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1 α ,20S-Dihydroxyvitamin D₃ Interacts with Vitamin D Receptor: Crystal Structure and Route of Chemical Synthesis

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1 α ,20S-Dihydroxyvitamin D₃ [1,20S(OH)₂D₃], a natural and bioactive vitamin D₃ metabolite, was chemically synthesized for the first time. X-ray crystallography analysis of intermediate **15** confirmed its 1 α -OH configuration. 1,20S(OH)₂D₃ interacts with the vitamin D receptor (VDR), with similar potency to its native ligand, 1 α ,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] as illustrated by its ability to stimulate translocation of the VDR to the nucleus, stimulate VDRE-reporter activity, regulate VDR downstream genes (VDR, CYP24A1, TRPV6 and CYP27B1), and inhibit the production of inflammatory markers (IFN γ and IL1 β). However, their co-crystal structures revealed differential molecular interactions of the 20S-OH moiety and the 25-OH moiety to the VDR, which may explain some differences in their biological activities. Furthermore, this study provides a synthetic route for the synthesis of 1,20S(OH)₂D₃ using the intermediate 1 α ,3 β -diacetoxy pregn-5-en-20-one (**3**), and provides a molecular and biological basis for the development of 1,20S(OH)₂D₃ and its analogs as potential therapeutic agents.

The classical pathway of vitamin D₃ (D₃) activation involves two key steps: 25-hydroxylation to produce 25-hydroxyvitamin D₃ [25(OH)D₃], and 1 α -hydroxylation by cytochrome CYP27B1 to produce the active 1 α ,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] (Fig. 1)¹. This natural ligand of the vitamin D receptor (VDR) regulates expressions of various genes including that encoding catabolic CYP24A1 through the VDR. Other activities mediated via the VDR include anti-inflammation, anti-proliferation, pro-differentiation, pro-apoptosis, immunomodulation, mineral homeostasis and anti-angiogenesis^{2–4}. In addition, D₃ can also be activated by a novel metabolic pathway initiated by CYP11A1 (P450_{scc}) producing 20S-hydroxyvitamin D₃ [20S(OH)D₃] as the major product⁵. As an activation enzyme, CYP27B1 is able to hydroxylate 20S(OH)D₃ producing the natural metabolite 1 α ,20S-dihydroxyvitamin D₃ [1,20S(OH)₂D₃] and has been used to produce μ g amounts of this product *in vitro*^{6,7}. Alternative biosynthesis using CYP11A1 to 20S-hydroxylate commercially available 1 α -hydroxyvitamin D₃ [1(OH)D₃] increased the production of 1,20S(OH)₂D₃ to 0.5–1 mg⁷.

1,20S(OH)₂D₃ has been found to upregulate the expression of CYP24A1 mRNA, suggesting that it can modulate the expression of genes downstream of the VDR⁷. It also inhibits cell growth and shows potent anti-leukemic and anti-melanoma activities, while displaying less calcemic (toxic) effect than 1,25(OH)₂D₃^{7–10}. In addition, 1,20S(OH)₂D₃ was found in human epidermis suggesting an endogenous role in the skin¹¹. However, the lack of detailed information on the interactions between 1,20S(OH)₂D₃ and VDR makes it difficult to understand its

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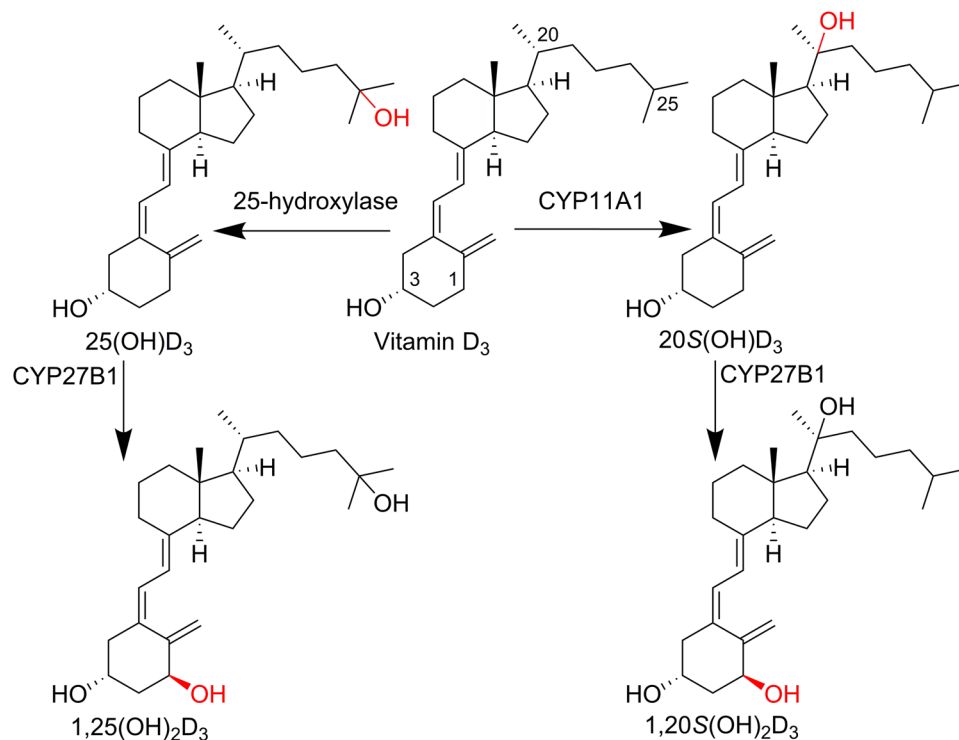


Figure 1. Classical (left) and novel (right) metabolic pathways of vitamin D₃ activation.

