_{Msm} was sequenced using the primers pUC/M13 F, pUC/M13 R, *rel* Msm seq 1 & 2 shown in Table S1. Plasmids were purified from *E. coli* BMH *pMind* and BMH pTZ57R-*rel*_{Msm} strains via alkaline lysis method, digested by NdeI and PacI restriction enzymes and separated by gel electrophoresis. Ligation, electroporation and selection procedures were performed by standard techniques and purified resultant vector *pMind-rel*_{Msm} was verified by restriction analysis with BamHI, PCR with Up *pMind* & Low *pMind* primers and sequencing. Original or derived plasmids (*pMind*-0 or *pMind-rel*_{Msm}) were both transformed into *M. smegmatis* mc²155, mc²155 Δ *rel*_{Msm}, mc²155 Δ *rel*_{Msm} Δ *relZ* to study antibiotic targeting and *E. coli* RO91 harboring *rpoS:lacZ* chromosome fusion (Table S1) to verify the actual role of *pMind-rel*_{Msm} plasmid in overexpression of *Rel*_{Msm} protein, whose product (p)ppGpp is known to upregulate *rpoS* expression.

Mycobacterial expression plasmid capable of producing *RelZ* was obtained in a similar fashion but without an intermediate vector. PCR-generated fragment synthesized with SAS NdeI and SAS PacI primers and mc²155 as a template was directly cloned into *pMind* plasmid. The resulting *pMind-relZ* plasmid was purified, tested by PCR and sequenced using Up/Low *pMind* and SAS seq

primers, and then used for transformation of *M. smegmatis* wild-type strain as well as knockout strains mentioned above.

pET23b-rel_{Msm} (His)

To isolate Rel_{Msm} protein, the overexpression plasmid pET23b-*rel_{Msm}* (His) was generated as follows. Primers rel His NdeI and rel His HindIII (Table S1) were used to amplify *rel_{Msm}* gene coding sequence by PCR with pTZ57R-*rel_{Msm}* as a template. The pET23b plasmid and produced DNA fragment were processed with NdeI and HindIII restriction endonucleases. Restriction products were purified from the agarose gel after electrophoresis, mixed with T4 DNA ligase and transferred into *E. coli* BMH. Resulting constructions were verified by PCR and sequencing with primers T7 promoter and T7 terminator (Novagen pET System Manual), as well as rel Msm seq 1&2. T7 polymerase-expressing *E. coli* BL21(DE3) strain was transformed with the purified plasmid for overexpression.

rel_{Msm}::luxAB reporter fusion.

The *rel_{Msm}* promoter is fused with the reporter genes *luxAB*, including Shine-Dalgarno sequence and start codon, based on identified promoter sequence (Sureka et al., 2008). The *rel_{Msm}p::luxAB* fusion was constructed on the basis of pKW08-Lx-Int plasmid that, along with *luxAB*, also included *int* gene from mycobacteriophage L5 enabling the plasmid integration into the mycobacterial chromosome. We developed rel fus PciI and rel fus BamHI primers containing restriction sites (Table S1) to amplify *rel_{Msm}* promoter region via PCR. The resulting DNA fragment was digested with appropriate restriction enzymes. To produce a vector with compatible ends, pKW08-Lx-Int plasmid containing double BamHI site was sequentially treated with BamHI and PciI restriction enzymes, resulting in three fragments:(i) PciI-BamHI (4 kb) containing plasmid origin and hygromycin resistance cassette; (ii) BamHI-BamHI (3,56 kb) containing *lux* genes; iii) PciI-BamHI (1kb) containing *tetRO* cluster. Then the first fragment was used as a vector to clone the previously obtained PCR fragment. The resulting plasmid possessed a unique BamHI site but lacked *lux* genes. After BamHI digestion this plasmid was processed with alkaline phosphatase (CIAP) to prevent self-ligation and then ligated with the second fragment that contained *lux* genes. Produced transformants were selected by hygromycin resistance, and the correct orientation of the insert was verified by PCR with Low pMind and luxA Low primers. Resulting plasmid designated as pKW08-rel-Lx-Int was purified, verified by sequencing via luxA Low primer and transformed into *M. smegmatis* mc²155 cells resulting in mc²155 *rel_{Msm}p::lux* strain.

Δrel_{Msm} and Δrel_{Msm} ΔrelZ knockout strains

The widely used two-step strategy was utilized to produce an unmarked *rel_{Msm}* deletion strain of *M. smegmatis* (Parish and Stoker, 2000). This method involves several stages: generation of suicide plasmid vector containing *rel_{Msm}* deletion cassette cloned into p2NIL/pGOAL backbone, chromosome homologous recombination of non-replicating delivery plasmid introduced in mycobacteria, SacB negative selection for unmarked antibiotic-sensitive mutants. To produce *rel_{Msm}* deletion cassette, *rel_{Msm}* upstream and downstream areas were amplified by PCR with rel knock HindIII/rel knock SalI and rel knock NotI/rel knock PacI primers (Table S1), digested with restriction enzymes, sequentially cloned into p2NIL vector and PCR checked with Up/reUp p2NIL and Low/reLow p2NIL primers. The plasmid was purified from *E. coli* BMH cells and both insert areas were sequenced to avoid nucleotide replacements using Up/reUp/Low/reLow p2NIL primers. After that pGOAL19-PacI cassette was cloned to result in final suicide delivery plasmid p2NIL-*rel_{Msm}* [3/3]. This plasmid was analyzed by BamHI restriction, purified and transformed into *M. smegmatis* mc²155. Single cross-over transformants were selected by kanamycin and hygromycin and examined by X-Gal Blue-white screening. Sucrose resistant double cross-overs were selected by Blue-white screening, tested for antibiotic susceptibility, analyzed by PCR and sequencing with rel fus PciI and rel Msm Low primers, resulting in *M. smegmatis* mc²155 Δrel strain. Knockout locus was amplified by PCR and sequenced using rel fus PciI and relMsm Low primers to confirm the presence of deletion and absence of nucleotide replacements in flanking genes.

