Structural studies of bacterial steroid-hydroxylating cytochrome P450 monooxygenases

Jóźwik, Ilona
Chapter 3

Characterization of cytochrome P450 CYP109E1 from Bacillus megaterium as a novel vitamin D₃ hydroxylase

Ammar Abdulmughni¹, Ilona K. Jóźwik², Natalia Putkaradze¹, Elisa Brill¹, Josef Zapp³, Andy-Mark W.H. Thunnissen², Frank Hannemann¹ and Rita Bernhardt¹

¹ Department of Biochemistry, Saarland University, Saarbrücken, Germany
² Laboratory of Biophysical Chemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands
³ Pharmaceutical biology, Saarland University, Saarbrücken, Germany

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In this study the ability of CYP109E1 from Bacillus megaterium to metabolize vitamin D₃ (VD₃) was investigated. In an in vitro system using bovine adrenodoxin reductase (AdR) and adrenodoxin (Adx₂₋₁₀₈), VD₃ was converted by CYP109E1 into several products. Furthermore, a whole-cell system in B. megaterium MS941 was established. The new system showed a conversion of 95% after 24 h. By NMR analysis it was found that CYP109E1 catalyzes hydroxylation of VD₃ at carbons C-24 and C-25, resulting in the formation of 24(S)-hydroxyvitamin D₃ (24S(OH)VD₃), 25-hydroxyvitamin D₃ (25(OH)VD₃) and 24S,25-dihydroxyvitamin D₃ (24S,25(OH)₂VD₃). Through time dependent whole-cell conversion of VD₃, we identified that the formation of 24S,25(OH)₂VD₃ by CYP109E1 is derived from VD₃ via the intermediate 24S(OH)VD₃. Moreover, using docking analysis and site-directed mutagenesis, we identified important active site residues capable of determining substrate specificity and regio-selectivity. HPLC analysis of the whole-cell conversion with the I85A-mutant revealed an increased selectivity towards 25-hydroxylation of VD₃ compared with the wild type activity, resulting in an approximately 2-fold increase of 25(OH)VD₃ production (45 mg l⁻¹day⁻¹) compared to wild type (24.5 mg l⁻¹day⁻¹).
Introduction

Cytochromes P450 (P450s) are heme-containing enzymes found in all domains of life [1]. They are involved in many metabolic processes, including the biosynthesis of steroids and fatty acids, the metabolism of drugs and the detoxification of xenobiotics [2]. P450 monooxygenases are gaining importance as enzymes for industrial biotechnology since they have the ability to introduce oxygen into non-activated C–H bonds of various compounds in a regio- and stereo-selective manner under mild conditions [3,4].

Vitamin D₃ (VD₃) is a fat-soluble prohormone, which is synthesized in the presence of ultraviolet radiation from the precursor 7-dehydrocholesterol [5,6]. The activation of VD₃ is achieved by different P450s: the mitochondrial CYP27A1 mediates hydroxylation of VD₃ at carbon 25, producing 25-hydroxyvitamin D₃ (25(OH)VD₃), which is then further hydroxylated by CYP27B1 resulting in the most active form of VD₃, i.e., 1α,25-dihydroxyvitamin D₃ (1α-25(OH)₂VD₃) [7,8]. It was found that other P450s such as the microsomal CYP2R1, CYP3A4 and CYP2J3 can also hydroxylate VD₃ at C-25 [9,10].

The active form of VD₃, 1α-25(OH)₂VD₃, is involved in the regulation of the calcium and phosphate metabolism, amongst other physiological processes [11-13]. However, sufficient levels of the precursor 25(OH)VD₃ are required for the regulatory action of 1α-25(OH)₂VD₃ [14]. Moreover, 25(OH)VD₃ represents the most abundant VD₃ circulating metabolite and, therefore, is used clinically as an indicator for the VD₃ status of patients [15].

During the last years there has been a growing interest in the biotransformation of VD₃ to its active metabolites, 25(OH)VD₃ and 1α-25(OH)₂VD₃. Thereby, recent research activity focused on microbial P450s [16]. However, only few bacterial P450s are known to produce these active metabolites such as CYP105A1 from S. griseolus [17] and CYP107 (Vdh) from P. autotrophica [18]. Therefore, the identification of new microbial P450s with 1α- and/or 25-hydroxylation activity towards VD₃ is of great interest.

Recently, CYP109E1 from Bacillus megaterium DSM319 was identified and characterized in our group [19]. It was shown that CYP109E1 has a 16β-hydroxylation activity towards testosterone. In addition, the X-ray crystal structures of CYP109E1 were solved for substrate-free protein and in complexes with testosterone or corticosterone. In the absence of bound steroids, CYP109E1 contains a large, open active site pocket at the distal side of the heme. The testosterone-bound CYP109E1 structure shows a different conformation, in which the active site pocket is more narrow (closed state of CYP109E1) [19], which likely reflects the protein’s functionally relevant state and therefore was applied in this study for vitamin D₃ docking calculations.

In this study, the substrate specificity of CYP109E1 was investigated for VD₃. Herein, for the first time we present the results demonstrating that CYP109E1 from B. megaterium exhibits hydroxylation activity towards VD₃. In addition, whole-cell conversion of VD₃ was carried out using B. megaterium. Furthermore, site-directed mutagenesis based on docking simulations of CYP109E1 and VD₃ was performed in order to optimize the regio-selectivity of CYP109E1 towards 25-hydroxylation. The effect of the mutations on the conversion of VD₃ was examined in the whole-cell system.
Results

Bioconversion of vitamin D₃ by CYP109E1

CYP109E1 from *B. megaterium* was previously cloned and characterized in our laboratory [19]. In order to identify new substrates for this enzyme, screening of a focused library consisting of different steroids was carried out. Hereby, VD₃ was identified as new substrate for CYP109E1. In an *in vitro* reconstituted system containing CYP109E1, AdR and Adx₄₄₁₀₈, about 90% of 200 µM VD₃ was converted within 1 h into 7 products with the following distribution: 3%, 15%, 8%, 22%, 42%, 5% and 5% of product 1 to product 7 (P1-P7), respectively (Figure 1A). Over time, it was observed that the increase in the formation of P2 is related to a corresponding decrease of P5. Therefore, we assumed that P5 is converted by CYP109E1 into P2. Through comparison of the retention time ($t_R$) of the detected products with that of available authentic standards, P1 ($t_R = 4.8$ min) and P4 ($t_R = 11.6$ min) were identified as 1α-25(OH)₂VD₃ and 25(OH)VD₃, respectively (data not shown).

