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22*S*-Butyl-1α,24*R*-dihydroxyvitamin D_3: Recovery of vitamin D receptor agonistic activity☆

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ABSTRACT

We recently reported that 22*S*-butyl-1α,24*R*-dihydroxyvitamin D_3 **3** recovers the agonistic activity for vitamin D receptor (VDR), although its 25,26,27-trinor analog **2** is a potent VDR antagonist. To investigate the structural features involved in the recovery of agonism, we crystallized the ternary complex of VDR-ligand-binding domain, ligand **3** and coactivator peptide, and conducted X-ray crystallographic analysis of the complex. Compared with the complex with **2**, the complex with **3** recovered the following structural features: a pincer-type hydrogen bond between the 24-hydroxyl group and VDR, the conformation of Leu305, the positioning of His301 and His393, the stability of the complex, and intimate hydrophobic interactions between the ligand and helix 12. In addition, we evaluated the potency of both compounds for recruiting RXR and coactivator. The results indicate that the complex with **3** generates a suitable surface for coactivator recruitment. These studies suggest that the action of **2** as an antagonist is caused by the generation of a surface not suitable for coactivator recruitment due to the lack of hydrophobic interactions with helix 12 as well as insufficient hydrogen bond formation between the 24-hydroxyl group and VDR. We concluded that the action of **3** as an agonist is based on the elimination of these structural defects in the complex with **2**.

1. Introduction

Most biological actions of 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) **1** are mediated through the actions of its nuclear receptor (vitamin D receptor, VDR) [1]. Upon ligand binding, VDR undergoes a conformational change that promotes RXR-VDR heterodimerization, recruitment of coactivator, and initiation of transcription.

We have investigated the mechanism of biological actions of both VDR agonists and antagonists [2–4], and recently reported that 22*S*-butyl-25,26,27-trinor-1α,24-dihydroxyvitamin D_3 **2**, a synthetic vitamin D_3 analog, is a potent VDR antagonist [5]. Compound **2** possesses a unique side chain where terminus three carbons are removed while 22*S*-butyl group is added. X-ray crystallographic analysis of the ligand-binding domain (LBD) of VDR complexed with **2** demonstrated that **2** induces the formation of an extra cavity in the binding site to accommodate the 22*S*-butyl group, and eliminates the hydrophobic interactions between the side chain terminus and helix 12 of VDR-LBD. Moras's group reported the same cavity, observed in zebrafish VDR-LBD/GEMINI complex [6,7]. It is interesting that GEMINI and compound **2** induce a common and characteristic cavity to accommodate the second side chain, but GEMINI works as a VDR agonist whereas **2** works as a VDR antagonist. These results imply that the antagonistic activity of compound **2** is related to decreased interactions with helix 12 [5,8]. We also found that 22*S*-butyl-1α,24*R*-dihydroxyvitamin D_3 **3**, which contains the three carbons absent from the side chain of **2**, showed the agonistic activity, albeit weakly [5]. We speculated that the agonistic activity of **3** is due to restored hydrophobic interactions with helix 12. To investigate the structural features responsible for the recovery of agonism of compound **3**, we crystallized the VDR-LBD/**3**/peptide complex and conducted X-ray crystallographic analysis. We also evaluated the potency of compounds **2** and **3** in recruiting RXR and coactivator (Scheme 1).

2. Materials and methods

2.1. Crystallization and X-ray analysis

Crystallization and X-ray crystallographic analysis of the VDR-LBD/**3**/peptide complex was conducted as reported previously [5]. Briefly, the rat VDR-LBD (residues 116–423, Δ165–211) was over-

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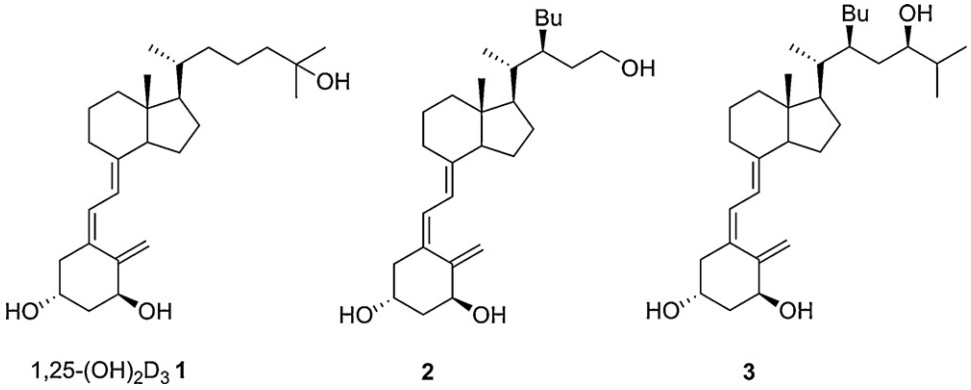