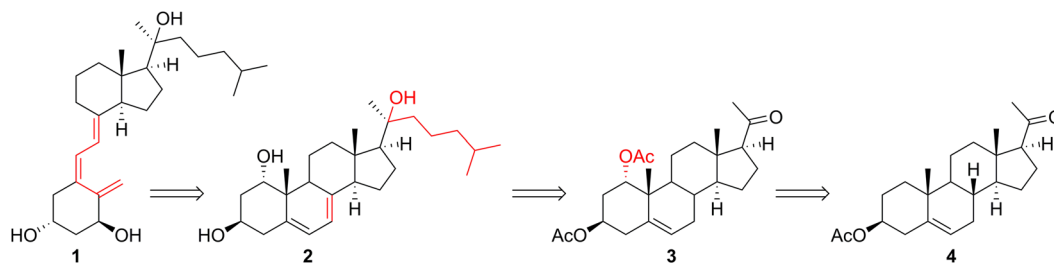


Figure 2. Retrosynthesis of 1,20S(OH)₂D₃.

mechanism of action, and some of the differential effects of 1,20S(OH)₂D₃ and 1,25(OH)₂D₃. Here we present the high resolution X-ray crystal structure of 1,20S(OH)₂D₃ in complex with the VDR, as well as further characterization of its biological activities. Importantly, while 1,20S(OH)₂D₃ has great potential as a therapeutic agent, the production of 1,20S(OH)₂D₃^{6,7} and its analogs^{12,13} has been limited to date by the need for purified enzymes, CYP27B1 or CYP11A1, for their biosynthesis. Now we report the first chemical synthesis of 1,20S(OH)₂D₃ facilitating its production for further testing of its biological activities.

Results and Discussion

Retrosynthesis of 1,20S(OH)₂D₃. A retrosynthetic strategy including a common 1 α -OH intermediate **3** was proposed (Fig. 2). The D₃-like structure could be obtained from UVB transformation of **2**^{12–14}, of which the 20S-OH and side chain could be achieved by Grignard reaction of **3**^{12–15}. Introduction of 1 α -OH to **4** could be carried out by a multi-step conversion following the synthesis of androstenolone¹⁶.

Synthesis of 1,20S(OH)₂D₃. The synthesis (Fig. 3) started with deacetylation and TBS protection of pregnenolone acetate (**4**) to give intermediate **6**. NaBH₄ treatment of **6** selectively afforded the 20*R* epimer as a major product which was then protected with an acetyl group to go through the DDQ oxidation safely (75% yield) to produce intermediate **10**. After replacing 20-OAc with 20-OTBS, a 1 α ,2 α -epoxide group was introduced by adding KOH and H₂O₂ solution to afford intermediate **13** (73%), followed by Birch reduction to give 1 α ,3 β -diol **14** (61%) as a major product^{16–18}. To confirm the 1 α -OH formation, **14** was protected with an acetyl group to produce **15**, which was characterized by 1D and 2D NMR spectrometry, and crystallized from hexane for X-ray structure analysis (see Supplementary Fig. S2). After removal of 20-OTBS, intermediate **16** was oxidized by DMP to 1 α ,3 β -diacetoxypregn-5-en-20-one (**3**, 95%), which was then transformed into the 5,7-diene 7DHC intermediate