The same protocol was used to construct *relZ* deletion cassette. Flanking areas were amplified by PCR with SAS knock HindIII_2/ SAS knock BamHI_3 and SAS knock BglII/ SAS knock PacI primers, digested with restriction enzymes mentioned in primer names, cloned sequentially into p2NIL vector, the fidelity of the insert was verified by PCR and sequencing with Up/reUp p2NIL primers for the upstream fragment and with SAS NdeI and Low p2NIL for the downstream fragment. Then pGOAL19-PacI cassette was cloned to result in final suicide delivery plasmid p2NIL-*relZ* [3/3], the clones were selected by both kanamycin and hygromycin. This plasmid was analyzed by BamHI restriction, purified, and transformed to *M. smegmatis* mc²155 and mc²155 Δ rel to produce single and double knockout strains. Knockout loci in both strains were amplified by PCR and sequenced using SAS His HindIII primer to confirm their genotypes.

Cell Viability and Persister Cell Number

Samples were regularly taken from batch culture of *M. smegmatis* mc²155 grown under the standard conditions (See section Media and Growth Conditions); cells from these samples were spot-plated onto LB agar either immediately to count the total number of culturable cells or after preliminary exposure of the cells to an antibiotic for 24 h to count persister cells. For the latter, the samples (180 μ L) were transferred to wells of sterile 96-well polystyrene microtiter plates and 20 μ L solutions of either DMNP in methanol or clinical antibiotics in water were added to the final concentrations (mM): 1.17 rifampicin (Rif), 0.094 tetracycline (Tetr), 0.0034 streptomycin (Str), 1.06 mM DMNP. After 24 h cultivation at 37°C, the cultures treated with antibiotics were diluted with saline and spot-plated. CFUs grown on plates after incubation at 37°C for 72 h were counted as persister cells. To study the character of killing curves specific for DMNP and antibiotics depending on the growth phase of cultures (Fig. 2), *M. smegmatis* cells were grown under the standard conditions (See section Media and Growth Conditions) to reach the exponential phase ($A_{600}\approx 0.5$) or the stationary phase ($A_{600}\approx 3.2$). The cells sampled at these growth phases (180 μ L) were transferred to wells of sterile 96-well polystyrene microtiter plates and 20 μ L solutions of either DMNP in methanol or clinical antibiotics in water were added to the final concentrations (mM): 1.17 rifampicin (Rif), 0.094 tetracycline (Tetr), 0.003 streptomycin (Str), 1.06 mM DMNP. The cells were further cultivated for 72 h at 37°C. At the designated time points (Figure 3) the samples were taken, diluted with saline and spot-plated. CFUs grown on plates after incubation at 37°C for 72 h were counted as survival cells.

Overexpression Assays

M. smegmatis mc²155 strains transformed with pMind-*rel*_{Msm} or pMind-*relZ* plasmids were grown overnight under the standard conditions (See section Media and Growth Conditions), except 50 μ g/mL kanamycin was added to the medium along with ampicillin. The grown cultures of each strain (50 mL) were transferred to two Erlenmeyer flasks (250 mL). To induce *rel*_{Msm} or *relZ* overexpression, 42 nM solution of tetracycline was added to one of two flasks of each strain (induced cells), whereas the second was kept as the control (uninduced cells). Cells taken in the volume of 180 μ L from both induced and uninduced batch cultures of each strain at the onset of cell transition to stationary phase (6 h of observation) were transferred to the wells of microtiter plates. Then 20 μ L solutions of either DMNP diluted in methanol or clinical antibiotics (where necessary) diluted in water were added to both of them at final concentrations designated in Figures. *M. smegmatis* culture with added 20 μ L of methanol served as the control for DMNP samples. The microtiter plates were then cultivated at 37°C for 24 h and the cells taken from the wells were diluted with saline and spot-plated onto LB agar to enumerate CFUs grown after 72 h of cultivation.

Cell Energy State

Cell energy state in batch cultures of *M. smegmatis* was characterized by cell adenylate pools (ATP, ADP and AMP) determined by HPLC-UV method specially developed for this object (Akhova and Tkachenko, 2019). Adenylate energy charge (EC) was calculated using equation $EC = [ATP] + 0.5[ADP] / [ATP] + [ADP] + [AMP]$ (Chapman and Atkinson, 1977).

Gene Expression

Gene expression in *E. coli* strains harboring *lacZ* gene fusions was detected by the β -galactosidase activity in cells treated with SDS and chloroform by Miller's method (Miller, 1972).

Gene expression in *M. smegmatis* pKW08-rel-Lx-Int cells harboring *rel_{Msm} p::luxAB* fusion was detected by bioluminescent reporter gene assay. For this, samples of culture in triplicate were analyzed for their luciferase activity (Williams et al., 2010). 1-mL samples were centrifuged and resuspended in 0.5 mL of buffered saline PBS supplemented with Tween 80. 100 μ L of resulting cell suspension were then transferred to wells of white plate, supplemented with 5 μ L of 1% n-decyl aldehyde (Sigma) in ethanol and vortexed for 10 s. Luminescence of samples diluted 10-fold in PBS was detected in microplate reader Infinite M200 (Tecan, Austria). The luminescent output was normalized to culturable cell number and expressed as Relative Light Units (RLU) per cell number.

