Improving rifamycin production in *Amycolatopsis mediterranei* by expressing a *Vitreoscilla* hemoglobin (*vhb*) gene fused to a cytochrome P450 monooxygenase domain.

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Abstract

Expression of the vhb gene, encoding hemoglobin from Vitreoscilla stercoraria in several organisms, clearly enhances oxygen-dependent product formation. In a previous work, we expressed the vhb gene that encodes hemoglobin from V. stercoraria in Amycolatopsis mediterranei, resulting in an increase (oxygen-dependent formation) in rifamycin B production. In the present work, we first confirm; by heterologous expression in Escherichia coli, that rif-orf5 from the rifamycin biosynthetic gene cluster, really encodes a cytochrome P450 enzyme, which is the key step for oxygen incorporation in the final biosynthetic product. Likewise, we fused rif-orf5 to the vhb gene, as part of a genetic engineering strategy. The fused genes were used to generate an Amycolatopsis mediterranei transformant (Msb-HbCYP5). Interestingly, the fermentation of Msb-HbCYP5 manifested 1.5-fold higher rifamicin B production than the transformant with only the hemoglobin gene, and 2.2-fold higher than the parental strain.

Key words: *Actinomycetes/Amycolatopsis*/cytochrome P450/hemoglobin/rifamycin/*Vitreoscilla.*

Introduction

The re-emergence of tuberculosis as a major health problem has increased demand for new and efficacious antibiotics (Al Yaquobi et al., 2018). Rifamycin and its synthetically modified derivatives are the principal chemotherapeutic agents used for combating tuberculosis and other mycobacterial infections (Davies et al., 2008). Consequently, much research has focussed on improving *in vivo* production yields of rifamycin, and also on using genetic engineering to generate rifamycin derivatives (Xu et al., 2018). Fortunately, the rifamycin biosynthetic gene cluster is one of the best characterized ansamycin gene clusters and its biosynthesis is known to involve 5 putative cytochrome P450 monooxygenases (CYP) (*rif-orf0*, *rif-orf4*, *rif-orf5*, *rif-orf13*, and *rif-orf16*), (Kaur et al., 2001).

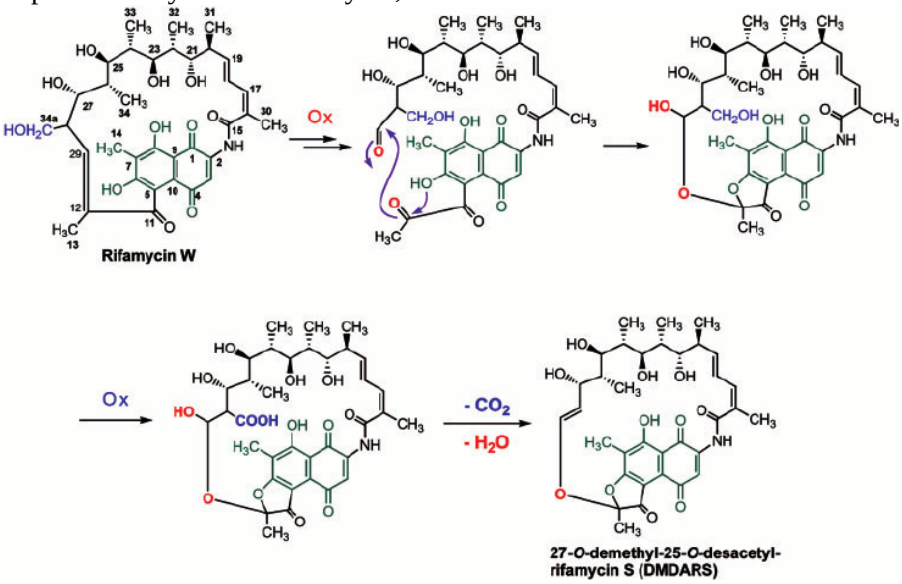


Figure 1. Possible participation of P450 enzyme Rif-Orf5, in the oxidative cleavage of rifamycin W (Xu et al., 2005).