Scheme 1.

produced as an N-terminal His_6-tagged fusion protein in *Escherichia coli* C41. Affinity chromatography on a Ni-NTA column, tag removal by thrombin digestion, and gel filtration on a Superdex200 column, yielded purified rVDR-LBD protein. The VDR-LBD/**3**/peptide complex was crystallized by the vapor diffusion method. Diffraction data sets were collected at 100 K in a stream of nitrogen gas at beamline BL-6A of KEK-PF (Tsukuba, Japan). The structure of the complex was solved by molecular replacement with the program CNS [9] using a rat VDR-LBD coordinates (PDB code: 1RK3) [10]. The coordinates of the complex will be deposited in the Protein Data Bank with accession number 3AFR.

2.2. RXR and SRC-1 recruitment

Recruitment of RXRα and SRC-1 to VDR by 22*S*-butyl compounds (**2** and **3**) and 1,25-$(OH)_2D_3$ (**1**) were assessed using HEK293 cells. The activities were evaluated by a dual luciferase assay [11] using VP16-VDR expression plasmid (pCMX-VP16-hVDR), RXRα or SRC-1 expression plasmid (GAL4-RXRα or GAL4-SRC-1), a reporter plasmid containing four copies of GAL4 response element (MH100x4-TK-Luc), and the internal control plasmid containing sea pansy luciferase expression construct (pRL-CMV). All experiments were carried out in triplicate.

3. Results and discussion

In contrast to the poor quality of the VDR-LBD/**2**/peptide complex (3.0 Å resolution) [5], the quality of the VDR-LBD/**3**/peptide complex was much better (2.0 Å resolution, Table 1). A ribbon-tube representation of the rVDR-LBD/**3**/peptide complex is shown in Fig. 1c, and the rVDR-LBD/**1**/peptide and rVDR-LBD/**2**/peptide complexes are shown in Fig. 1a and b. The overall structure of the VDR-LBD complexed with **3** is similar to those complexed with **1** and **2**. The coactivator peptide is tightly bound to the activation function 2 (AF2) surface and occupies the typical agonist position (Fig. 1c). The crystal structure demonstrated that an additional cavity is produced in the same region observed in the rVDR-LBD/**2**/peptide complex, although the cavity is smaller (Fig. 1f). This cavity is formed because the terminus of the 22-butyl group of **3** pushes the Leu305 side chain away to rearrange the ligand-binding pocket (LBP) (Fig. 1k).

Table 1
Summary of data collection statistics and refinement.

Data set	rVDR-LBDdel + **3** + Peptide
X-ray source	KEK-PF BL-6A
Wavelength [Å]	0.97800
Space group	*C*2
Unit cell dimensions [Å]	$a = 153.45$, $b = 43.39$, $c = 41.99$
[°]	$\alpha = 90.00$, $\beta = 95.34$, $\gamma = 90.00$
Resolution range[Å][a]	50.00–2.00 (2.07–2.00)
Total number of reflections	67380
No. of unique reflections	19085
% completeness[a]	97.5 (88.9)
R_{merge}[a,b]	0.064 (0.211)
Refinement statistics	
Resolution range [Å][a]	50.00–2.00 (2.07–2.00)
R factor (R_{free}/R_{work})[a,c]	0.264 (0.348)/0.216 (0.339)

[a] Values in parentheses are for the highest-resolution shell.
[b] $R_{merge} = \Sigma|(I_{hkl} - \langle I_{hkl}\rangle)|/(\Sigma I_{hkl})$, where $\langle I_{hkl}\rangle$ is the mean intensity of all reflections equivalent to reflection *h k l*.
[c] R_{work} (R_{free}) = $\Sigma||F_{obs}|-|F_{calc}||/\Sigma|F_{obs}|$, where 10% of randomly selected data were used for R_{free}.