![Figure 1. HPLC chromatogram of the CYP109E1-catalyzed VD₃ conversion.](image)

**Figure 1.** HPLC chromatogram of the CYP109E1-catalyzed VD₃ conversion. (A) *in vitro* VD₃ conversion, using bovine Adx₄₄₁₀₈ (20 µM) and AdR (3 µM) and CYP109E1 (1 µM). The reaction was carried out in a final volume of 250 µl at 30 °C for 1 h. (B) CYP109E1-dependent whole-cell conversion of VD₃ in *B. megaterium* MS941. The reaction was carried out in 50ml TB medium for 24h at 30 °C. Substrate was added at a final concentration of 200 µM. The peaks of detected products and substrate are labeled with (P1–P7) and VD₃, respectively. The authentic standard of 200 µM VD₃ (grey) was detected with the same HPLC method.

Whole-cell conversion of VD₃ in *B. megaterium*

After successful *in vitro* conversion of VD₃, a whole-cell conversion system was established. Figure 1B shows an HPLC profile of VD₃ whole-cell conversion in *B. megaterium* demonstrating a similar pattern as for the *in vitro* conversion. After 24 h, 95% conversion of 200 µM substrate was achieved (Figure 2). The product distribution after 24 h conversion was as follows: 6%, 37%, 14%, 33%, 6%, 2% and 2% for products P1-P7, respectively.

For further characterization, we analyzed the time course of product formation during whole-cell conversions, which showed that the product distribution changed over time (Figure 2). In the first 2 h of the reaction, P5 was found to be the main product with 44% of total share (40 mg l⁻¹ day⁻¹). Afterwards, P5 constituted only 5% of total share (4.3 mg l⁻¹ day⁻¹). Accompanied by the decrease of P5, an increase of P2 was observed (Figure 2). The results obtained thus indicate that P2
formation is dependent on the action of CYP109E1 on P5. Therefore, we hypothesized that CYP109E1 has the potency to hydroxylate VD₃ at different positions. To further test, this we decided to identify the main products by nuclear magnetic resonance (NMR) spectroscopy.

Figure 2. Product distribution of VD₃ whole-cell conversion in B. megaterium. Reactions were carried out in 50 ml TB medium for 48 h at 30 °C. Samples of the cultures were taken after defined time points (2, 4, 6, 8, 24 and 48 h) and analyzed by HPLC as described in “Materials and methods”. Labeling of the products (P1–P7) corresponds to that in Figure 1. The vertical bars indicate the standard deviation values of the mean from two independent experiments.

Large scale conversion of VD₃ and product identification

In order to obtain sufficient amounts of VD₃ metabolites for further characterization by NMR spectroscopy, whole-cell conversions with a total culture volume of 750 ml were performed. Three products were obtained with sufficient purity and amounts (5–25 mg) for structural characterization via NMR spectroscopy.

In contrast to vitamin D₃, its conversion product P4 showed resonances of an additional tertiary hydroxyl group in the ¹³C NMR spectrum (δC 71.15) and the resonances of the methyl groups C-26 and C-27 appeared both as singlets (δH 1.19s, 6H) in the ¹H NMR spectrum. This clearly indicated hydroxylation at position C-25 and led to the structure of 25(OH)VD₃ for P4. The data were in accordance with those reported in literature [20,21]:

¹H NMR (CDCl₃, 500 MHz): δ 0.52 (s, 3xH-18), 0.92 (d, J = 6.5 Hz, 3xH-21), 1.02 (m, H-22a), 1.06 (m, H-22b), 1.19 (s, 6H, 3xH-26 and 3xH-27), 1.20 (m, H-23a), 1.24 (m, H-16a), 1.28 (m, H-17), 1.29 (m, H-12a), 1.36 (m, H-24a), 1.39 (m, H-23b), 1.45 (m, 2H, H-11a and H-24b), 1.51 (m, H-20), 1.52 (m, 2H, H-11b and H-15a), 1.60 (m, H-2a), 1.63 (m, H-15b), 1.69 (m, H-9a), 1.86 (m, H-16b), 1.94 (m, H-14), 1.96 (m, H-2b), 1.98 (m, H-12b), 2.16 (dd, J = 13.5, 8.5 and 5.0 Hz, H-1a), 2.26 (dd, J = 13.0 and 7.5 Hz, H-4a), 2.38 (ddd, J = 13.5, 7.5 and 4.6 Hz, H-1b), 2.55 (dd, J = 13.0 and 4.0 Hz, H-4b), 2.82 (m, H-9b), 3.92 (m, H-3), 4.80 (d, J = 2.5 Hz, H-19a), 5.03 (m, H-19b), 6.01 (d, 11.3 Hz, H-7), 6.21 (d, 11.3 Hz, H-6). ¹³C NMR (CDCl₃, 125 MHz): δ 11.99
(CH₃, C-18), 18.81 (CH₃, C-21), 20.82 (CH₂, C-23), 22.24 (CH₃, C-11), 23.56 (CH₃, C-15), 27.67 (CH₂, C-16), 29.07 (CH₂, C-9), 29.29 (CH₃, C-26), 29.34 (CH₃, C-27), 31.92 (CH₂, C-1), 35.16 (CH₂, C-2), 36.10 (CH₂, C-22), 36.40 (CH, C-20), 40.53 (CH₂, C-12), 44.39 (CH₂, C-24), 45.85 (C, C-13), 45.92 (CH₂, C-4), 56.33 (CH, C-14), 56.53 (CH, C-17), 69.21 (CH, C-3), 71.15 (C, C-25), 112.40 (CH₂, C-19), 117.51 (CH, C-7), 122.45 (CH, C-6), 135.00 (C, C-5), 142.89 (C, C-8), 145.09 (C, C-10).