There are reports of several CYP enzymes participating in the macrolide biosynthesis of different species of the industrially important antibiotic-producing actinomycetes (Bhattacharya et al., 2018). In 2004 (Lee et al., 2004), Lee and co-workers inactivated one of the five genes from the rifamycin gene cluster (*rif-orf0*), while Xu and co-workers inactivated the four remaining genes in 2005 (Xu et al., 2005). Their results indicated that inactivation of *rif-orf5* inhibits rifamycin B production and causes the accumulation of rifamycin W, a rifamycin analogue that has been proposed as a biosynthetic intermediary of rifamycin B. This transformation is intimately related to oxygen availability in the culture (Mejía et al., 2003). Similarly, when the culture reaches stationary phase, *A. mediterranei*, like other filamentous microorganisms, exhibits high viscosity that provokes low levels of dissolved oxygen. As a result, unwanted rifamycins such as rifamycin W accumulate in the culture medium. In order to solve this problem, we expressed the *vhb* gene encoding hemoglobin from *Vitreoscilla stercoraria*, in *A. mediterranei* (Guerra et al., 2008). This strategy successfully improved the biosynthesis of the oxygen-dependent product rifamycin B. However, even though the concentration of rifamycin W decreased, we continued to detect some accumulation of this biosynthetic intermediary. The main hypothesis regarding the remaining rifamycin W suggested inner oxygen limitation. We considered the fact that despite the inner oxygen increase related to the initial strategy, *A. mediterranei* was driving the source in the primary metabolism, thus restricting the rifamycin biosynthetic route. To solve this problem, we proposed that the inclusion of a hemoglobin (*vhb*) bound to the cytochrome P450 (*rif-orf5*) would facilitate the incorporation of oxygen into the biosynthetic rifamycin pathway, at the limiting step. Therefore, our first objective in the present work was to confirm experimentally that the *rif-orf5* gene actually codes for a cytochrome P450. Secondly, a fusion between the *vhb* gene from *V. stercoraria* with *rif-orf5*, was constructed, cloned into the plasmid pULVK2, introduced into *A. mediterranei* and measured its effect on the production of rifamycin B. It is apparent that the *in vivo* antibiotic yields improved when the fusion protein transformed the bacterial host.

Materials and Methods

Microorganisms, media and culture conditions

In the present work, we used the following strains of *A. mediterranei*: Msb2 (a mutant strain from M18 ATCC 21789; Mejía et al. 1998); Msb-sag (Msb strain containing pUAMSAG1; Priscila et al. 2008); *S699* strain derived from the *ME/83 ATCC 13685* type strain to obtain the *rif-orf5* gen. *S699* strain was provided by Professor Heinz G. Floss (Department of Chemistry, University of Washington, Seattle, U.S.A.), who used this strain to define the sequence of the rifamycin biosynthetic gene cluster.

Escherichia coli DH5α strain as recipient strain for plasmids; *BL21 pLys* was used for the over-expression of *orf5*. For storage purposes, *A. mediterranei* strains were either lyophilized, frozen at –20 °C or maintained in Bennett agar medium. *E. coli DH5α* and *BL21[DE]pLysS* strains were stored at –80 °C. *E. coli* strains and plasmid containing transformants were grown at 37 °C in Luria-Bertani medium. Growth media were supplemented with 100 μg/mL of ampicillin and 50 μg/mL of chloramphenicol. Bennett agar was used to grow *A. mediterranei*, which is a medium composed of: glucose 10g, meat extract 1g, NZ-amine A 2g, yeast extract 1g, and agar 20g in 1.0 litre tap water. *A. mediterranei* strains were grown in liquid medium containing the following components: glucose 20g, soybean meal 20g, CaCO3 2.5g, $MgSO_4.7H_2O$ 0.4g, $FeSO_4.7H_2O$ 0.01g, $ZnSO_4.7H_2O$ 0.05g and $CoCl_2.6H_2O$ 0.003g in 1.0 litre tap water. Seed cultures were grown in a shaking incubator (150 rpm) at 25 °C for 84 h. The Lee production medium was composed of: glucose 120g, bacto-peptone 10g, yeast extract 5g pH 7.2 in 1.0 litre tap water. This medium was inoculated with 20% of the seed culture and incubated at 25 °C in a 250 mL Erlenmeyer flask with 25 mL production medium and incubated in a shaking incubator at 250 rpm.

Primers and plasmids

Table 1. Oligonucleotides used in this work. *Restriction endonuclease cleavage sites are underlined.