His-tag Rel_{Msm} Protein Purification and DMNP Activity Assays

The overexpression of *rel_{Msm}* was induced by the supplementation of *E. coli* BL21DE3 pET23b-*rel_{Msm}* cells with IPTG to a final concentration of 1.0 mM. Recombinant His-tag protein was purified with FPLC chromatography in accordance with the standard procedures (Coligan, 1996). To study effect of purified Rel_{Msm} protein on DMNP activity, *M. smegmatis* mc²155 cells were grown under the standard conditions. Aliquots of cells (180 μ L) were taken from the culture after 6, 12 and 24 h of cultivation, transferred to the wells of sterile 96-well polystyrene microtiter plates, supplemented (Rel⁺) or not (Rel⁻) with a 5 μ L of a purified Rel_{Msm} protein solution (1 μ g/mL), then a methanol solution of DMNP (20 μ L) was added to both at the final concentrations (mM) 0.86, 0.96, 1.06. The bovine serum albumin (BSA) was used as an unspecific control at the same concentration as Rel_{Msm}. The microtiter plates were then cultivated at 37° C for 24 h, the cells taken from the wells were diluted with saline and spot-plated onto LB agar to enumerate CFUs grown after 72 h of cultivation.

Rel_{Msm} (p)ppGpp Synthesizing Activity Depending on DMNP Assays

In vitro (p)ppGpp synthesizing activity of purified Rel_{Msm} was measured under the standard conditions with no supplementation or with the following supplementations: 5% methanol or DMNP dissolved in 5% methanol to the final concentrations specified in Figure 5. The (p)ppGpp synthesizing activity was assayed using a slightly modified method by Wexselblatt et al. (Wexselblatt et al., 2010). 5x reaction mixture containing 2.0 mM GTP, 20 mM ATP, 200 mM Tris-HCl (pH 7.4), 5 mM DTT, 50 mM MgCl₂, 50 mM KCl, 135 mM (NH₄)₂SO₄ was freshly prepared. 50-200 μ g/mL Rel_{Msm} together with 1x reaction mixture were mixed in a total volume of 0.5-1.0 mL. The reactions were incubated at 37° C for a period of 1-4 hours and stopped by the addition of 0.4 N HClO₄. The samples were analyzed by HPLC-UV method (UV detection at 254 nM) developed by us (Akhova and Tkachenko, 2019), except the mobile phase used consisted of acetonitrile (A) and 50 mM aqueous monobasic potassium phosphate (pH 4.6) (B) in a ratio of 0.5(A)/99.5(B). Rel_{Msm} activity is quantified by the decrease in the concentration of the substrate (GTP) per 1 μ mole of the protein per 1 hour. To verify the DMNP effect on the (p)ppGpp synthetase activity, it was added to the reaction mixture in the concentrations specified in the legends to Figures.

Molecular Docking Assay

Since the substrate interaction data are not yet available for Rel_{Mtb} structure, GTP pose in the Rel_{Mtb} active site was calculated using AutoDock Vina (Trott and Olson, 2010). The lack of data regarding the amino acids that contribute to the binding of GDP/GTP molecules in the active site of Rel_{Mtb} protein prompted us to screen for such an information in Rel proteins from different species. We used the X-ray structure data showing GDP interaction with Rel_{Seq} from *Streptococcus dysgalactiae* subsp. *equisimilis* (PDB ID 1VJ7) (Hogg et al., 2004), which showed several amino acid residues contributing to the GDP-Rel_{Seq} binding through Rel proteins ClustalX alignment (Larkin et al., 2007). The corresponding amino acids from Rel_{Mtb} were utilized to localize GTP/GDP binding site. The binding pocket has been centered using AutoDock Vina to take into account amino acid residues Y300, Y309, H313, Q327 (Mtb numbers), which deemed crucial for the Rel_{Mtb} interaction with GTP/GDP. These include Y309 and H313 from Syn3 responsible for guanosine moiety interaction in various species (Steinchen and Bange, 2016) and Y300 and Q327 according to the crystal structure of Rel_{Seq} where GDP interactions have been shown. Thus, the box center was set to 147, 154, -14 for x, y, z respectively and box size was set at 20 Å for all directions with 0.375 Å spacing parameter to cover all the amino acids mentioned and to include adjacent residues only. We run a series of blind docking calculations using Achilles Blind Docking Server (Sanchez-Linares et al., 2012), a tool specialized for such purposes, to find yet unknown DMNP binding sites. Protein-Ligand Interaction Profiler (Salentin et al., 2015) was utilized to analyze interactions in protein-ligand complexes (Fig 5B & 5D). PyMOL was used for visual analysis of the docking site and for merging results of the substrate and the inhibitor dockings. To reveal the similarities of the DMNP binding sites in Rel_{Mtb} and RelP proteins, we performed BLASTp alignment of their amino acid sequences. To visualize the structure of Rel_{Msm} protein, we merged the Rel_{Mtb} N-terminal domain from the crystal structure (PDB ID 5XNX) with the C-terminal domain structure calculated using GalaxyTBM (Ko et al., 2012) based only on Rel_{Msm} amino acid sequence. A similar approach was applied to predict *M. smegmatis* RelZ structure: we merged *S. aureus* RelP structure (PDB ID 6EWZ) with computationally obtained structure of RNase HII domain from RelZ.

Antibiotic Susceptibility and Biofilm-Eradication Assays

MIC, MBC, MBEC

To determine the Minimal Inhibitory Concentration (MIC), Minimal Bactericidal Concentration (MBC) and Minimal Biofilm Eradication Concentration (MBEC) for tetracycline, streptomycin, rifampicin, isoniazid, ethambutol, and DMNP (Table S2), the Calgary system with 96-well plates MBECTM Biofilm Inoculator (Innovotech, Canada) was used according to the instructions from the manufacturer and the method by Garrison and Huigens (Ceri et al., 1999; Garrison and Huigens, 2017).