The NMR spectra of P5 revealed an additional secondary hydroxyl group (δ₉ 3.34 m, δs 77.41 CH). Its position at C-24 was obvious by vicinal correlations of its proton to the isopropyl proton H-25 (δ₉ 1.69 m) in the HHCOSY and to the methyls C-26 (16.69, CH₃) and C-27 18.93, CH₃ in the HMBC. Comparison of the data with those of an authentic sample [22] supported these findings. Especially the chemical shifts of C-1 to C-19 fitted perfectly to each other, whereas the resonances for the side chain slightly differed. This might originate from a different stereochemistry at C-24 in our sample and its epimer from literature. Unfortunately the authors gave no hint to the stereochemistry at C-24 and our sample decomposed within one day in solution. Therefore, the assignment of the absolute configuration at C-24 could not be solved by subsequent NMR measurements, such as Mosher’s method. But as was found for closely related 24-hydroxylated steroids the 24(R)- and 24(S)-isomers showed characteristic differences in the ¹³C NMR [23]. Applying these observations to our problem led to the identification of the 24(S)-form for our molecule and to the 24(R)-form for the epimer reported by [22]:

**NMR (CDCl₃, 500 MHz):** δ 0.57 (s, 3xH-18), 0.92 (d, J = 6.9 Hz, 3xH-26), 0.95 (d, J = 6.9 Hz, 3xH-27), 0.97 (d, J = 6.5 Hz, 3xH-21), 1.08 (m, H-22a), 1.28 (m, H-23a), 1.33 (m, H-16a and H-17), 1.34 (m, H-12a), 1.43 (m, H-20), 1.51 (m, H-11a), 1.56 (m, H-15a), 1.58 (m, H-23b), 1.59 (m, H-11b), 1.64 (m, H-22b), 1.68 (m, H-2a), 1.69 (m, H-25), 1.70 (m, H-15b), 1.72 (m, H-9a), 1.93 (m, H-16b), 1.96 (m, H-2b), 2.01 (m, H-14), 2.03 (m, H-12b), 2.20 (dd, J = 13.5, 8.5 and 5.0 Hz, H-1a), 2.30 (dd, J = 13.0 and 7.5 Hz, H-4a), 2.42 (ddd, J = 13.5, 7.8 and 4.6 Hz, H-1b), 2.60 (dd, J = 13.0 and 4.0 Hz, H-4b), 2.86 (m, H-9b), 3.34 (m, H-24), 3.97 (m, H-3), 4.86 (d, J = 2.5 Hz, H-19a), 5.07 (dt, J = 2.5 and 1.3 Hz, H-19b), 6.06 (d, 11.3 Hz, H-7), 6.26 (d, 11.3 Hz, H-6). **¹³C NMR (CDCl₃, 125 MHz):** δ 12.00 (CH₃, C-18), 16.69 (CH₃, C-26), 18.93 (CH₃, C-27), 19.05 (CH₃, C-21), 22.23 (CH₂, C-11), 23.55 (CH₂, C-15), 27.62 (CH₂, C-16), 28.99 (CH₂, C-9), 30.74 (CH₂, C-23), 31.91 (CH₂, C-1), 32.16 (CH₂, C-22), 33.15 (CH, C-25), 35.14 (CH₂, C-2), 36.29 (CH, C-20), 40.50 (CH₂, C-12), 45.84 (C, C-13), 45.90 (CH₂, C-4), 56.30 (CH, C-14), 56.37 (CH, C-17), 69.20 (CH, C-3), 77.41 (CH, C-24), 112.44 (CH₂, C-19), 117.51 (CH, C-7), 122.43 (CH, C-6), 135.07 (C, C-5), 142.21 (C, C-8), 145.05 (C, C-10).

P2 was found to be a dihydroxylated conversion product of vitamin D₃. The NMR spectra revealed resonances for a supplementary secondary (δs 79.55 CH) and a tertiary (δs 73.15C) hydroxyl function. 2D NMR HHCOSY, HSOQCED and HMBC measurements revealed their positions as immediate neighbours at C-24 and C-25. According to P5, C-24 in P2 was expected to be in (S)-configuration. However, we wanted to prove this assumption in an independent manner. Both epimers were known from literature but no comparative NMR studies were available. Therefore, we performed a comparison of the NMR data of their synthetic precursors, the 24(R)- and 24(S)-forms of de-A,B-cholesta-8,24,25-triol [24]. Analysis of the ¹³C NMR data for the side chain of P2 and comparison with those of the C-24 epimeric de-A,B-cholestanes gave a clear and unambiguous evidence for 24S,25(OH)₂VD₃ as structure for P2:

**NMR (CDCl₃, 500 MHz):** δ 0.55 (s, 3xH-18), 0.95 (d, J = 6.5 Hz, 3xH-21), 1.04 (m, H-22a), 1.14 (m, H-23a), 1.17 (s, 3xH-26), 1.22 (s, 3xH-27), 1.28 (m, H-17), 1.29 (m, H-16a), 1.31 (m, H-12a), 1.41 (m, H-20), 1.49 (m, 2H, H-11a and H-11b), 1.54 (m, H-15a), 1.57 (m, H-23b), 1.68 (m, H-2a and H-15b), 1.69 (m, H-9a), 1.77 (m, H-22b), 1.89 (m, H-16b), 1.94 (m, H-2b), 1.98 (m, H-14), 2.00 (m, H-12b), 2.21 (m, H-1a), 2.30 (dd, J = 13.0 and 7.5 Hz, H-4a), 2.41 (ddd, J = 13.5, 7.8 and 4.6 Hz, H-1b), 2.58 (dd, J = 13.0 and 4.0 Hz, H-4b), 2.83 (m, H-9b), 3.29 (d, J = 10.1 and 2.0 Hz, H-24), 3.94 (m, H-3), 5.05 (dt, J = 2.5 and 1.3 Hz, H-19b), 4.82 (d, J = 2.5 Hz, H-19a), 6.03 (d, 11.3 Hz, H-7), 6.23 (d, 11.3 Hz, H-6). **¹³C NMR (CDCl₃, 125 MHz):** δ 12.00 (CH₃, C-
These results confirmed our assumption, that CYP109E1 can hydroxylate VD$_3$ at different positions. As shown above, the ratio of P2 (24S,25(OH)$_2$VD$_3$) increased inversely proportional to the ratio of P5 (24S(OH)VD$_3$) over time, indicating that CYP109E1 has 25-hydroxylation activity towards VD$_3$ as well as 24S(OH)VD$_3$. The reaction pathway of VD$_3$ conversion by CYP109E1 is shown in Figure 3.