Name	Sequence (5'-3' direction)*
Rorf5F	TGCCTTCATATGACCACCACTGCCGAGACT

Rorf5R	AAGGCATTCGAATCA**GTGATGGTGATG**GGACGCCGGCCGGGCGAG
PvhbF	TATAAGCTTATGCGAGTGTCCGTTCGAGTGGC
PvhbR	TTCAACCGCTTGAGCGTACAAATCTGCTT CC
Porf5F	ATGACCACCACTGCCGAGACTTCGGCCG
Porf5R	GAATTCTCAGTGATGGTGATGGGACGCCGGCC

Table 2. Plasmids.

Name	Description
pET17b	*E. coli* expression vector (Novagen®)
pULVK2	AAGGCATTCGAATCA**GTGATGGTGATG**GGACGCCGGCC
pUAMSAG1	Derivated from pULVK2 with the promoter P*erm*E* linked to the *vhb* gene
pGEMrif-orf5	Vector (Promega®) with *rif-orf5* gene.
pULVK2vhb-cyp	Vector for the expression of the fusion protein VHb-P450

RNA isolation.

For isolation of RNA, *A. mediterranei S699* was cultured in 25 mL of the production medium and incubated at 25 °C and 250 rpm for 72 h. Every 24 h, a single flask of culture was removed and the contents loaded into centrifuge tubes, where biomass was separated by centrifugation at 3,381 ×g for 10 min at 4 °C. We then washed the resultant pellet and froze it with liquid nitrogen. The pellet from each sample was ground in a diethyl pyrocarbonate-treated mortar and pestled under liquid nitrogen. Total RNA was isolated from about 100 mg of cell lysate using the RNeasy Mini Kit (Qiagen Inc., Chatsworth, CA, USA). In order to remove DNA contamination from the RNA samples, DNAse was added to the sample (10 µg/mL). RNA was purified using an equal volume of phenol/chloroform. RNA samples were then resuspended in 10 – 20 µL RNAse free-water and stored at –80 °C.

Cloning, heterologous expression and protein isolation.

Purified P450 DNA fragments were cloned into the *E. coli* expression vector pET17b (Novagen). Each expression construct was verified by complete sequence analysis and then transformed into competent *E. coli* BL21[DE]pLysS (Novagen). Expression was performed at 25 °C with shaking at 190 rpm for 24 h with the heme precursor δ-aminolevulinic acid added to a final concentration of 2 mM for optimal CYP synthesis. Following expression, *E. coli* cultures were pelleted by centrifugation at 5,000 rpm for 20 min at 4 °C and then resuspended in 25 ml of cold potassium phosphate buffer (pH 7.4, 0.1 M). These suspensions were frozen overnight at -80 °C. Subsequently, the cells were broken by thawing at room temperature. The cytosolic fraction was separated from the membrane fraction by ultracentrifugation at 100,000 x g spin for 1 h at 4 °C. Heterologous proteins were isolated using a Ni^{2+}-nitriloagarose affinity column (Qiagen).

Construction of hemoglobin-P450 fusion plasmids, *A. mediterranei* transformation and screening.

The plasmid pULVK2vhb-cyp for the expression of the fusion protein VHb-P450 containing the DNA of *vhb* from *V. stercoraria* linked to the cDNA of *rif-orf5* was constructed, as described below. Plasmid pUAMSAG1 was used as a template for the promoter P*erm*E* linked to the *vhb* gene, while pGEMrif-orf5 was used for amplifications of *rif-orf5* gene. Purified DNA fragments (Malhotra et al., 2007) were digested with *Hind*III and *Eco*RI and cloned directly into pULVK2, digested with the same enzymes. We identified correct plasmid by enzyme restriction mapping and authenticated reading frames related

to the expression cassette, by DNA sequencing. *A. mediterranei* strains were made competent and transformed by biolistic transfection (vacuum 20 inches Hg, 6 cm target distance, 900 psi, BioRad M5 tungsten particle). The treated cells were seeded on plates with Bennett agar. After incubation for 6 h at 30 °C, the plates were covered with soft agar with erythromycin (10 µg/ml). Transformants were selected according to their ability to grow in this medium. We compared the performance of the transformed strain with the fused genes of *vhb-cyp* in *A. mediterranei* and evaluated the production of rifamycin B and W production in shake-flasks under low aeration (Figure 4). In control experiments, this strain was also compared with the transformant Msb-sag1 containing pUAMSAG1 (vector with *vhb* gene), Msb-ulvk (Msb strain transformed with the empty vector pULVK2) and the parental strain.