Complex effect of DMNP and clinical antibiotics on *M. smegmatis* survival assays

M. smegmatis mc²155 cells grown in 5mL of Middlebrooke medium with addition of 5 µg/mL ampicillin and 0.05 % Tween 80 in thermo-shaker GFL1092 (GFL, Germany) (200 rpm, 37°C) to optical density of 1.5 (A₆₀₀) were transferred in the volume of 180 µL to the wells of sterile 96-well polypropylene microtiter plates, to which streptomycin or rifampicin were added in the volume of 20 µL to final concentration (x MIC) of 0.0; 1.0; 2.0; 5.0 for streptomycin (at MIC = 0.137 µM) and 0; 1; 5; 10; 20 for rifampicin (at MIC= 5.83 µM). The cultures with no addition of DMNP (control) or with an addition of 20 µL of methanol (met) were used as the controls. The cultures in wells were then cultivated in the microplate reader Infinite M200 (Tecan, Austria) under 37°C with every hour agitation (15 sec) and measuring the optical density. After 24 h the samples from each of the wells were taken and spot-plated to encounter the number of survival cells. Then 10 µL of the methanol DMNP solution were added to the same wells to achieve the final concentrations designated in Figures and the cultures were further cultivated for 24 h under the same conditions. The cultures treated with antibiotics were diluted with saline and spot-plated. CFUs grown on plates after incubation at 37°C for 72 h were counted as persister cells.

Effects of DMNP or clinical antibiotics on biofilm formation

M. smegmatis mc²155 cells grown under the standard conditions were washed with fresh Middlebrook 7H9 with no addition of Tween 80. To produce biofilm, washed cells were inoculated into the same medium and the resultant culture (180 μ L) was transferred to the wells of sterile 96-well polypropylene microtiter plates. Methanol DMNP solution or water solutions of clinical antibiotics were added to the cultures in 20- μ L volumes to the final concentrations specified in Figures. The samples (180 μ L) of *M. smegmatis* culture with no additions (contr) or with the addition of 20 μ L of methanol (met) served as the controls. Plates were then cultivated at 37°C for 48 h with no agitation. To assess the antibiotic effects on biofilm formation, liquid planktonic cultures were carefully removed from under the pellicles to avoid their damaging. Pellicles were then dried and the levels of biofilm formation were assessed via the conventional staining with 0.1% crystal violet. Optical density (A_{570}) of the stained ethanol extracts was then measured using microplate reader Infinite M200 (Tecan, Austria). Growth conditions and antibiotic concentrations for studying planktonic cells were the same as for biofilm formation, except the Middlebrook 7H9 medium supplemented with Tween 80. To assess the cell survival, cells taken from the planktonic cultures grown for 48 h were spot-plated onto LB agar and the CFU number was calculated.

Effect of DMNP on *M. smegmatis* Pellicle Formation

M. smegmatis forms architecturally complex biofilms (pellicles) at the air/liquid interface of standing cultures. To investigate the pellicle structure and susceptibility of biofilm formation to DMNP, cells of parental strain *M. smegmatis* mc²155 (WT) and its *rel_{Msm}* and *relZ* knockout- and overexpressing derivatives grown with the standard procedure were washed out by centrifugation (16g X 5 min) with Middlebrook 7H9 medium with no Tween 80 and transferred to 5 mL (final volume) of the same medium containing DMNP in concentrations designated in figures to initial optical density of 0.1 (A_{600}) measurement. Resultant medium with inoculated cells was then placed in preliminary weighted polystyrene plates ($\varnothing$ 40 mm). Cells were grown with no agitation at 37°C for 72 h. Plates were photographed, liquid planktonic cultures were carefully removed from under the pellicles to avoid their damaging and pellicles inside the plates were then dried at 37°C to constant weights that were used as the values of pellicle mass (p.m.). Just after removing planktonic cultures their biomass and cell viability were assessed by optical density (A_{600}) measurement and spot-plating, respectively.

Cell Sliding Motility and Colony Morphology

To evaluate DMNP activity against *M. smegmatis* mc²155 sliding motility, cells were grown overnight under the standard conditions. The culture was then brought with the same medium to optical density of 0.4 (A_{600}) and 3 μ L of resultant cell suspension were inoculated onto the surface of Middlebrook 7H9 medium solidified with 0.3% agarose (w/v) into the center of plate ($\varnothing$ 40 mm). Then either DMNP dissolved in methanol to the final concentrations specified in the Figures or pure methanol (control) were added in the volume of 50 μ L to the medium during its preparation. Plates were cultivated at 37°C for 40 h and photographed with a digital camera (Olympus, Japan) to process the grown sliding colonies. The colony density was described by colony integrated optical density. Processing included the measuring of the sliding colony area (mm²) and its optical density (absorbance units (a.u.)). A colony area was measured by counting the pixel number within the colony region. The values of a specific optical density (SOD) calculated for sliding areas as a ratio of optical density to colony area (a.u./mm²) were used to differentiate the inhibition of sliding motility from the killing effect of DMNP (explanations are given in section Results/). To evaluate probable effect of DMNP on colony morphology, *M. smegmatis* mc²155 parent strain (WT) and its knockout derivatives (Δrel_{Msm} or $\Delta rel_{Msm} \Delta relZ$) were grown as above (this section) and each of cultures in the volume of 2 μ L (A_{600} = 1.0) were inoculated into the center of the medium surface of each of two plates ($\varnothing$ 40 mm) with agarized (0.3%) Middlebrook 7H9 medium, one of which contained 0.02 mM DMNP, while another kept with no additions (control), cultivated (37°C) for 72 h. To make the macrophoto of colonies, Olympus c-3040 zoom camera (Japan) was used with

maximal multiplication. Micro photos of the colony edges were produced with microscope MICMED 6 (LOMO, Russia) equipped with digital camera MC-6.3 (LOMO, Russia).

Zeta Potential

To determine the bacterial cell surface charge (Bayer and Sloyer, 1990), cells were washed with 1mM PBS, brought with the 10 mM potassium phosphate buffer to optical density of 0.3 (A_{650}) and Zeta potential (ζ) of cell suspension was measured with zetameter Zetasizer Nano S (Malvern Instruments Ltd, UK).