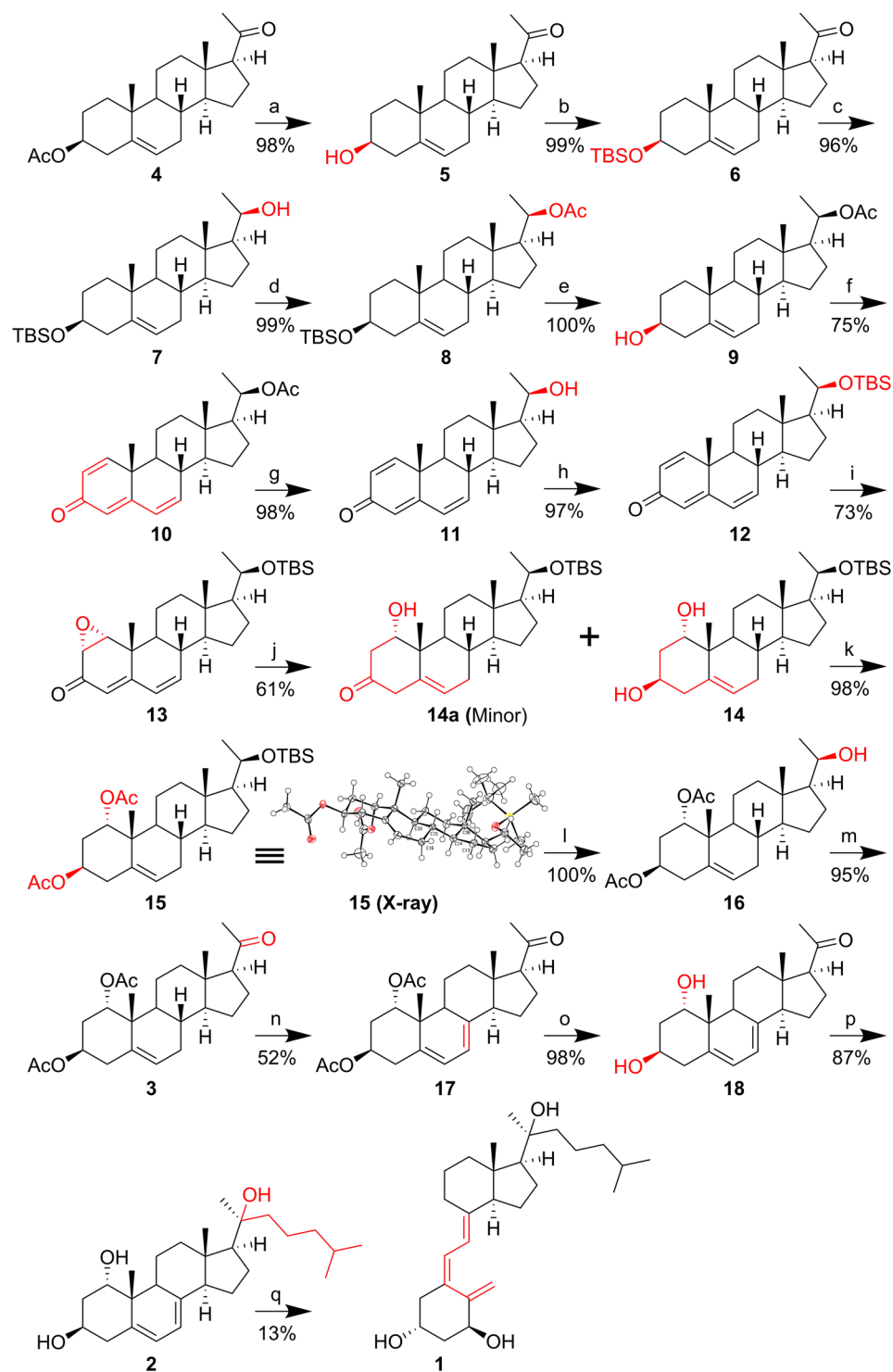


Figure 3. Synthesis of 1 α ,20S-dihydroxyvitamin D₃. Reagents and conditions: (a) K₂CO₃, MeOH, r.t., overnight. (b) TBSCl, imidazole, DMF, r.t., overnight. (c) NaBH₄, DCM: MeOH (1:1), 0°C - r.t., overnight. (d) Ac₂O, Et₃N, DMAP, DCM, r.t., overnight. (e) TBAF, THF, r.t., 12 h. (f) DDQ, 1,4-dioxane, reflux, 4 h. (g) KOH, MeOH, r.t., 3 h. (h) TBSCl, imidazole, DMF, r.t., overnight. (i) KOH in MeOH, 30% H₂O₂, MeOH, -40°C - 0°C, 12 h. (j) Li, NH₃ (liquid), -80°C, 30 min; addition of starting material in THF, -80°C, 2 h; -40°C, 1 h; NH₄Cl, -80°C, 2 h. (k) Ac₂O, Et₃N, DMAP, DCM, r.t., overnight. (l) TBAF, THF, r.t., 48 h. (m) DMP, DCM, r.t., 12 h. (n) Dibromantoin, AIBN, benzene: hexane (1:1), reflux 20 min; TBAB, THF, r.t., 75 min; TBAF, r.t., 50 min. (o) K₂CO₃, MeOH, r.t., overnight. (p) i) Mg, I₂, 1-bromo-4-methylpentane, THF, reflux, 1 h; ii) **18**, THF, 0°C - r.t., overnight. (q) UVB irradiation, Et₂O, 50°C, 15 min; r.t., 10 d; HPLC, MeCN:H₂O. AIBN, azobisisobutyronitrile; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; DMP, Dess-Martin periodinane; DMAP, 4-dimethylaminopyridine; HPLC, high-performance liquid chromatography; TBAB, tetra-*n*-butylammonium bromide; TBAF, tetra-*n*-butylammonium fluoride; TBSCl, *tert*-butyldimethylsilyl chloride.

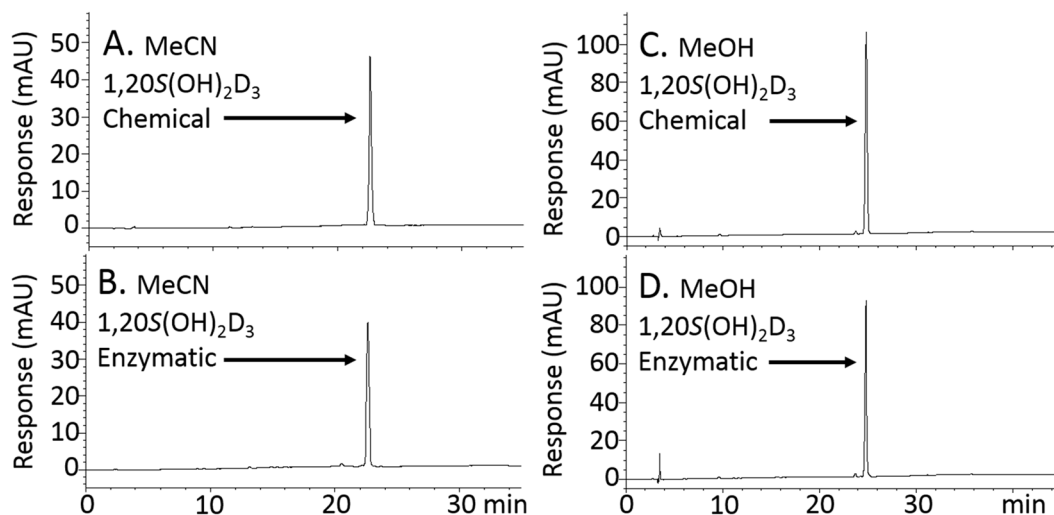


Figure 4. Comparison of HPLC retention times of chemical and enzymatic $1,20S(OH)_2D_3$. Chemically synthesized (A and C) and enzymatically produced (B and D) $1,20S(OH)_2D_3$ was analysed under MeCN: water condition (A and B, 0.25 μ g) and MeOH: water condition (C and D, 0.5 μ g).

(17, 52%) following a well-established procedure^{12–14}. To avoid potential separation problems caused by acetyl protection after Grignard reaction, ester hydrolysis was carried out prior to Grignard reaction (87%) to afford $1\alpha,20S$ -7DHC (2) where the formation of 20S confirmation was discussed in previous reports^{12–14}. UVB irradiation of 2 in ethyl ether followed by pre-vitamin D_3 isomerization afforded the desired product 1 (13%), which was compared with its enzymatic counterpart after HPLC separation.