Protein-Protein Docking and molecular simulation

Hemoglobin and cytochrome P450 initial structures were built using the iTasser server (Zhang et al., 2008) (http://zhanglab.ccmb.med.umich.edu/I-TASSER). We then assigned all hydrogens to both structures and minimized energy with 100 steepest descent (SD) steps using charmm c35b2 with charmm 27 parameters. The standalone Zdock version (Chen et al., 2003) was used to dock both structures. All pdb structures generated by Zdock were converted to charmm structures, using Simulaid software (Mezei et al., 2010) and read into charmm. 100 SD minimization steps to all hydrogen, keeping heavy atoms fixed first and a final 100 SD minimization steps to all atoms were applied to each structure to relieve any close contact. The structures were sorted according to the distance between their carboxyl terminal (C atom) of hemoglobin and the cytochrome P450 amino terminal (N atom). The final structure joined both proteins and was soaked in a water box of 100 Å3 with 0.15 M KCl. The solvent was energy minimized with 50 SD steps followed by 50 adopted basis Newton-Raphson method, keeping protein fixed. A 200 ps NPT molecular dynamics simulation was undertaken with periodic boundary conditions at 298K with NAMD version 2.7b2 (Phillips et al., 2005). All structures were visualized using VMD software.

Analytical methods.

Cell growth was monitored by measuring dry weight. The rifamycin B and W contents of the culture were determined as previously described (Mejía et al., 1998). Reduced carbon monoxide difference spectra for identification of cytochrome P450 content were measured, following the spectral method by Omura and Sato (1964) (Sambrook et al., 2001). Biomass samples, containing 2 mg of protein per ml of 0.1 M phosphate buffer (pH 7.0), were placed in both the sample and reference cells. After recording the base-line, the content of sample cell was treated with various reagents, and the spectral difference thereby induced was measured. When CO was used, it was carefully bubbled through the sample for about 20 seconds; this was sufficient to saturate the sample with the gas. Reduction of samples with dithionite was done with a few milligrams of solid $Na_2S_20_4$. In some experiments the sample and reference cells were treated differently, and the difference spectrum was measured. All spectrophotometric measurements were made at room temperature (20-25º).

Results and Discussion

Confirmation that *rif-orf 5* encodes a cytochrome P450

cDNA from *rif-orf5* was cloned in pET-17b and heterologously expressed in *E. coli BL21 pLysS.* Recombinant protein from this open reading frame showed the reduced carbon monoxide difference spectra, typical for P450 cytochromes. A peak at 420 nm indicating the production of an inactive derivative of P450 and another close to the typical 450 nm (Fig. 2).