Hydrophobicity Assay

The protocol for the hydrophobicity assay was adapted from that of Rosenberg (Rosenberg, 2006). Briefly, *M. smegmatis* mc²155 cells grown under the standard conditions to optical density of 0.8 (A_{600}) were exposed to antibiotics at 37°C for 5 h, washed in PBS, and 300 μ L of cell suspensions were mixed with 30 μ L of hexadecane. The mixture was then vortexed for 30 sec and allowed to settle at room temperature for 15 min. The aqueous phase (150 μ L) was then carefully removed, and its optical density (A_{600}) was measured in microplate reader Infinite M200 (Tecan, Austria). The percentage of cell hydrophobicity was calculated as follows: Cell hydrophobicity = $[1 - (A_{600} \text{ final} / A_{600} \text{ initial})] \times 100$.

Chemical synthesis

Materials and general methods

The NMR spectra were recorded in CDCl_3 , ^1H at 400 MHz and ^{13}C at 100.6 MHz with CDCl_3 (δ 7.26, ^1H ; δ 77.0, ^{13}C ;) as an internal standard unless otherwise indicated. Various 2D-techniques and DEPT experiments were used to establish the structures and to assign the signals. The mass spectra were measured on a Thermo Exactive (Orbitrap), where the spectra were recorded in a positive or negative ion mode using electrospray ionization (ESI) from methanol or acetonitrile. All reactions were performed under an atmosphere of dry, oxygen-free nitrogen in oven-dried glassware. Yields are given for isolated products showing one spot on a TLC plate and no impurities detectable in the NMR spectrum. The identity of the products prepared by different methods was checked by comparison of their NMR spectra.

Reaction progress was monitored by GC/MS analysis and thin layer chromatography (TLC) on aluminum backed plates with Merck Kiesel 60 F254 silica gel. TLCs were either visualized by UV irradiation at 254 nm or stained by exposure to an ethanolic solution of phosphomolybdic acid. Purification by column chromatography was carried out using Merck Kiesel 60 H silica adsorbent. All solvents and reagents for the reactions were of reagent grade and were dried and distilled under argon immediately before use.

(*E*)-3-(2-bromo-3-methylphenyl)-2-methylacrylaldehyde (**3**) and Z-crotyltrichlorosilane (**4**) were synthesized following published protocol by Malkov (Incerti-Pradillos et al., 2016); 1,6-Dimethyl-1,2-dihydronaphthalene and methyl (*E*)-2-diazo-3-pentenoate were synthesized by the method of Davies (Davies and Walji, 2005). Catalyst MAKDIOX was synthesized by the method of Malkov (Fukazawa et al., 2019; O'Hora et al., 2015) All spectral data were in agreement with literature. Copies of ^1H and ^{13}C NMR spectra of (–)-**2** are presented in Table S7.

Asymmetric synthesis of (–)-(R)-4-((1*S*,4*R*)-4,7-dimethyl-1,2,3,4-tetrahydronaphthalen-1-yl)pentanoic acid (DMNP).

(–)-(3*R*,4*S*,*E*)-1-(2-bromo-3-methylphenyl)-2,4-dimethylhexa-1,5-dien-3-ol (**5**): A 100 mL round bottom flask fitted with a magnetic stirring bar was flame-dried, evacuated and backfilled with N_2 . The flask was charged with 30 mL of dry propionitrile and successively with a solution of MAKDIOX (80.4 mg, 0.1 mmol, 2 mol%), aldehyde **3** (1.25 g, 5.2 mmol, 1 eq.) and Hünig's base (1.8 mL, 10.3 mmol, 2 eq.) in 1 mL of dry propionitrile each. The solution was cooled at –60 °C

and a solution of Z-crotyltrichlorosilane **4** (1.7 g, 8.9 mmol, 1.7 eq.) in 1 mL of dry propionitrile was added dropwise to the reaction mixture. The mixture was stirred at this temperature for two days. After that time, it was quenched with a saturated solution of NaHCO₃ (10 mL), washed with 20 mL of NaHCO₃ and extracted with DCM (3 x 60 mL). Combined organic layers were dried over Na₂SO₄ and solvent was removed in *vacuo*. Flash chromatography on *silica* with a gradient eluent system (100% hexane to 95:5, hexane/ethyl acetate) afforded the target homoallylic alcohol (–)-**5** as a colourless oil (1.10 g, 72 % yield). ¹H NMR (400 MHz, CDCl₃): δ_H 1.13 (d, *J* = 6.8 Hz, 3H); 1.70 (s, 3H); 1.73 (s_{broad}, 1H); 2.43 (s, 3H); 2.53 (q, *J* = 6.8 Hz, 1H); 4.08 (d, *J* = 6.4 Hz, 1H); 5.06-5.15 (m, 2H); 5.83-5.91 (m, 1H); 6.53 (s, 1H); 7.04-7.06 (m, 1H); 7.11-7.17 (m, 2H). In agreement with literature. (Incerti-Pradillos et al., 2016) Chiral HPLC (Chiralpak IA-3, hexane/2-propanol = 98:2, 0.75 mL/min, UV detection at 225 nm) showed 99:1 e.r. (*t_R* = 19.3 min (major), *t_S* = 20.8 min (minor)).

In the racemic variant: Z-crotyltrichlorosilane **4** (11.34 g, 60 mmol, 1.2 eq.) was added to a solution of **3** (11.9 g, 50 mmol, 1 eq) in DMF (150 mL) and Hünig's base (20.86 mL, 120 mmol, 2 eq.) under N₂ atmosphere. The reaction mixture was stirred and monitored by TLC until completion. Then it was quenched with saturated NaHCO₃ (100 mL) and extracted with ethyl acetate (3 x 200 mL). The organic layers were dried over Na₂SO₄ and concentrated in *vacuo*. Flash chromatography on *silica* with a gradient eluent system (100% hexane to 95:5, hexane/ethyl acetate) afforded the target homoallylic alcohol (±)-**5** as a colourless oil (12.5 g, 85 %). ¹H NMR data matched those in the chiral version.