Pregnenolone acetate (4) has often been used as the starting material for $20S(OH)_2D_3$ analogs^{12–14}, in which 1α -hydroxylation was necessary to display potent stimulation of the VDR^{12,13}. Owing to the lack of appropriate 1α -OH intermediates, the production of 1α -OH derivatives of $20S(OH)_2D_3$ analogs was dependent on the purification of recombinant CYP27B1. The limited amount of 1α -OH derivatives that could be made was thus a hurdle for extensive biological testing. The production of $1\alpha,3\beta$ -diacetoxy-pregn-5-en-20-one (3) in this report enables production of various analogs of $1,20S(OH)_2D_3$ for future studies.

We experienced inconsistent yields during the Birch reduction of epoxide 13 in the initial trials. In fact, the addition of NH_4Cl (a quenching step) is the key to the success of this reaction. Quick addition (<10 min) of NH_4Cl gave predominantly intermediate 14a, whereas slow addition (>2 h) afforded mainly the desired product 14. To our knowledge, 14a as a semi-reduced intermediate was obtained and characterized for the first time.

HPLC showed matched retention times of chemical and enzymatic $1,20S(OH)_2D_3$. In addition to the UV spectra and NMR identification (Supplementary Information), the chemically synthesized $1,20S(OH)_2D_3$ was analysed by HPLC under two different solvent systems, either an acetonitrile in water gradient or a methanol in water gradient. We conclude that the chemically synthesized $1,20S(OH)_2D_3$ and the enzymatically produced counterpart are identical on the basis of their UV and NMR spectra, as well as their HPLC retention times (Fig. 4). Co-migration of the chemically and enzymatically synthesized $1,20S(OH)_2D_3$ was further confirmed by chromatography of a mixture of the two (see Supplementary Information).

Identification of 15 as having a 1α -OH by NMR analysis. To identify the formation of the 1α -hydroxyl, the structure of intermediate 15 was characterized from its NMR spectra (Supplementary Information). The NOESY spectrum of 15 gave a strong NOE integral (0.42, see Supplementary Fig. S1) of $1H_3$ to $19-CH_3$, using the NOE integral of $1H_3$ to $2H_3$ as an internal reference. In contrast, the NOE signal of $1H_3$ to $2H_\alpha$ was not observed, suggesting the presence of 1α -OAc group in 15.

Confirmation of 15 by X-ray crystallographic analysis. To confirm the structure of 15, crystals were produced in hexane for X-ray crystallographic analysis (Supplementary Information). The X-ray structure of 15 (CCDC code: 1527430, Fig. 3) confirmed its absolute structure as the desired product reported in the Fig. 3.

Transcriptional activity. The ability of $1,20S(OH)_2D_3$ to activate the VDR was analysed in three cell lines (HaCaT, Caco-2 and Jurkat) transduced with a lentiviral vitamin D response element (VDRE) reporter (luciferase)^{12–15}. Compared with $1,25(OH)_2D_3$ and 22-oxa- $1,25(OH)_2D_3$ (22-Oxa), two known VDR agonists, $1,20S(OH)_2D_3$ showed potent transcriptional activity with EC_{50} s of 450 nM in HaCaT cells, 285 nM in Caco-2 cells and 19.1 nM in Jurkat cells (Table 1). Although less potent than 22-Oxa in all three cell lines, $1,20S(OH)_2D_3$ is equally potent to (HaCaT and Caco-2 cells) or less potent than (Jurkat cells) $1,25(OH)_2D_3$, the native ligand of the VDR.

X-ray crystallographic analysis of the zVDR ligand binding domain in complex with $1,20S(OH)_2D_3$. To characterize molecular interactions in order to understand the mechanisms underlying the

Compound	VDRE stimulation (nM)			Cytokine level	
	HaCaT	Caco-2	Jurkat	IFN γ	IL1 β
Control	NA	NA	NA	710 \pm 9	123 \pm 2
1,20S(OH) $_2$ D $_3$	450.4 \pm 14.9	284.8 \pm 13.2	19.1 \pm 0.9	383 \pm 3	90 \pm 2
1,25(OH) $_2$ D $_3$	421.9 \pm 3.1	300.2 \pm 9.2	2.1 \pm 0.1	353 \pm 11	121 \pm 3
22-Oxa	10.5 \pm 2.6	154.5 \pm 0.8	1.2 \pm 0.1	258 \pm 2	91 \pm 2

Table 1. Stimulation of VDRE-reporter activity and inhibition of cytokine production by 1,20S(OH) $_2$ D $_3$. Note: VDRE stimulation activity = EC $_{50}$ \pm standard deviation, cytokine level in splenocyte cultures = value \pm standard error of the mean (pg/mL).

differential VDRE stimulatory effects, the *Danio rerio* VDR (zVDR) ligand binding domain (LBD) was crystallized in the presence of 1,20S(OH) $_2$ D $_3$ or 1,25(OH) $_2$ D $_3$. The overall structure of VDR-1,20S(OH) $_2$ D $_3$ (PDB code: 5MX7) is highly homologous to the VDR-1,25(OH) $_2$ D $_3$ structure, adopting the canonical active conformation. When compared to the zVDR LBD-1,25(OH) $_2$ D $_3$ structure^{19,20}, the C α atoms of the zVDR LBD-1,20S(OH) $_2$ D $_3$ complex have a root mean square deviation of 0.25 Å over 238 residues. The ligand binds similarly to 1,25(OH) $_2$ D $_3$ with the notable difference being that the 20S-OH forms a weak H-bond with His305 (3.42 Å) and does not interact with His397 (note that the residues numbers correspond to hVDR). The H-bond with His305 causes a ligand-induced conformational change in the receptor where His305 (loop6-7) is shifted by 0.63 Å to enable this H-bonding interaction. (Fig. 5). The 1 α -OH and 3 β -OH form similar hydrogen bonds to the zVDR to those seen with 1,25(OH) $_2$ D $_3$.