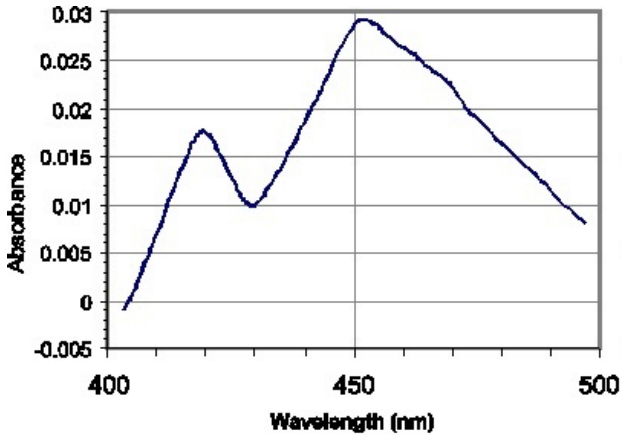


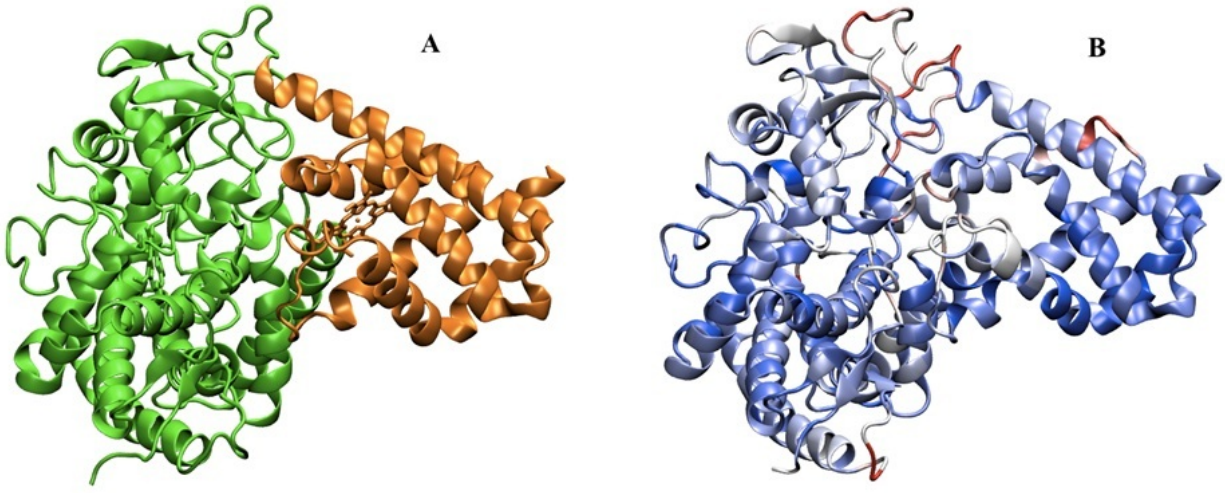
Figure 2. Reduced carbon monoxide difference spectra of heterologous proteins from *rif-orf5* expressed in *E. coli* BL21 pLysS. The absorbance difference at 450 nm between reduced haemoprotein (Fe^{2+}) *versus* reduced haemoprotein bound to carbon monoxide (Fe^{2+}·CO) was measured using a cytosolic fraction prepared from BL21[DE] pLysS cells expressing heterologous proteins.

These results concur with the role for this gene defined by Xu and co-workers (2005). This study thus confirms that *rif-orf5* gene codes for a cytochrome P450. Likewise, the accumulation of rifamycin W that was observed to result from the inactivation of the *rif-orf5* gene (Xu et al. 2005), implies that this CYP is the key enzyme responsible for halting rifamycin B biosynthesis with low oxygen transfer (Mejía et al., 2003). This result, promoted the design of a chimeric protein, consisting of hemoglobin from *V. stercoraria* linked to the CYP domain from rif-orf5.

Molecular modelling and design of a hemoglobin-P450 fusion protein

In order to obtain a functional fusion protein, we devised an *in silico* model to predict any possible steric impediment. Hemoglobin and P450 models were obtained from 3600 protein complexes with Zdock. The best complex was characterised as that manifesting lower interaction energy between each protein and the shorter distance between the hemoglobin carboxyl and the P450 amino terminal. The proposed model (Figure 3A) meets both requirements and was simulated by 200 ps of molecular dynamics to test its stability. The final structure (Figure 3B) had a root mean square deviation (RMSD) of 2.1 Å (backbone atoms) when compared to the initial structure.

Figure 3. Predicted structure of fusion protein consisting of hemoglobin from *V. stercoraria* linked to the CYP domain from *rif-orf5*. A) Chimeric protein model of *V. stercoraria* hemoglobin (orange) and *Amycolatopsis*



mediterranei cytochrome P450 (green) used in this work. B) Final structure of the molecular dynamics (200 ps). Red regions are those with a higher contribution to RMSD when compared against the initial structure and the opposite is true for blue regions. Figures were created using VMD

The structural effect produced by the insertion of glycine amino acids was also studied, which due to their small size may function as a hinge between both protein molecules. Results from geometrical analysis of minimized chimeric structures suggest that the presence of a glycine hinge, in any size studied, destabilizes the system by separating out both molecules. This destabilisation may cause the effective interchange of material between ligand and active sites in the chimeric structure. For all molecular conformations obtained from dynamic simulations, the chimeric structure without a glycine hinge manifested greater molecular interaction (data not shown), suggesting a better chain coupling for the experimental merged protein. Consequently, we decided to link both protein molecules directly to the chimeric structure, in the absence of a glycine amino acid between them. The model proposed for both proteins in this work quite adequately resembles that of other family members of each protein (data not shown). The chimeric protein model proposed and studied here was stable during the time simulated. Based on this result, we may hypothesize that the chimeric protein is stable and more importantly is functional *in vivo* as shown here by the experimental data. Using simulation, we tested the stability of the chimeric protein, over a long period.