(2E,4R,5S,7E)-Methyl 5-(2-bromo-3-methylphenyl)-4-methylnona-2,7-dienoate (**6**): A 30% suspension of KH in mineral oil (2.2 g, 55.5 mmol, 3 eq.) was washed with anhydrous DME (3 x 10 mL). Anhydrous DME (45 mL) was added to the KH. Alcohol (–)-**5** (1.10 g, 3.7 mmol, 1 eq.) and 18-crown-6 (0.97 g, 3.7 mmol, 1 eq.) were successively added to the solution and the mixture was stirred for two days at rt. The solution was cooled to –78 °C and quenched with a 1M solution of 2,4,6-tri-*tert*-butylphenol in dry DME (3.5 eq). The solution was poured into a saturated solution of NH₄Cl (50 mL) and extracted with DCM (3 x 50 mL). The combined organic layers were dried over Na₂SO₄ and concentrated in *vacuo*. The resulting crude aldehyde obtained as a yellow oil was used immediately in the next step.

LiCl (186.5 mg, 4.44 mmol, 1.2 eq.) was weighted in a 50 mL flame-dried round bottom flask. The flask was evacuated and backfilled with N₂. Dry CH₃CN (10 mL) was added followed by a solution of Hünig's base (0.8 mL, 4.44 mmol, 1.2 eq.) and trimethyl phosphonoacetate (0.9 mL, 4.44 mmol, 1.2 eq.) in 2 mL of dry CH₃CN each. After stirring the mixture for 5 minutes, the crude aldehyde (3.7 mmol, 1 eq.) in 2 mL of anhydrous CH₃CN was added dropwise. The reaction mixture was stirred overnight at rt and after that time it was quenching with a saturated solution of NH₄Cl (20 mL). The mixture was extracted with DCM (3 x 50 mL), the organic phases dried over Na₂SO₄ and the solvents removed under reduced pressure. The crude mixture of diastereoisomers was separated by flash column chromatography on *silica* eluting with a gradient system (100% petroleum ether to 30:1 petroleum ether/ethyl acetate) to yield in order of elution *epi*-**6** as a colourless oil (0.3 g, 23 %) and **6** as a colourless oil (0.7 g, 51% yield over two steps).

6: ¹H NMR (400 MHz, CDCl₃): δ_H 1.05 (d, *J* = 6.8 Hz, 3H), 1.56 (dd, *J* = 6.3, 1.4 Hz, 3H), 2.37 (t, *J* = 6.7 Hz, 2H), 2.45 (s, 3H), 2.72 (m, 1H), 3.54 (m, 1H), 3.73 (s, 3H), 5.25 (m, 1H), 5.40 (m, 1H), 5.77 (dd, *J* = 15.7, 1.4 Hz, 1H), 7.00 (m, 2H), 7.13 (m, 2H). ¹³C NMR (100 MHz, CDCl₃): δ_C 14.6, 17.3, 24.1, 33.1, 39.5, 47.5, 50.8, 119.7, 125.8, 126.5, 127.8, 128.2, 138.1, 141.1, 152.3, 166.6

epi-**6**: ¹H NMR (400 MHz, CDCl₃): δ_H 0.97 (d, *J* = 6.8 Hz, 3H), 1.50 (dd, *J* = 6.3, 1.4 Hz, 3H), 2.18 (m, 1H), 2.43 (s, 3H), 2.53 (m, 1H), 3.41 (m, 1H), 3.74 (s, 3H), 5.15 (m, 1H), 5.26 (m, 1H), 5.82 (dd, *J* = 15.7, 1.4 Hz, 1H), 6.96 (m, 2H), 7.09 (m, 1H), 7.16 (m, 1H). ¹³C NMR (100 MHz, CDCl₃): δ_C 17.3, 17.5, 24.1, 37.7, 41.8, 48.2, 50.9, 120.2, 125.0, 126.1, 127.4, 128.1, 138.0, 141.8, 152.4, 166.4

The racemic variant: A 30% suspension of KH in mineral oil (16.4 g, 123 mmol, 3 eq.) was washed with anhydrous THF (3 x 50 mL). Anhydrous THF (200 mL) was added to the KH. Alcohol ($\pm$)-**5** (12 g, 41 mmol, 1 eq.) and 18-crown-6 (10.82 g, 41 mmol, 1 eq.) were successively added to the solution and the mixture was stirred for 48 h at rt. The solution was cooled to $-78\text{ }^{\circ}\text{C}$ and quenched with a 1M solution of 2,4,6-tri-*tert*-butylphenol (3.5 eq) in dry DME. The mixture was poured into a saturated solution of NH_4Cl (200 mL) and extracted with DCM (3 x 200 mL). The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. The resulting crude aldehyde

obtained as a yellow oil was used immediately in the next step. LiCl (2.09 mg, 49.2 mmol, 1.2 eq.) was weighted in a 250 mL flame-dried round bottom flask. The flask was evacuated and backfilled with N_2 . Dry CH_3CN (100 mL) was added followed by a solution of Hünig's base (8.57 mL, 49.2 mmol, 1.2 eq.) and trimethyl phosphonoacetate (7.96 mL, 49.2 mmol, 1.2 eq.) in 20 mL of dry CH_3CN each. After stirring the mixture for 5 minutes, the crude aldehyde (1 eq.) in 50 mL of anhydrous CH_3CN was added dropwise. The reaction mixture was stirred overnight at rt and after that time it was quenching with a saturated solution of NH_4Cl (100 mL). The mixture was extracted with DCM (3 x 200 mL), the organic phases dried over Na_2SO_4 and the solvents removed under reduced pressure. The crude mixture was purified by flash column chromatography on *silica* eluting with a gradient system (100% petroleum ether to 30:1 petroleum ether/ethyl acetate) to yield ($\pm$)-**6** as a colourless oil (7.6 g, 53 % yield over two steps). ^1H NMR data matched those in the chiral version.