An additional difference in the structures is that the 20S-OH forms a Van der Waals interaction with Val300. While most of the Van der Waals interactions are maintained, the side chain and terminal methyl groups that are differently positioned to interact differently with some of the residues (Fig. 5). Weaker interactions are formed with Leu227 (4.1 Å instead of 3.8 Å with C26) and Tyr399 (4.1 Å instead of 3.8 Å with C27), interactions compensated by stronger interactions with Val234 (3.9 Å instead of 4.2 Å with C22), and Leu412 (3.9 Å instead of 4.2 Å with C27). Overall, the hydrogen bonding interaction of 20S-OH with His305 and hydrophobic contacts formed by the ligand explains its agonist activity, however, with less potency than that of 1,25(OH) $_2$ D $_3$.

VDRE translocation activity. 1,25(OH) $_2$ D $_3$ binds to cytosolic or membrane-associated VDR³, then translocation of 1,25(OH) $_2$ D $_3$ -bound VDR from the cytoplasm to the nucleus is a key step to exert its gene-regulatory effects^{1,3}. In SKMEL-188 melanoma cells transduced with pLenti-CMVVDR-EGFP-pgk-puro²¹, both 1,20S(OH) $_2$ D $_3$ and 1,25(OH) $_2$ D $_3$ showed stimulatory effects on this translocation with EC $_{50}$ values of 2.14 \times 10 $^{-9}$ and 7.87 \times 10 $^{-9}$ M (Fig. 6A), respectively. The results indicate that 1,20S(OH) $_2$ D $_3$ induces VDR translocation in a similar fashion to 1,25(OH) $_2$ D $_3$.

Regulatory activity of 1,20S(OH) $_2$ D $_3$ on VDR downstream genes. To investigate how 1,20S(OH) $_2$ D $_3$ affects VDR target genes through VDR activation, expression of *VDR*, *CYP24A1*, *TRPV6* and *CYP27B1* genes at the mRNA level was determined in HaCaT cells (Fig. 6B). 1,20S(OH) $_2$ D $_3$ was capable of mildly upregulating the expression (1.6-fold) of the gene encoding its own receptor, the VDR, while being moderately stronger than 1,25(OH) $_2$ D $_3$ (1.3-fold) and comparable to 22-Oxa (1.7-fold). 1,25(OH) $_2$ D $_3$ is known to induce expression of the vitamin D catabolic enzyme, *CYP24A1*^{14,22}. Similarly, 1,20S(OH) $_2$ D $_3$ strongly stimulates *CYP24A1* mRNA levels 34-fold, as compared to 10-fold for 1,25(OH) $_2$ D $_3$ and 78-fold for 22-Oxa. In addition, *TRPV6* encoding an intestinal calcium channel is also a well-known target of VDR for mineral homeostasis^{14,23}. The mRNA levels of *TRPV6* were increased by 1.4-, 1.4- and 2.6-fold for 1,20S(OH) $_2$ D $_3$, 1,25(OH) $_2$ D $_3$ and 22-Oxa, respectively. Moreover, VDR activation induced by its agonists inhibits the expression of the vitamin D activation enzyme, *CYP27B1*^{24,25}. Although less than 1,25(OH) $_2$ D $_3$ and 22-Oxa, 1,20S(OH) $_2$ D $_3$ slightly but significantly inhibited the expression of *CYP27B1*. These results indicate that 1,20S(OH) $_2$ D $_3$ is able to activate the VDR, and exert its effects through regulating VDR target genes in a similar manner to 1,25(OH) $_2$ D $_3$. Since 1,20S(OH) $_2$ D $_3$ affected the expression of *VDR*, *TRPV6* and *CYP27B1* weakly (~2-fold), further investigation on their protein levels will be beneficial to confirm the actions of 1,20S(OH) $_2$ D $_3$.

Anti-inflammatory activity. The anti-inflammatory effect of 1,20S(OH) $_2$ D $_3$ was determined in mouse splenocytes stimulated by lipopolysaccharide prior to treatments with the secosteroids. The concentrations of IFN γ and IL1 β in the culture media were significantly reduced by 1,20S(OH) $_2$ D $_3$, compared with the control (Table 1). The effect of 1,20S(OH) $_2$ D $_3$ (1.0 nM) was comparable with or slightly weaker than that of 1,25(OH) $_2$ D $_3$ but weaker than 22-Oxa for reduction of IFN γ production. In contrast, 1,20S(OH) $_2$ D $_3$ (100 nM) showed equal efficacy to 22-Oxa, and higher than that of 1,25(OH) $_2$ D $_3$ for reduction of IL1 β production. These studies suggested that 1,20S(OH) $_2$ D $_3$, acting similarly to 1,25(OH) $_2$ D $_3$ and 22-Oxa, is a potent anti-inflammatory agent.

Conclusions

Similar to 1,25(OH) $_2$ D $_3$, 1,20S(OH) $_2$ D $_3$ can interact with the VDR with high potency, as evidenced by its ability to stimulate its translocation to the nucleus, regulate VDR downstream genes (including but not limited to *VDR*, *CYP24A1*, *TRPV6* and *CYP27B1*), and exert strong anti-inflammatory activity. The crystal structure of 1,20S(OH) $_2$ D $_3$ bound to the VDR reveals differences from the 1,25(OH) $_2$ D $_3$ bound form with respect to their interactions, including the important role of the H-bond between the 20S-OH and His305 that shifts the position of this residue compared to the 1,25(OH) $_2$ D $_3$ -bound form. This difference may contribute to their differential