Rifamycin Production Evaluation

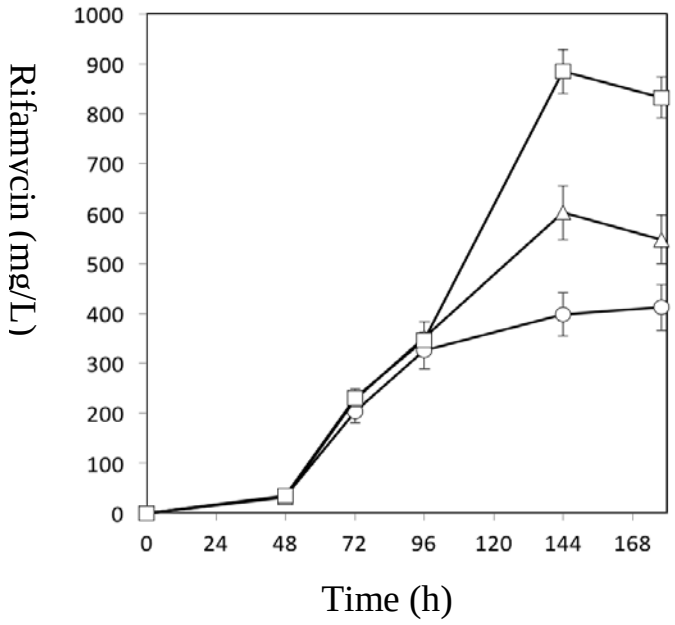


Figure 4. Time course of rifamycin B production. Transformed strain (Msb-sag) with *vhb* gene (Δ). Transformed strain (Msb-Lq2) with fused genes (*vhb-cyp*) (□) and the parental strain (o). 250 ml Flasks containing 70 ml of production medium at 26 ºC, 200 rev/min.

The performance of transformed strain of *A. mediterranei* with the fused genes of *vhb-cyp* was compared with Msb-sag1, Msb-ulvk and parental strain. Results revealed that under low aeration conditions (Mejía et al., 2003), rifamycin B production increased by approximately 42% and 118% in relation to the Msb-sag strain and parental strain, respectively. Same performance was observed between Msb-ulvk and parental strain. Notably there was no indication of significant differences in growth, indicating that specific production maintained the same pattern. An explanation for these positive results may be that two different biochemical properties were linked in a single protein, with two-domains containing N-terminal hemoglobin and C-terminal monooxygenase. VHb has an unusually high affinity for oxygen brought about by a moderate association constant, coupled with an extremely low dissociation constant. Although the affinity constant of the cytochrome P450 from the rif-orf5 gene is unknown, results from this study strongly suggest a synergistic effect between their activities or else some other enhancement of the activity of the limiting cytochrome P450. This is corroborated by the lower rifamycin W production found in the transformed strain. Some other examples of the synergistic effect of CYP proteins can be found in recent reports. One case is presented by Matthews and co-workers, where they fused an alditol oxidase from *S. coelicolor* (AldO) to a cytochrome P450 as a chimeric system. With this strategy, the oxidase produces the H_2O_2 needed by the cytochrome, thus facilitating its use for efficient fatty acid decarboxylation during the production of alkenes with industrial importance in biofuels application (Matthews, et al., 2017).

Conclusion

Our results revealed that the beneficial effect of VHb expression on biosynthesis of rifamycin can be substantially improved by expressing the fusion protein (VHb-CYP). The expression of this chimeric protein resulted in a 1.5-fold increase in rifamycin B production compared to the VHb-expressing strain and a 2.2-fold increase with respect to the parental strain. VHb activity may induce a local increase of oxygen concentration; possibly increasing the activity of cytochrome P450 monooxygenase and the fusion construct enhanced catalytic efficiency for the biosynthesis of rifamycin B. This technique has potential application for industrial strains, where oxygen transference is important.

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Conflict of interest

On behalf of all authors, the corresponding author states that there is not conflict of interest.

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