Ligand **3** was accommodated into the VDR-LBP in a manner similar to compounds **1** and **2**. The hydroxyl groups at the 1α- and 3β-positions form pincer-type hydrogen bonds, evident in the VDR-LBD/**1**/peptide complex (Fig. 1j). Additionally, the 24-hydroxyl group forms hydrogen bonds with His393 and His301 (Fig. 1j). It should be noted that, in the complex with antagonist **2**, the 24-hydroxyl group formed a hydrogen bond with His393 but not with His301 [5]. Fig. 1k and l are superpositions of the complexes with **1**, **2** and **3**. As shown in Fig. 1l, His393 in the complex with **3** is shifted compared with the complex with **1** but His301 is not, whereas both His393 and His301 are shifted in the complex with **2**. The B-factors for the Cα of His393 and His301 in the complex with **2**, but not with **1** and **3**, are rather larger than those of other unrelated residues (see PDB code: 3AFR). These results indicate that the complex with **3** recovered the almost correct hydrogen bond network composed of His393, His301 and the side chain hydroxyl group of the ligand. In addition, compound **2** does not interact directly with helix 12 (Fig. 1h) but compound **3** restores the intimate hydrophobic interactions with Val414 and Phe418 on helix 12 (Fig. 1i). However, the restoration of the hydrogen bond network and hydrophobic interactions in the complex with **3** is not complete, which may explain the weak agonist activity of **3**. The differences mentioned above are likely thought to be the source of the agonism of **3** and antagonism of **2**. It should be noted that in the complex of VDR with GEMINI, which is an agonist, the side chain hydroxyl group at C(25) forms hydrogen bonds with the corresponding two histidines and the side chain terminus of the ligand interacts with hydrophobic residues of VDR in a manner similar to that of the complex with **1** [6,7].

We evaluated ligand-dependent recruitment of RXRα and a coactivator peptide, SRC-1, by VDR using a mammalian two-hybrid assay in HEK293 cells. As shown in Fig. 2a and b, compounds **2** and **3** recruited RXRα weakly in a concentration-dependent manner. Fig. 2c shows that compound **2** slightly inhibited RXRα recruitment induced by natural hormone **1**, whereas compound **3** did not. These results demonstrate that both **2** and **3** can heterodimerize with RXRα, albeit weakly. In the recruitment of SRC-1, compound