(*R,E*)-methyl 4-((1*S*,4*R*)-8-bromo-4,7-dimethyl-1,2,3,4-tetrahydronaphthalen-1-yl)pent-2-enoate (**7**): Methanesulfonic acid (0.37 mL, 5.7 mmol, 3 eq.) was added to a solution of **6** (693 mg, 1.9 mmol, 1 eq.) in CHCl_3 (3 mL) and the reaction mixture was stirred at $40\text{ }^{\circ}\text{C}$ for two days. The reaction was quenched with saturated NaHCO_3 (5 mL) and extracted with CH_2Cl_2 (3 x 20 mL). The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. Purification by column chromatography on *silica* (eluting with 95:5 petroleum ether/ethyl acetate) gave the *title compound* **7** as a colourless oil (570 mg, 83% yield). ^1H NMR (400 MHz, CDCl_3): δ_{H} 0.93 (d, $J = 6.9\text{ Hz}$, 3H), 1.26 (d, $J = 6.9\text{ Hz}$, 3H), 1.92 (m, 4H), 2.40 (s, 3H), 2.79 (m, 1H), 3.08 (m, 1H), 3.54 (m, 1H), 3.73 (s, 3H), 5.77 (dd, $J = 15.8, 1.6\text{ Hz}$, 1H), 7.11 (m, 4H). ^{13}C NMR (100 MHz, CDCl_3): δ_{C} 14.0, 21.1, 21.8, 23.6, 28.7, 31.7, 39.2, 41.4, 50.7, 119.2, 124.9, 127.7, 135.6, 137.5, 142.8, 152.8, 166.5.

In the racemic variant: Methanesulfonic acid (4.12 mL, 64.3 mmol, 3 eq.) was added to a solution of ($\pm$)-**6** (7.5 g, 21.4 mmol, 1 eq.) in CHCl_3 (30 mL) and the reaction mixture was stirred at $40\text{ }^{\circ}\text{C}$ for two days. The reaction was quenched with saturated NaHCO_3 (50 mL) and extracted with CH_2Cl_2 (3 x 200 mL). The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. Purification by column chromatography on *silica* (eluting with 95:5 petroleum ether/ethyl acetate) gave the *title compound* ($\pm$)-**7** as a colourless oil (6.4 g, 87% yield).

Methyl (*R*)-4-((1*S*,4*R*)-4,7-dimethyl-1,2,3,4-tetrahydronaphthalen-1-yl)pentanoate ((-)-**8**): Palladium on carbon (10 mol%) and NaHCO_3 (360 mg, 4.3 mmol, 3 eq.) were added to a solution of alkene **7** (0.5 g, 1.43 mmol) in methanol (45 mL) and the mixture was pressurized to 30 psi of hydrogen for 3 h. The reaction mixture was then filtered through a pad of celite, washed with diethyl ether and concentrated under reduced pressure to give (-)-**8** as a clear oil (0.384 g, 98% yield). ^1H NMR (400 MHz, CDCl_3): δ_{H} 0.70 (d, $J = 6.8\text{ Hz}$, 3H), 1.29 (d, $J = 6.9\text{ Hz}$, 3H), 1.36 (m, 1H), 1.79 (m, 4H), 1.95 (m, 1H), 2.16 (m, 1H), 2.32 (s, 3H), 2.43 (m, 2H), 2.76 (m, 1H), 2.90 (m, 1H), 3.72 (s, 3H) 6.97 (d, $J = 7.9\text{ Hz}$, 1H), 7.02 (s, 1H), 7.16 (d, $J = 7.9\text{ Hz}$, 1H). ^{13}C NMR (100 MHz, CDCl_3): δ_{C} 13.7, 20.5, 21.0, 21.3, 29.7, 31.0, 32.1, 36.5, 41.0, 50.9, 125.6, 126.0, 127.5, 134.2, 138.7, 139.8, 173.7. HRMS (ESI) 275.2011 ($\text{C}_{17}\text{H}_{26}\text{O}$ $[\text{M}+\text{H}]^+$ required 275.2006). $[\alpha]_{\text{D}}^{25} = -37.2$ (c 0.1, CHCl_3).

In the racemic variant: Palladium on carbon (10 mol%) and NaHCO_3 (4.54 g, 54 mmol, 3 eq.) were added to a solution of alkene ($\pm$)-**7** (6.3 g, 18 mmol, 1 eq.) in methanol (400 mL) and the mixture was pressurized to 30 psi of hydrogen for 3 h. The reaction mixture was then filtered through a pad

of celite, washed with diethyl ether and concentrated under reduced pressure to give (±)-**8** as a clear oil (4.83 g, 98% yield).

(R)-4-((1S,4R)-4,7-dimethyl-1,2,3,4-tetrahydronaphthalen-1-yl)pentanoic acid ((-)-DMNP **2**). LiOH (96 mg, 4.0 mmol) was added to a solution of ester **8** (0.274 g, 1.0 mmol) in a mixture of THF:MeOH:H₂O (2:1:1). The reaction mixture was heated to 50°C for 3 hours, and then allowed to cool to room temperature. A 10% aqueous hydrochloric acid was added dropwise to the reaction mixture until the pH of the solution reached pH 3. The aqueous layer was then extracted with diethyl ether, dried using MgSO₄, filtered and the solvent was removed under reduced pressure to give (-)-DMNP **2** as a light oil (255 mg, 98% yield). ¹H NMR (400 MHz, CDCl₃): δ_H 0.72 (d, *J* = 6.8 Hz, 3H), 1.31 (d, *J* = 6.9 Hz, 3H), 1.37 (m, 1H), 1.95 (m, 1H), 2.19 (m, 1H), 2.33 2.48 (m, *J* = 9.2, 2H), 2.77 (m, 1H), 2.92 (m, 1H), 6.98, 7.03 (s, 1H), 7.17 (m, 1H), 9.82 (br.s., 1H). ¹³C NMR (100 MHz, CDCl₃): δ_C 13.7, 20.5, 21.0, 21.3, 29.4, 31.0, 32.2, 36.4, 41.1, 125.7, 126.0, 127.5, 134.2, 138.6, 139.8, 179.2. HRMS (ESI) 261.1855 (C₁₇H₂₅O₂ [M+H]⁺ required 261.1855). [α]_D²⁵ = -29.0 (c 0.1, CHCl₃).