Figure 3. Reaction pathway of VD$_3$ by CYP109E1 from B. megaterium.

**Molecular docking of VD$_3$ to CYP109E1**

Unfortunately soaking/co-crystallization trials to obtain the structure of the CYP109E1-VD$_3$ complex proved unsuccessful, therefore docking of the VD$_3$ molecule was performed to identify potential substrate-binding residues. A structural comparison of CYP109E1 to other P450s converting VD$_3$ found in the PDB, revealed that the closed conformer of CYP109E1 (PDB: 5L94) is highly similar to the closed state observed for CYP107 (Vdh) crystallized in complex with VD$_3$ (PDB: 3A50, [25], r.m.s.d of 1.23 Å for 347 Cα atoms). Therefore, the closed conformation of CYP109E1 likely reflects the functionally relevant conformational state of the protein and was chosen in this study for the substrate docking calculations. Among the calculated VD$_3$ conformations, the one showing a suitable distance of the C-25 atom to the heme iron and the
lowest predicted free energy of binding was chosen for further analysis. The docked pose places the aliphatic side chain of VD₃ close to the heme iron, productively for 25-hydroxylation (C-25-Fe distance of 3.7 Å), and is well in agreement with the crystallographically observed VD₃ binding mode in CYP107 (Vdh) (C-25-Fe distance of 4.6 Å). The only hydrophilic group (3β-OH group at the A-ring) of VD₃ is solvent exposed, predicted not to interact with any of the protein side chains; the rest of the VD₃ molecule interacts with hydrophobic residues lining the active site pocket, similarly as in Vdh. Since VD₃ hydroxylation is of outstanding importance for sustainable biotransformation process of the production of active VD₃, we were interested to improve the yield of 25(OH)VD₃. Four amino acids predicted to interact with VD₃ were mutated to alanine to determine their roles in CYP109E1 activity and selectivity towards VD₃: I85 (BC-loop, substrate recognition site 1, SRS1), I168 and V69 (F-helix, SRS2) and I241 (I-helix, SRS4) Additionally, one more residue was chosen for mutagenesis, K187 (SRS3), since its mutation to alanine in CYP109E1 was previously shown to cause a slight decrease in testosterone conversion activity. Thus we considered the possibility that this flexible residue, located at the top of the active site (G helix), might also take part in VD₃ binding (Figure 4).

**Figure 4. Docking model of VD₃ in the active site of CYP109E1.** The suitable conformation of VD₃ for 25-hydroxylation is shown (in magenta), and compared to the crystallographically observed VD₃ binding mode in CYP107 (Vdh) (in green). Heme is in red coloured sticks. Amino acids selected for site-directed mutagenesis are shown in orange sticks.

**Conversion of VD₃ by CYP109E1 mutants**

To investigate the effect of selected mutations on activity and regio-selectivity of CYP109E1 towards VD₃ directly in the whole-cell system, *B. megaterium* cells were transformed with the plasmid pSMF2.1.CYP109E1 containing the corresponding CYP109E1 mutations. The results clearly showed that all CYP109E1 mutants still maintained VD₃ hydroxylation activity. However, activity or/and selectivity of CYP109E1 was affected by the amino acid replacements (Figure 5).

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Figure 5. HPLC chromatograms of VD₃ whole-cell conversions by *B. megaterium* expressing different variants of CYP109E1. The reactions were carried out in 50 ml TB medium for 24 h at 30 °C. The substrate was added at a final concentration of 200 µM. The authentic standard of 200 µM VD₃ (grey) was detected with the same HPLC method.
While the K187A mutant showed the same conversion ratio (97%) as compared with wild type, the activity of CYP109E1 was changed by selected mutations (Figure 6). All other mutants showed decreased activity in the first 8 h of the reaction. However, the activity in case of I85A, I168A, V169A and I241A mutants was increased afterwards. After 24h conversion, the I168A mutant exhibited a comparable conversion (96%) as the wild type and the I85A mutant showed a maximum conversion of 87%. On the other hand, a significant decrease of the activity was observed in case of V169A and I241A mutants, displaying only 76% and 54% conversion, respectively.

**Figure 6.** Effect of selected mutations in CYP109E1 on the conversion of VD₃. Reactions were performed using *B. megaterium* MS941 in 50 ml TB medium at 30 °C. The substrate was added at a final concentration of 200 µM. Samples of the cultures were taken after defined time points (2, 4, 6, 8, 24 and 48 h) and analyzed by HPLC as described in “Materials and methods”. The vertical bars indicate the standard deviation values of the mean from three independent whole-cell experiments.

Additionally, the effect of the point mutations on the regio-selectivity of CYP109E1-dependent VD₃ conversion was studied (Table 2). Compared to wild type, the formation of 24S(OH)VD₃ was decreased over time in case of the I85A and I168A mutants, which, as a consequence, led to reduced amounts of the derived product 24S,25(OH)₂VD₃. On the other hand, the 25-hydroxylation activity towards VD₃ was strongly preferred in the reactions catalyzed by the I85A and I168A mutants resulting in 63% and 49% of total products, respectively (Table 2). Moreover, mutant I85A displayed a significant reduction in the number of products. Consequently, an increase of the absolute 25(OH)VD₃ production was observed with these mutants, compared to the wild type (Figure 7). These results indicate that the side chains of amino acids I85 and I168 are essential for determining the regio-selectivity of CYP109E1 towards VD₃. Compared to wild type, the regio-selectivity of the K187A mutant was also slightly changed towards 25-hydroxylation (Table 2). Furthermore, it was observed that the 24S,25(OH)₂VD₃ production decreased in the whole-cell conversion with the K187A mutant, compared to wild type. In contrast to the decrease in the 24S,25(OH)₂VD₃ production, an accumulation of 24S(OH)VD₃ was observed, suggesting that the K187A mutant has less specificity towards 24S(OH)VD₃, compared to wild type.