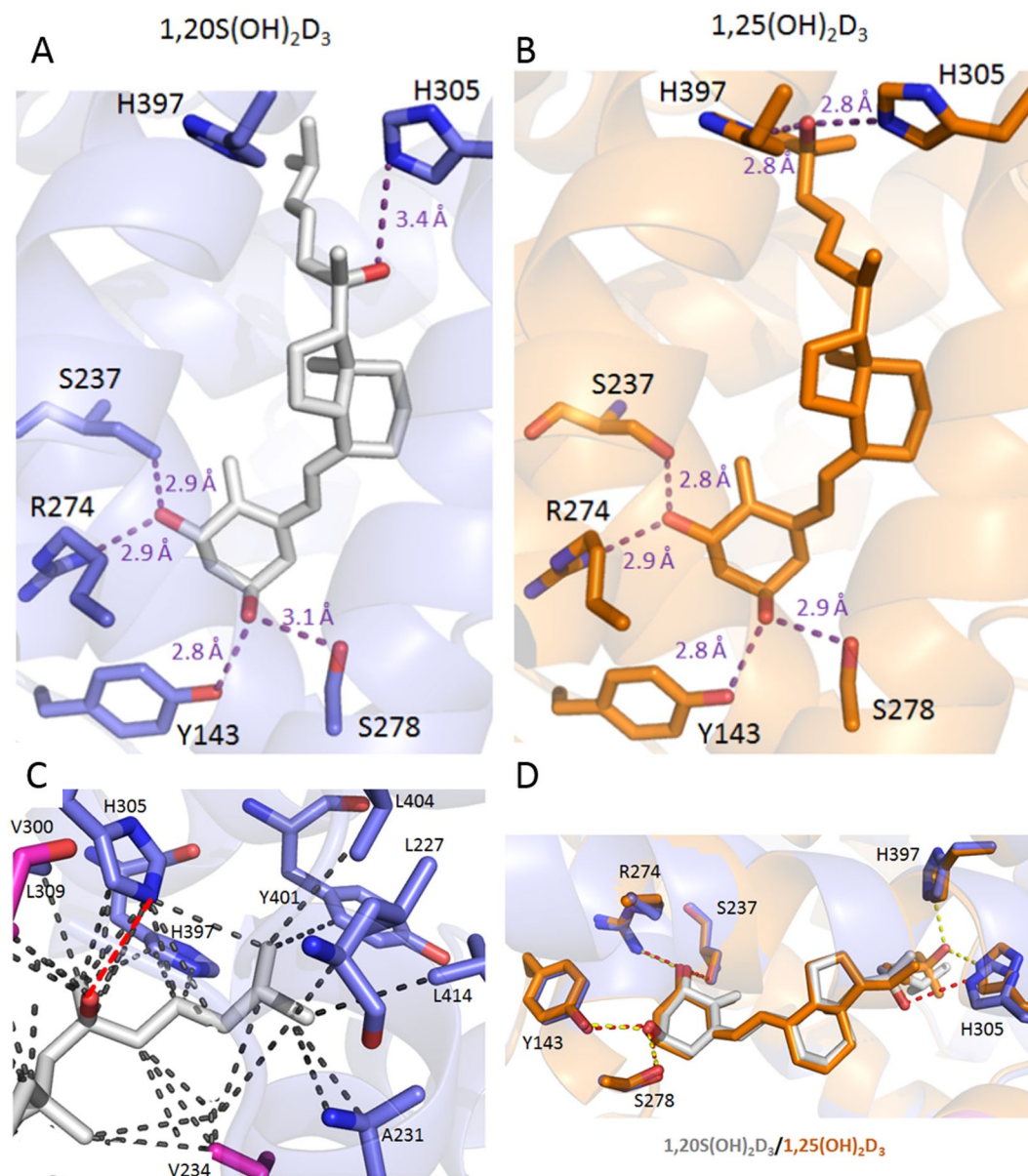


Figure 5. X-ray crystal structures of 1,20S(OH)₂D₃ and 1,25(OH)₂D₃ in complex with zVDR LBD. **(A)** Binding pose and interactions of 1,20S(OH)₂D₃ in in complex with zVDR LBD. **(B)** Binding pose and interactions of 1,25(OH)₂D₃ in in complex with zVDR LBD. **(C)** Details of the interactions mediated by the side chain of 1,20S(OH)₂D₃ with residues of the zVDR LBD at a 4.0 Å distance cutoff. The residues numbers correspond to hVDR. **(D)** Overlay of 1,25(OH)₂D₃ (carbon atoms in orange and oxygen atoms in red) with 1,20S(OH)₂D₃ (grey) within zVDR LBD complexes with the indication of the hydrogen bonds formed by the ligands. The Hydrogen bonds are shown by red or yellow dashed lines and Van Der Waals interactions are shown by grey dashed lines. Hydrogen bonds are shown by dashed lines, and hydrogen-bonding residues are labelled.

activities of these secosteroids such as the lower calcemic activity of 1,20S(OH)₂D₃ compared to 1,25(OH)₂D₃⁸. This study provides a molecular basis for the rational design and practical synthesis of novel 1,20S(OH)₂D₃ analogs that interact with VDR for future drug development. 1,20S(OH)₂D₃ was successfully chemically synthesized for the first time, providing ample material for further characterization of its biological activities, including animal studies in the future. The 1 α ,3 β -diacetoxyregn-5-en-20-one (**3**) intermediate can serve as a common precursor for production of other 1,20S(OH)₂D₃ analogs which will facilitate the synthesis of similar secosteroids containing a 1 α -OH group.