In the racemic variant: A solution of ester (±)-**8** (4.5 g, 16.4 mmol) was prepared in a mixture of THF:MeOH:H₂O (2:1:1), to which LiOH (1.57 g, 65.6 mmol) was added. The reaction mixture was heated to 50°C for 3 hours, and then allowed to cool to room temperature. 10% aqueous hydrochloric acid was added dropwise to the reaction mixture until the pH of the solution reached pH 3. The aqueous layer was then extracted with diethyl ether, dried using MgSO₄, filtered and the solvent was removed under reduced pressure to give (±)-DMNP **2** as a light oil (4.17 g, 98% yield).

Asymmetric synthesis of (+)-(S)-4-((1S,4R)-4,7-dimethyl-1,2,3,4-tetrahydronaphthalen-1-yl)pentanoic acid (+)-DMNP.

Methyl (S)-4-((1R,4S)-4,7-dimethyl-1,2,3,4-tetrahydronaphthalen-1-yl)pentanoate ((+)-**8**): To a flame dried 25 mL round bottom flask with a magnetic stir bar was added 1,2-dihydro 1,6-dimethylnaphthalene(±) (0.5 g, 3.15 mmol) and Rh₂(R-DOSP)₄ (250.0 mg), dissolved in 2,2-DMB (13 mL) under argon at room temperature. In a 20 mL syringe a solution of methyl (*E*)-2-diazo-4-methyl-3-butenolate (0.9 g, 13 mmol) was prepared in 2,2-DMB (20 mL). The solution of diazo compound was added via syringe pump at a rate of 10 mL/h (~2 h addition time). After the diazo addition was complete the reaction mixture was allowed to stir for an additional 0.5 h, the solvent was removed under reduced pressure. The crude reaction mixture was taken up in methanol (100 mL) and palladium on carbon (Pd/C) (1.0 g, 10% Pd) was added. The reaction mixture was pressurized with H₂ gas at 30 psi and allowed to shake for 4 h. The crude mixture was passed through a pad of celite and washed with diethyl ether and concentrated under reduced pressure. Purification by column chromatography (SiO₂, petroleum ether : ethyl acetate (99.5 : 0.5) gave ((+)-**8** as a light oil (292 mg 34 % yield, over 2 step): ¹H NMR (400 MHz, CDCl₃): δ_H 0.70 (d, *J* = 6.8 Hz, 3H), 1.29 (d, *J* = 6.9 Hz, 3H), 1.36 (m, 1H), 1.79 (m, 4H), 1.95 (m, 1H), 2.16 (m, 1H), 2.32 (s, 3H), 2.43 (m, 2H), 2.76 (m, 1H), 2.90 (m, 1H), 3.72 (s, 3H) 6.97 (d, *J* = 7.9 Hz, 1H), 7.02 (s, 1H), 7.16 (d, *J* = 7.9 Hz, 1H). ¹³C NMR (100 MHz, CDCl₃): δ_C 13.7, 20.5, 21.0, 21.3, 29.7, 31.0, 32.1, 36.5, 41.0, 50.9, 125.6, 126.0, 127.5, 134.2, 138.7, 139.8, 173.7. HRMS (ESI) 275.2011 (C₁₇H₂₆O [M+H]⁺ required 275.2006). [α]_D²⁵ = +37.2 (c 0.1, CHCl₃).

(S)-4-((1R,4S)-4,7-dimethyl-1,2,3,4-tetrahydronaphthalen-1-yl)pentanoic acid ((+)-DMNP): A solution of ester (+)-**8** (0.274 g, 1.0 mmol) was prepared in a mixture of THF:MeOH:H₂O (2:1:1), to which LiOH (96 mg, 4.0 mmol) was added. The reaction mixture was heated to 50°C for 3 hours, and then allowed to cool to room temperature. A 10% aqueous hydrochloric acid was added dropwise to the reaction mixture until the pH of the solution reached pH 3. The aqueous layer was then extracted with diethyl ether, dried over MgSO₄, filtered and the solvent was removed under reduced pressure to give (+)-DMNP **2** as a light oil (255 mg, 98% yield). ¹H NMR (400 MHz, CDCl₃): δ_H 0.72 (d, *J* = 6.8 Hz, 3H), 1.31 (d, *J* = 6.9 Hz, 3H), 1.37 (m, 1H), 1.95 (m, 1H), 2.19 (m, 1H), 2.33 2.48 (m, *J* = 9.2, 2H), 2.77 (m, 1H), 2.92 (m, 1H), 6.98, 7.03 (s, 1H), 7.17 (m, 1H), 9.82 (br.s., 1H). ¹³C NMR (100 MHz, CDCl₃): δ_C 13.7, 20.5, 21.0, 21.3, 29.4, 31.0, 32.2, 36.4, 41.1,

125.7, 126.0, 127.5, 134.2, 138.6, 139.8, 179.2. **HRMS (ESI)** 261.1855 ($\text{C}_{17}\text{H}_{25}\text{O}_2$ $[\text{M}+\text{H}]^+$ required 261.1855). $[\alpha]_{\text{D}}^{25} = +29.0$ (c 0.1, CHCl_3).

Quantification and Statistical Analysis

Statistica for Windows 5.0 (StatSoft, Inc., 1995) software was used for processing of the experimental data, presented as mean $\pm$ SEM; n equals the number of assay replicates (independent experiments). Statistical significance of the differences between the mean values of the compared groups was assessed by the unpaired Student's t -test. The differences were considered significant at $p < 0.05$

Declaration of Interests

The authors declare no competing interests.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7