Furthermore, significant changes of the specificity and regio-selectivity of CYP109E1 were determined in whole-cell conversions with the V169A and I241A mutants. Compared to the wild
type, it was observed that reactions of variant V169A showed a decreased 25-hydroxylation activity of 24S(OH)VD₃, whereas the 25-hydroxylation of VD₃ was enhanced (Table 2). In addition, no formation of P1, P3 and P7 was observed with this mutant. In whole-cell conversions with the V169A mutant, the product distribution was as follows: 24S(OH)VD₃ (40%), 25(OH)VD₃ (47%), 24S,25(OH)₂VD₃ (3%) and P6 (10%). It was further observed that the substitution of I241 to alanine shifts the regio-selectivity of CYP109E1 towards 24-hydroxylation (60% of total products). In contrast, a decrease of 24S,25(OH)₂VD₃ was observed (only 3% of total products) indicating that the 25-hydroxylation activity of this mutant of CYP109E1 towards 24S(OH)VD₃ is dramatically decreased.

Table 2. Comparison of products distribution of 24 h whole-cell conversions of VD₃ by different variants of CYP109E1.

<table>
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<th>Enzyme variant</th>
<th>Activity [%]</th>
<th>Number of products</th>
<th>Major product(s) [%]*</th>
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<td>Wild type</td>
<td>95</td>
<td>7</td>
<td>24S,25(OH)₂VD₃ (37%)</td>
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<td></td>
<td></td>
<td></td>
<td>25(OH)VD₃ (33%)</td>
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<td>I85A</td>
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<td>25(OH)VD₃ (63%)</td>
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<td>I168A</td>
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<td>V169A</td>
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<td>24S(OH)VD₃ (58%)</td>
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* Only products with a ratio of ≥ 20% of total products are defined here as major products.

Figure 7. Production of 25(OH)VD₃ in B. megaterium overexpressing different variants of CYP109E1. Reactions were performed in 50 ml TB medium for 24 h at 30 °C. The substrate was added at a final concentration of 200 µM. The amount of 25(OH)VD₃ was measured by HPLC as described in “Materials and methods“.

The vertical bars indicate the standard deviation values of the mean from three independent whole-cell experiments.
Discussion

During the past years bioconversion processes, including specific hydroxylations, have gained increasing interest, since chemical synthesis often requires complex procedures and environmentally unfriendly conditions. The ability of P450s to hydroxylate a broad range of compounds makes them suitable as versatile biocatalysts [2,3].

It is known that VD$_3$ is activated in kidneys and liver by different P450s such as mitochondrial cytochromes CYP27A1 and CYP27B1 as well as the microsomal enzymes CYP2R1, CYP3A4 and CYP2J3. However, low activity and stability of mammalian P450s compared with those of bacterial origin are important factors limiting their industrial applications [26]. As a result of such limitations, bacterial P450s constitute an attractive alternative for the industrial production of different valuable products, including VD$_3$ metabolites. Until now, only a few studies have reported the usage of bacterial P450s for the production of VD$_3$ metabolites such as CYP105A1 and CYP107 (Vdh) [16]. Therefore, the identification of new bacterial P450s with hydroxylation activity towards VD$_3$ is of a great interest for the industry. In particular, *B. megaterium* offers a great potential, both as a source and expression host, for such new bacterial P450s. It has been used since many decades for the production of different industrial enzymes [27,28]. It offers an advantage that replicative plasmids are stable and maintained [29]. In addition, *B. megaterium* lacks external alkaline proteases and, therefore, is suitable for heterologous protein expression. Moreover, *B. megaterium* can grow on simple media utilizing a broad spectrum of carbon sources [30].

Therefore, the establishment of *B. megaterium*-based whole cell system using a bacterial P450 as biocatalyst for the production of active VD$_3$ metabolites, such as 25(OH)VD$_3$, is an interesting alternative for the industrial production. Recently, *B. megaterium* was used in our laboratory for the conversion of the steroid hormone precursor cholesterol to pregnenolone and for the hydroxylation of 11-keto-ß-boswellic acid (KBA) [31-33]. Moreover, a CYP27A1-based whole-cell system was established in *B. megaterium* allowing for efficient production of valuable pharmaceuticals such as 27-hydroxycholesterol, 26/27-hydroxy-7-dehydrocholesterol, 25(OH)VD$_3$ and 25-hydroxy-7-dehydrocholesterol [34].

In this study, we demonstrated for the first time the conversion of VD$_3$ by a member of the CYP109 family, namely CYP109E1 from *B. megaterium* DSM319. We showed that CYP109E1 can convert VD$_3$ into different products, in both enzyme-based and whole-cell-based assays. The new whole-cell system converts more than 90% of the added substrate (200 µM) within 24h supporting its promising potential as VD$_3$ hydroxylase. The two main products of CYP109E1-dependent conversion of VD$_3$ were identified by NMR analysis as 25(OH) VD$_3$ (33%) and 24S, 25 (OH)$_2$VD$_3$ (37%) with yields of 24.5 mg l$^{-1}$ day$^{-1}$ and 28.6 mg l$^{-1}$ day$^{-1}$, respectively. In addition, the product P5 was identified by NMR analysis as 24S(OH)VD$_3$. For further characterization of product formation, time-dependent whole-cell conversions were performed. It was shown that both, 24S(OH)VD$_3$ (P5) and 25(OH)VD$_3$ (P4), are initially detected as major products (Figure 3). Nevertheless, a decrease in the 24S(OH)VD$_3$ ratio was observed in correlation with the formation of 24S,25(OH)$_2$VD$_3$ (P2). In contrast, the ratio of 25(OH)VD$_3$ was relatively constant over time (30–33% of total products). Thus we conclude that the formation of 24S,25(OH)$_2$VD$_3$ from VD$_3$ proceeds via the intermediate 24S(OH)VD$_3$. This is to the best of our knowledge a novel reaction pathway of VD$_3$ leading to 24S,25(OH)$_2$VD$_3$ via 24S(OH)VD$_3$. In addition, these results demonstrate that CYP109E1 has a 25-hydroxylation activity towards VD$_3$ as well as 24S(OH)VD$_3$.