Methods

General procedures. Reagents and solvents for the synthesis were anhydrous (purchased or self-dried) to ensure good product yield. Solvents used for separations were ACS chemical grade, purchased from commercial sources and used upon arrival. NH₄Cl was sublimed in our lab for Birch reduction. Reactions for light sensitive

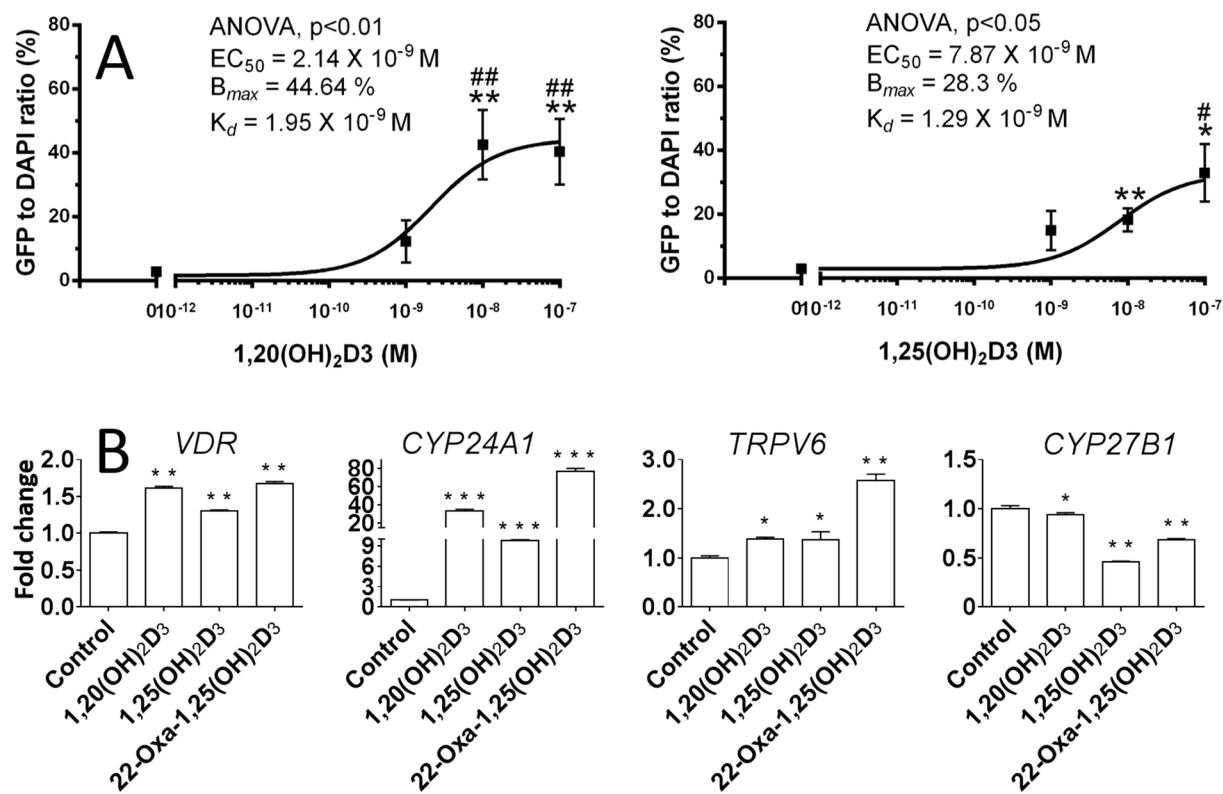


Figure 6. VDR translocation and gene regulation activities of 1,20S(OH)₂D₃. (A) The effect on vitamin D receptor (VDR) translocation from the cytoplasm to the nucleus. Data are mean \pm SEM ($n \geq 3$). The dose-dependent stimulation of VDR translocation was analysed by one-way ANOVA with $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$. The differences between control and treatment were analysed with Student's *t*-test, where $^*p < 0.05$ and $^{**}p < 0.01$. (B) 1,20S(OH)₂D₃ regulates mRNA expression of genes VDR, CYP24A1, TRPV6 and CYP27B1 in HaCaT cells at 100 nM after 24 h treatment ($n = 3$). $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

compounds (7DHC or D₃ structures) were protected from light by wrapping flasks with aluminum foil, and were monitored under UV lights. Moisture-sensitive reactions were carried out under argon gas in flame-dried flasks. Reactions for non-UV active compounds were visualized on TLC by 5% phosphomolybdic acid in ethanol. All NMR data were collected on a Bruker Avance III 400 MHz NMR or a Varian Inova 500 MHz NMR. Samples were dissolved in 0.5 mL CDCl₃, methanol-*d*₄, DMSO-*d*₆ or actone-*d*₆, and NMR data were collected at r.t. Mass spectra of compounds were acquired using a Bruker LC-IT-MS system with an ESI source. High-resolution MS spectra and extracted ion chromatogram (EIC) were obtained using a Waters ACQUITY UPLC I-Class System equipped with a Xevo G2-S QToF mass spectrometer based on our previously reported conditions^{26,27}. Reaction mixtures were extracted with ethyl acetate, DCM or hexanes, washed with aqueous Na₂CO₃, brine, and water, and then dried over anhydrous Na₂SO₄. The solution was transferred to a round-bottom flask and dried by rotary evaporator. The purities of final products were determined by HPLC as $>98\%$ (Fig. 4).

Crystallization of intermediate 15. To a clean test tube (13 \times 100 mm), 18 mg of compound 15 powder and 3 mL anhydrous *n*-hexane were added. The tube was shaken until the solid was completely dissolved, then sealed with 5 layers of sealing film (Para film) membrane. The resulting solution was allowed to stand in a quiet environment for 10 days, by which time the hexane had evaporated, leaving crystals of 15 which were collected for crystallographic analysis (Supplementary Information).