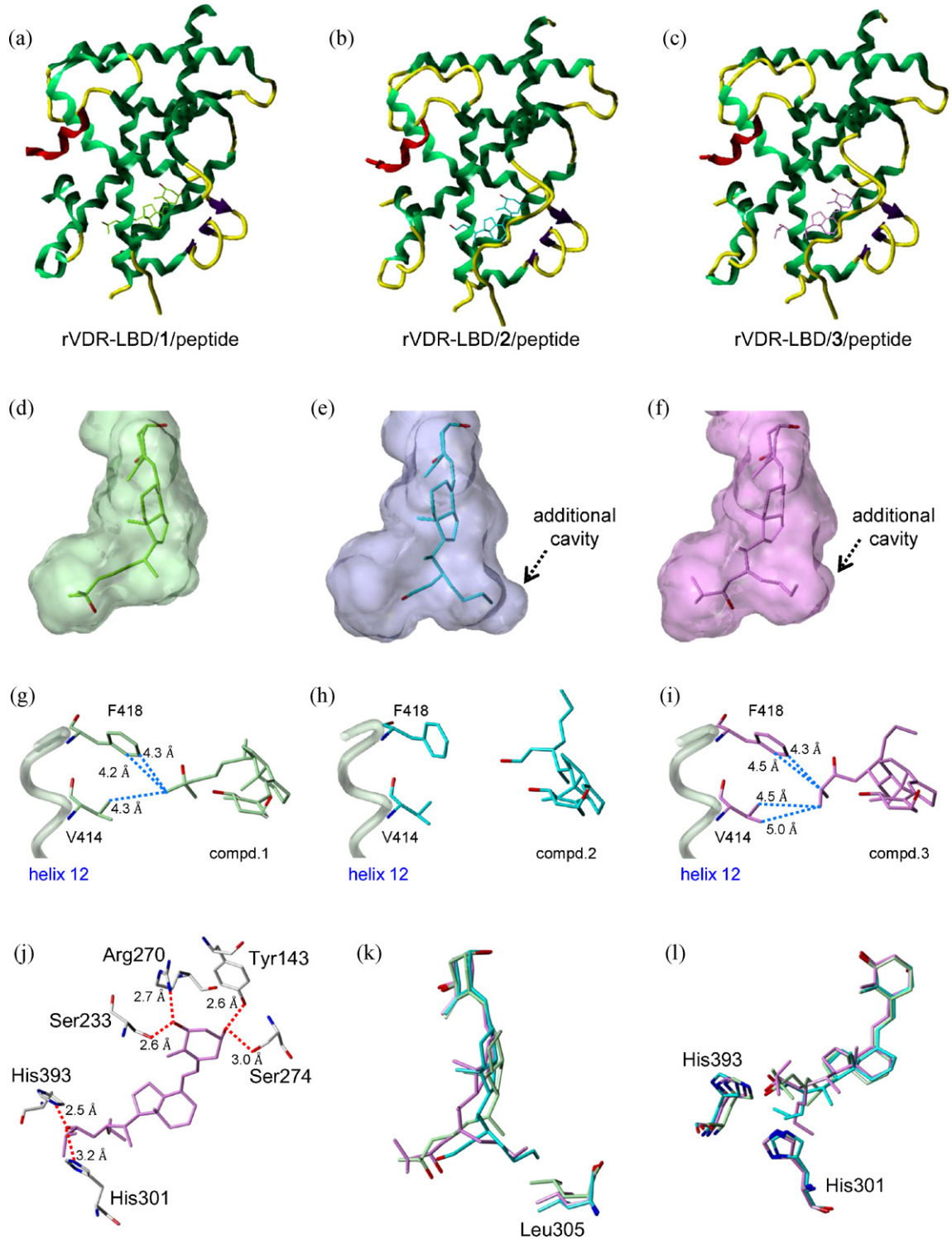

Fig. 1. X-ray crystal structure of rVDR-LBD/ligand/peptide complexes. ((a)–(c)) Overall view of rVDR-LBD/ligand/peptide complexes: helices, loops, β-sheets and coactivator peptide are shown in green, yellow, violet and red, respectively. (a) 1,25-$(OH)_2D_3$ (**1**) is shown as a stick model in green, (b) ligand **2** is shown as a stick model in cyan, (c) ligand **3** is shown as a stick model in magenta. ((d)–(f)) Connolly channel surfaces of the LBP of rVDR-LBD/ligand/peptide complexes. Ligands are 1,25-$(OH)_2D_3$ (**1**) (d), **2** (e), and **3** (f). ((g)–(i)) Interaction between helix 12 and the ligand. Hydrophobic interactions are shown as dotted blue lines. Ligands are 1,25-$(OH)_2D_3$ (**1**) (g), **2** (h), and **3** (i); (j) Hydrogen bonds between ligand **3** and the VDR. The bonds are shown as dotted red lines; ((k) and (l)) Superposition of three rVDR-LBD/ligand/peptide complexes. 1,25-$(OH)_2D_3$ (**1**) (green), **2** (cyan) and **3** (magenta). His301 of the complexes with **1** and **3** are almost superimpose, in contrast to His301 of the complex with **2**. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3 recruited SRC-1 weakly, whereas compound **2** did not (Fig. 2d and e). Compound **2** inhibited the recruitment of SRC-1 induced by natural hormone **1** but compound **3** did not, as shown in Fig. 2f. These results demonstrate that ligand **2** cannot induce VDR-LBD into a conformation appropriate for SRC-1 binding, whereas ligand **3** has moderate ability to induce the appropriate conformation. That both compounds **2** and **3** can heterodimerize with RXRα, and that compound **3** but not compound **2** can recruit SRC-1 to VDR, suggests