It is known that the hydroxylation of VD$_3$ at C-25 is the first step of the VD$_3$ activation [7,8,11]. The 25(OH)VD$_3$ is the best indicator of the nutritional status of VD$_3$ in the circulation [15]. This compound has been gaining importance in recent years as it was shown to be a better therapeutic agent for several diseases than VD$_3$ itself, due to its direct biological effect and better intestinal
absorption [35,36]. It was found that supplementation of 25(OH)D$_3$ has a preventive, therapeutic effect against diseases such as hyperglycemia, chronic kidney disease, Crohn’s and cholestatic liver disease [35,36]. In addition to applications of this metabolite for human health, it is used as supplement in animal feed [37]. Whereas specific biological activities for the natural VD$_3$ metabolite 24R,25(OH)$_2$VD$_3$ were identified [38-40], so far no functions are known for its unnatural epimer 24S,25(OH)$_2$VD$_3$, which was identified here in the CYP109E1-based whole cell conversion of VD$_3$.

Our aim in the next step of this study was the enhancement of the 25(OH)VD$_3$ production yield by CYP109E1. Since the crystal structure of a VD$_3$-bound CYP109E1 complex is not available, the putative substrate-binding residues were predicted with docking simulations. It is anticipated that VD$_3$ binds with its aliphatic side chain close to the heme iron and the C-25 atom is in a suitable distance for hydroxylation (Figure 4). High similarity of the CYP109E1-VD$_3$ model with the experimental CYP107 (Vdh)-VD$_3$ crystal structure gives credence to the reliability of our docking results. A similar set of hydrophobic residues is predicted to interact with VD$_3$ in CYP109E1, including I85, I241, I168 and V169, which correspond to I88, I235, L171, and V172 in Vdh [25]. To test their potential role in VD$_3$ binding and conversion in CYP109E1, these four residues were targeted for mutation to alanines. In addition, residue K187 was chosen for mutagenesis, to test whether this flexible residue may play a role in substrate binding by interacting with the 3β-OH group of VD$_3$. Previously, it was shown that mutation of this residue to an alanine slightly lowers the conversion rate of testosterone by CYP109E1 [19]. However, our results show that considering VD$_3$ hydroxylation, the mutation of K187 to alanine does not influence CYP109E1 activity (Figure 6). Thus, residue K187 plays no role in VD$_3$ binding by CYP109E1, in accordance with the docking results and with the lack of substrate binding interactions by the equivalent residue (K180) in the VD$_3$-bound crystal structure of Vdh.

Mutagenesis of the other four residues (I85, I168, V169 and I241) ultimately yielded an improved regio-selectivity of CYP109E1 towards VD$_3$. It has been found that all mutants, except I241A, exhibited a higher selectivity towards 25-hydroxylation than the wild type (Table 2). This enhancement of the regio-selectivity resulted in an increase of the 25(OH)VD$_3$ production upon whole-cell conversion of VD$_3$ (Figure 7). Compared to the wild type, approximately a 2-fold increase of the 25(OH)VD$_3$ yield was achieved with the I85A mutant, 45 mg l$^{-1}$ day$^{-1}$ compared to 24.5 mg l$^{-1}$ day$^{-1}$ in the literature, the conversion yield of 25(OH)VD$_3$ by the most widely investigated P450 for vitamin D$_3$ hydroxylation, CYP105A1, had been initially very low and was later significantly improved by protein engineering [17]. The most productive mutant of CYP105A1 (R73A/R84A) is reported to produce 8.3 mg l$^{-1}$ day$^{-1}$ of 25(OH)VD$_3$ [16,41]. Residue I85 of CYP109E1, as mentioned above, is located in SRS1, in the close vicinity of the heme, and is one of the most investigated residues in P450s. The corresponding position in different P450s was described to interact with the P450s ligands and, therefore, to affect the activity and selectivity of the enzyme [42]. The importance of this position was observed in case of CYP102A1 as the substitutions of F87 caused improved selectivity towards propylbenzene and terpene substrates, among others [42-44]. In addition, the substitution of S122 to threonine in CYP1A1 (corresponding position to I85 in CYP109E1 and F87 in CYP102A1) improved the 7-methoxy- and 7-ethoxyresorufin O-dealkylase activity [45].

Furthermore, our results showed that the substitution of V169 or I241 to alanine residues significantly diminished the conversion of the 24S(OH)VD$_3$ intermediate into 24S,25(OH)$_2$VD$_3$, as compared to reactions with the wild type enzyme. The functional importance of V169 and I241 was also previously determined for testosterone conversion by CYP109E1 [19]. It was shown that by substituting either residue to an alanine the activity of CYP109E1 towards testosterone is completely abolished. Therefore, we suggest that V169 and I241 of CYP109E1 are substrate specificity-determining residues.
Conclusions

This study describes the identification of a new VD₃ hydroxylase from B. megaterium, CYP109E1, with 25- and 24-hydroxylation activity. The established B. megaterium-based whole-cell system for the conversion of VD₃ has a promising potential for the biotechnological production of the valuable metabolite 25(OH)VD₃.

In addition, further investigations on CYP109E1 were performed in order to increase the production of 25(OH)VD₃. Based on docking studies, site-directed mutagenesis was performed at selected positions in the active site of the enzyme. We were able to demonstrate the importance of selected active site residues for VD₃ conversion. A change of the regio-selectivity and substrate specificity was observed for most of the mutants. A considerable increase of the production of the valuable metabolite 25(OH)VD₃ was achieved with the I85A mutant. In addition, two novel metabolites of VD₃, 24S(OH)VD₃ and 24S,25(OH)₂VD₃, have been identified, whose potential for further drug development needs to be investigated.

Materials and methods

**Chemicals.** VD₃, 25(OH)VD₃, 1α-25(OH)₂VD₃, (2-hydroxypropyl)-β-cyclodextrin and saponin (from quillaja bark) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-aminolevulinic acid were purchased from Carbolution chemicals (Saarbrücken, Germany). Bacterial media were purchased from Becton Dickinson (Heidelberg, Germany). All other chemicals were from standard sources and of highest purity available.

**Bacterial strains and plasmids.** Cloning experiments were carried out with E. coli Top10 (Invitrogen, San Diego, USA). The E. coli strain C43 (DE3) for the heterologous protein expressions was purchased from Lucigen Corporation (Wisconsin, USA). Whole-cell conversions were carried out using B. megaterium MS941 [29,46]. pET17b (Merck Bioscience, Bad Soden, Germany) and pSMF2.1 [32] were used for expression purposes in E. coli and B. megaterium, respectively.

**Cloning of CYP109E1.** The coding region for CYP109E1 (GenBank GeneID 9119265) was amplified by polymerase chain reaction (PCR) using genomic DNA of B. megaterium MS941 as template. The coding region of CYP109E1 was cloned into the SpeI and KpnI restriction sites of pSMF2.1, yielding pSMF2.1.CYP109E1. The gene of CYP109E1 with a hexahistidine tag at the C-terminus was cloned into NdeI and KpnI restriction sites of pET17b, yielding pET17b.CYP109E1.

**Site-directed mutagenesis.** The mutants of CYP109E1 were generated by the QuikChange site-directed mutagenesis method using the plasmid pSMF2.1.CYP109E1 as template and Phusion DNA polymerase (Thermo Fisher Scientific GmbH, Dreieich, Germany). The PCR primers (MWG-Biotech AG, Ebersberg, Germany) were designed to introduce point mutation at the desired positions. The oligonucleotide primers for mutagenesis are shown in Table 1. The reactions were performed in a 50 µl volume using a gradient cycler (PTC-200 DNA Engine cycler). 20 cycles were carried out as follows: initial denaturation at 95 °C for 30 s, denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 4 min. Correct generation of the desired mutations was confirmed by DNA sequencing, carried out by Eurofines-MWG (Ebersberg, Germany).
Table 1. Oligonucleotides used in PCR to generate CYP109E1 mutants.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>oligonucleotides</th>
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<tbody>
<tr>
<td>I85A-for</td>
<td>5’-ACGAGCCCTAGCTAAATATTGATCCGCTTAAG-3’</td>
</tr>
<tr>
<td>I85A-rev</td>
<td>5’-CTTAGGGCAATATAATTAGCTTAGGCTCGT-3’</td>
</tr>
<tr>
<td>I168A-for</td>
<td>5’-TGGATATTTGCGTAGCCGTCCTCTCTCAATAACGAACGT-3’</td>
</tr>
<tr>
<td>I168A-rev</td>
<td>5’-ACGGTCGTTATAGAAGGACCCGCTACGGCAATATCCGA-3’</td>
</tr>
<tr>
<td>V169A-for</td>
<td>5’-GATATTATCGAGCCGTCCTCTCTAAATACGAACGT-3’</td>
</tr>
<tr>
<td>V169A-rev</td>
<td>5’-ACGGTCGTTATAGAAGGACCCGCTACGGCAATATCCGA-3’</td>
</tr>
<tr>
<td>K187A-for</td>
<td>5’-CTCCAGCAGGAAGCATGAAAGCAATGAATGATGAC-3’</td>
</tr>
<tr>
<td>K187A-rev</td>
<td>5’-GCTCATATTGCGTCTTCTGCTGGAG-3’</td>
</tr>
<tr>
<td>I241A-for</td>
<td>5’-CTATTTTTGCTACTGGTCTGGAAACGGAACACCATACC-3’</td>
</tr>
<tr>
<td>I241A-rev</td>
<td>5’-GTGGTGTTCGTTCCAGCAGGACAGTAGCAAAATAG-3’</td>
</tr>
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**Heterologous expression in E. coli and purification.** To express CYP109E1 and its mutants, *E. coli* C43 (DE3) cells were transformed with the corresponding expression plasmids (pET17b.CYP109E1) and cultured overnight in Luria-Bertani (LB) medium containing ampicillin (100 μg ml⁻¹) at 37 °C and 140 rpm. The seed culture was used to inoculate a 200 ml Terrific Broth (TB) medium containing ampicillin 100 μg ml⁻¹ (1:100 dilution) in a 2-1 baffled flask. The main culture was grown at 37 °C and 140 rpm. When the OD₆₀₀ reached 0.5, the expression was induced with 1 mM IPTG. 0.5 mM delta-aminolevulinic acid served as heme precursor. The cultures were shifted to 30 °C and 120 rpm for 24h. The *E. coli* cells were harvested by centrifugation at 4500 rpm for 30 min, and the cell pellets were stored at −20 °C until purification. All purification steps were performed at 4 °C. The cell pellets were resuspended in 50 mM potassium phosphate buffer (pH 7.4) containing 300 mM NaCl and 20% glycerol. Phenylmethylsulphonyl fluoride (PMSF) was added to a final concentration of 1 mM and the suspension was sonicated with a T13 sonotrode for 15 min with an amplitude of 12% and consisted of repeated intervals of 15 s pulse and 15 s pause. Cell free extract was obtained by ultracentrifugation at 30,000 rpm for 30 min. The supernatant was applied to an immobilized metal ion affinity chromatography column (TALON, Takara Bio Europe, Saint-Germain-en-Laye, France) that had been equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 300 mM NaCl and 20% glycerol. The column was washed with 5 column volumes of 50 mM potassium phosphate buffer (pH 7.4) containing 300 mM NaCl, 20 mM imidazole and 20% glycerol. The tagged protein was eluted with 50 mM potassium phosphate buffer (pH 7.4) containing 300 mM NaCl, 150 mM imidazole and 20% glycerol. The bovine Adx₄₁₀₈ and AdR were expressed and purified as described elsewhere [47,48].

**Carbon monoxide (CO) difference spectroscopy.** The reduced CO difference spectra of P450 were measured with a double-beam spectrophotometer (UV-2101PC, Shimadzu, Japan). The concentration of P450 was estimated using a molar extinction coefficient of ε₄₅₀-₄₉₀= 91 mM⁻¹ cm⁻¹ referred to the method of Omura and Sato [49].