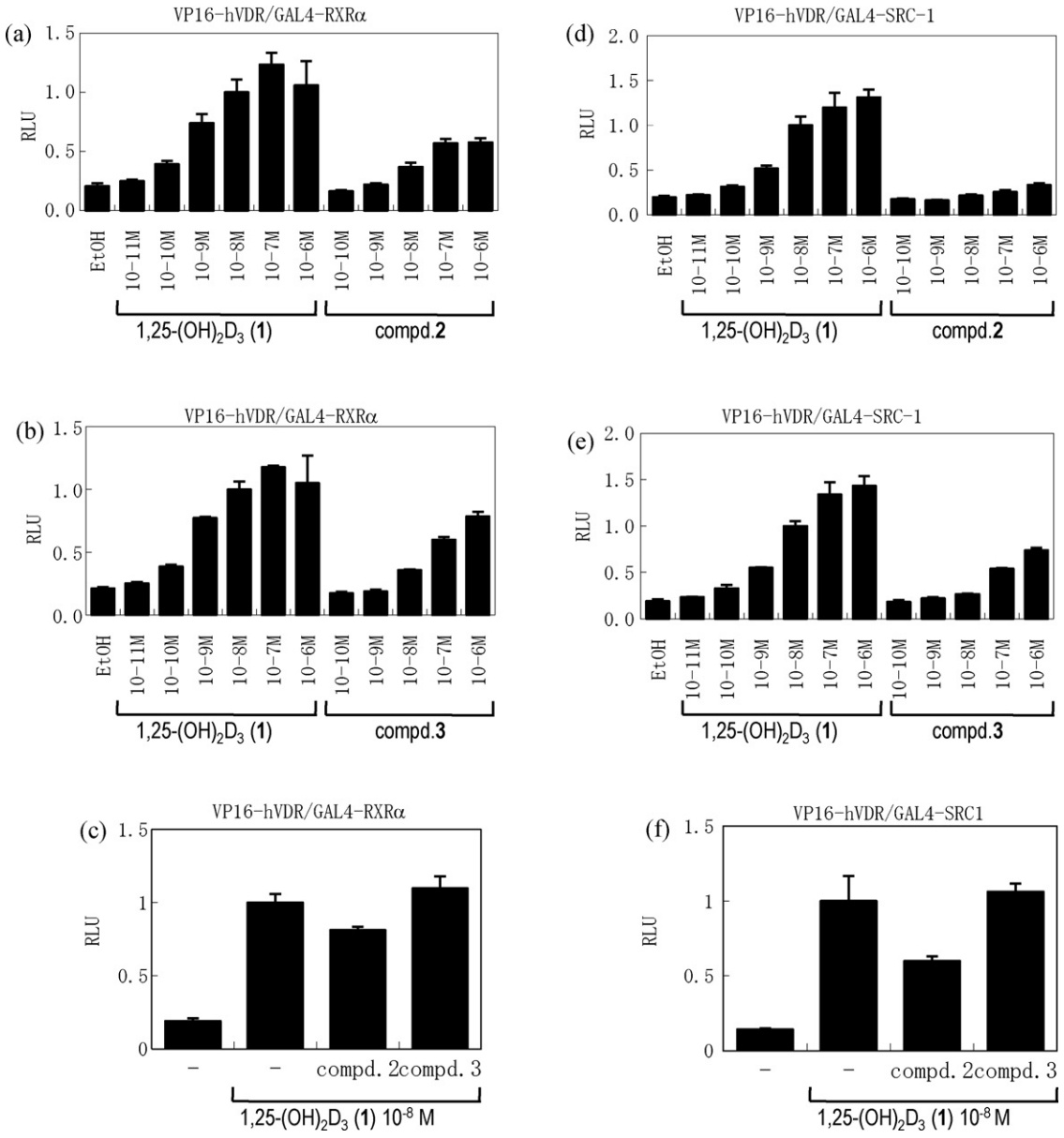

Fig. 2. RXR and SRC-1 recruitment to VDR by 1,25-$(OH)_2D_3$ (**1**) and 22*S*-butyl compounds (**2** and **3**) in HEK293 cells. The activities were evaluated by a dual luciferase assay using VP16-VDR expression plasmid (pCMX-VP16-hVDR), RXRα or SRC-1 expression plasmid (GAL4-RXRα or GAL4-SRC-1), a reporter plasmid containing four copies of GAL4 response element (MH100x4-TK-Luc), and the internal control plasmid containing sea pansy luciferase expression construct (pRL-CMV). All experiments were carried out in triplicate. RXRα recruitment by hormone **1** and **2** (a), by **1** and **3** (b), by **2** (10^{-6} M) and **3** (10^{-6} M) in the presence of 10^{-8} M of **1** (c); SRC-1 recruitment by hormone **1** and **2** (d), by **1** and **3** (e), by **2** (10^{-6} M) and **3** (10^{-6} M) in the presence of 10^{-8} M of **1** (f).

that the recovery of agonism by **3** is due to the construction of an AF2 surface suitable for coactivator recruitment.

Taken together, the results suggest that compound **3** shows agonistic activity because **3** restores the following structural features: a pincer-type hydrogen bond between the 24-hydroxyl group and VDR; the conformation of Leu305; the positioning of His301 and His393; the stability of the VDR complex; the intimate hydrophobic interaction with helix 12; the suitable surface for coactivator recruitment. From the present study we conclude that antagonist action of **2** is caused by a surface not suited for coactivator recruitment, due to lack of hydrophobic interactions with helix 12 in addition to insufficient hydrogen bonding of the 24-hydroxyl group with VDR; and that the action of **3** as an agonist is based on the removal of the structural defects observed in the complex with **2**.

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