**In vitro conversion of **VD₃. A reconstituted *in vitro* system containing CYP109E1 (1μM), AdR (3 μM), Adx₄₁₀₈ (20 μM), MgCl₂ (1 mM), and a cofactor regenerating system with glucose-6-phosphate (5mM) and glucose-6-phosphate dehydrogenase (1 U) was used in a final volume of 250 μl in potassium phosphate buffer (20 mM, pH 7.4). The substrate was dissolved in 2-hydroxypropyl-β-cyclodextrin (2.25% w/v) and added to a final concentration of 200 μM. The reaction was started by addition of 0.5 mM NADPH. After 1 h at 30 °C the reaction was stopped and extracted twice with 2 vol of ethyl acetate. The organic phases were combined, evaporated to dryness and prepared for analysis by high-performance liquid chromatography (HPLC).
Whole-cell conversion of VD$_3$. The whole-cell conversions were performed in *B. megaterium* MS941. The cells were transformed with the corresponding pSMF2.1.CYP109E1 plasmid using the polyethylene glycol-mediated protoplast transformation method [50]. The seed culture was prepared with LB medium (10 µg/ml tetracycline). The main culture (50 ml TB medium, 10 µg/ml tetracycline) was inoculated with 500 µl of seed culture (dilution 1:100) in 300 ml baffled flasks and incubated at 37 °C, 140 rpm. The culture was grown to OD$_{578}$ of 0.4 and recombinant gene expression was induced with xylose (5 mg/ml). The culture was grown further at 30 °C for 24h with shaking at 140 rpm. For whole-cell conversion experiments, VD$_3$ was dissolved in 45% 2-hydroxypropyl-β-cyclodextrin and 4% Quillaja Saponin as membrane solubilizing agent. After 24h of protein expression, 2.5 ml of the substrate solution were added to the 50 ml culture. A final substrate concentration of 200 µM was used for all conversion experiments. Afterwards, the conversion was performed for the indicated time at 30 °C and 120 rpm in 300 ml baffled flasks. 500 µl samples of the cultures were taken after defined time periods, extracted and prepared for HPLC analysis. Large scale whole-cell conversions for purification of VD$_3$ metabolites were performed in 2 l baffled flasks using 250 ml of main culture. Cultivation of bacteria as well as the whole-cell conversion and extraction were accomplished as mentioned above. After extraction, organic phases were dried using solvent evaporator and stored at −20 °C under protection from UV light until product purification by HPLC.

High-performance liquid chromatography (HPLC). The HPLC was carried out on a Jasco system (Pu-980 HPLC pump, AS-950 sampler, UV-975 UV/visible detector, LG-980-02 gradient unit; Jasco, Gross-Umstadt, Germany) equipped with a Nucleodur 100-5 C$_{18}$ column (125x4 mm; Macherey-Nagel, Düren, Germany). The column temperature was adjusted to 40 °C. The samples were dissolved in 200 µl acetonitrile. The flow rate was 1 ml/min with a linear gradient of 60–100% aqueous acetonitrile for 15 min followed by 100% acetonitrile for 15 min. The UV detection of the substrate and products was accomplished at 265 nm. The absorption properties of the products did not differ from the substrate and, therefore, the product formation was calculated from the relative peak area (area%) of the HPLC chromatograms, dividing each respective product peak area by the sum of all peak areas.

Product purification. Purification of the products was carried out with reversed-phase HPLC using a preparative column VP 250/8 NUCLEODUR 100-5 C$_{18}$ ec (Macherey-Nagel, Düren, Germany). First, the dried extract was dissolved in an acetonitrile/water mixture and filtered through the Rotilabo syringe filters (0.22 µm, Carl Roth GmbH, Karlsruhe, Germany). For purification of product P2, a linear gradient of 80–100% acetonitrile aqueous solution as a mobile phase for 17 min was applied (UV detection: 265 nm; flow rate: 3.5 – 4 ml/min; column temperature: 40 °C). Products P4 and P5 were purified isocratically using a 65% acetonitrile aqueous solution as a mobile phase for 40 min (UV detection: 265 nm; flow rate: 2.5 ml/min; column temperature: 40 °C). Collected product fractions were combined, evaporated to dryness and analyzed by NMR characterization.

NMR characterization of the metabolites. The NMR spectra were recorded in CDCl$_3$ with a Bruker Avance 500 NMR spectrometer at 298 K. The chemical shifts were relative to CDCl$_3$ at δ 7.26 (1H NMR) and CDC$_3$ at δ 77.00 (13C NMR) using the standard δ notation in parts per million. The 1D NMR (1H and 13C NMR) and the 2D NMR spectra (gs-HH-COSY and gs-HSQCED) were recorded using the BRUKER pulse program library.

Molecular docking. Vitamin D$_3$ (ligand) was docked into the active site of CYP109E1 (receptor) in its closed conformation (PDB: 5L94, CYP109E1-TES) with the use of Autodock Vina 1.1.2 [51]. The testosterone molecule and all waters were removed, and the resulting model was used as a template for the docking experiments. Coordinates for the ligand were taken form an available crystal structure (PDB: 3VRM); six bonds were kept rotatable as confirmed by manual inspection in AutoDockTools 1.5.6. Hydrogens and Gasteiger charges were also added in AutoDockTools 1.5.6. The protein was kept rigid during docking. The simulation cell was limited to a grid box centered at the heme iron with sides adjusted to cover the whole distal heme pocket (x:28 Å, y:34 Å, z:28 Å). The protein was kept rigid during docking. Hydrogens and Gasteiger charges were added in AutoDockTools 1.5.6.
Docking simulations were done in triplicate and twenty docking poses were generated for each simulation. The binding poses were analyzed according to lowest binding energies and distances of the target carbon atom (C-25) to the heme iron. Ligand binding residues were identified by analysis done with LigPlot+ [52] and further visualized with ViewDock tool in UCSF Chimera [53].

Competing interests

The authors declare that they have no competing interest.

Author contributions

AA designed and carried out the experiments, analyzed and interpreted the results and drafted the manuscript. IJ performed the docking simulations and assisted in drafting the manuscript. NP and EB purified the products for NMR analysis. EB carried out the substrate screening. JZ performed the NMR measurements and structure determination of vitamin D₃ metabolites. AT analyzed and interpreted the results, and assisted in drafting the manuscript. FH and RB designed the project, analyzed and interpreted the results, and assisted in drafting the manuscript. All authors read and approved the final manuscript.

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References