Crystallization and structural analysis of 1,20S(OH)₂D₃-VDR complex. cDNA encoding zVDR LBD (156–453 AA) was subcloned into pET28b vector to generate an *N*-terminal His-tag fusion protein. Purification was carried out as previously described, including metal affinity chromatography and gel filtration²⁸. The protein was concentrated using Amicon ultra-30 (Millipore) to 3–7 mg/mL and incubated with a two-fold excess of ligand and a three-fold excess of the coactivator SRC-1 peptide (686-RHKILHRLLEQEGSPS-698). Crystals were obtained in 50 mM Bis-Tris pH 6.5, 1.6 M lithium sulfate and 50 mM magnesium sulfate. Protein crystals were mounted in a fiber loop and flash-cooled under a nitrogen flux after cryo-protection with 20% glycerol. Data collection from a single frozen crystal was performed at 100 K on the ID23-1 beamline at ESRF (France). The raw data were processed and scaled with the HKL2000 program suite²⁹. The crystals belong to the space group P6₂22, with one LBD complex per asymmetric unit. The structure was solved and refined using BUSTER³⁰, Phenix³¹ and iterative model building using COOT³². Crystallographic refinement statistics are presented in Supplementary Table S9. All structural figures were prepared using PyMOL (www.pymol.org/).

Biosynthesis of 1,20S(OH)₂D₃. Enzymatic synthesis of 1,20S(OH)₂D₃ involved the 20S-hydroxylation of 1 α (OH)D₃ by recombinant bovine CYP11A1 and was carried out as described in detail before⁷. HPLC comparison was determined by using an Agilent HPLC 1100 system and a Phenomenex Luna-PPF C₁₈ column (5 μ m, 250 mm \times 4.6 mm, Torrance, CA) at 25 °C and a flow rate of 1.0 mL/min. MeCN: H₂O and MeOH: H₂O were used as mobile phases with a gradient comprising 50–100% organic solvent for 30 min. 263 nm was used to display chromatograms.

VDRE-reporter assay. HaCat, Caco-2 and Jurkat cells were transduced by lentiviral VDRE-reporter (luciferase) vector^{12–15}. Caco-2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin antibiotic solution (Ab) (Sigma-Aldrich, St. Louis, MO). HaCaT cells were grown in DMEM supplemented with 5% FBS and 1% Ab. Jurkat cells were grown in RPMI 1640 medium containing 10% FBS and 1% Ab. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. All cell lines were selected for at least one week by medium containing additional 1.0 μ g/mL puromycin before treatment with secosteroid. Each cell line was then plated in a 96-well plate (10,000 cells/100 μ L medium/well) using FBS-free media and incubated for 24 h. 1,20S(OH)₂D₃, 1,25(OH)₂D₃ and 22-Oxa at a series of concentrations in 10% DMSO were added separately to 96-well plates (1.0 μ L/well), while 10% DMSO was used as control. After 24 h incubation, 100 μ L of ONE-GloTM Luciferase Assay System (Promega, Madison, WI) was added to each well. After 5 min at r.t., the signal was recorded by a BioTek Synergy HT microplate reader (BioTek Instruments, Inc., Winooski, VT, US). All concentrations of secosteroids were tested in triplicate.

VDR translocation assay. The effects of 1,20S(OH)₂D₃ on VDR translocation from the cytoplasm to the nucleus were tested on the previously described SKMEL-188 cell model^{21,33}, using cells stably transduced with pLenti-CMV-VDREGFP-pgk-puro (VDR and EGFP expressed as fusion protein)³⁴. Cells were treated with secosteroids (up to 100 nM) for 90 min followed by analysis with Cytation 5 (BioTek, Winooski, VT, US). Translocation to the nucleus was determined by counting cells with a fluorescent nucleus and the results are presented as the percentage of the total cells that displayed nuclear staining, as described previously²¹. The data were obtained from at least two separate experiments, with images taken in the central area from at least three different wells and counted as described^{21,34}.

Real-time PCR assay. HaCaT cells were seeded in 60 mm dishes (1 million/dish) in 10 mL DMEM supplemented with 5% FBS and 1% Ab. After overnight incubation they were cultured in FBS-free medium for another 12 h to synchronize the cells. The media were then removed and secosteroids in DMEM (5% FBS and 1% Ab) with a concentration of 100 nM were added to the dishes. After 24 h incubation, media were removed, and 10 mL PBS was used to wash the dish. Cells were then detached by trypsin, centrifuged in Eppendorf tubes, washed with PBS (5 mL), and stored at –80 °C. Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA, USA) was used to isolate the RNA, and Transcriptor First Strand cDNA Synthesis Kit (Roche Inc., Mannheim, Germany) was used for reverse transcription (100 ng RNA/reaction). Real-time PCR was carried out using cDNA which was diluted 10-fold in sterile water and a SYBR Green PCR Master Mix. The forward reverse primers for VDR, CYP24A1, TRPV6 and CYP27B1 genes were designed based on the rat and mouse sequences using Primer Quest software (Integrated Device Technology, San Jose, CA, USA). Reactions ($n = 3$) were performed at 50 °C for 2 min, 95 °C for 10 min and then 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Data were collected and analyzed on a Roche Light Cycler 480. Using a comparative Ct method²⁵, the amount of the final amplified product was normalized to the amount of β -actin as a housekeeping gene.

IFN γ production assay. All animal experiments in this study were performed in accordance with the NIH animal use guidelines and protocol (protocol No.: 15–043.0) approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center (UTHSC, Memphis, TN). Splenocytes were isolated from 7-week old C57BL/6 female mice and cultured at 2×10^6 /mL 500 μ L/well for 72 h at 37 °C in a humidified atmosphere. Harvested supernatants were analyzed for levels of murine IFN γ by ELISA (R & D Systems Minneapolis, MN) according to the manufacturer's instructions. Results are expressed as mean [IFN γ] \pm SEM of triplicate determinations (pg/mL) of culture supernatant. The difference in [IFN γ] between EtOH vehicle + anti-CD3e MOAB (0.08 μ g/mL) and [IFN γ] in each of the cultures containing D₃ analog was analyzed by one way ANOVA Sigma. The amount of EtOH in the EtOH + PBS and EtOH + anti-CD3e MOAB culture was equal to that in the cultures of the D₃ analogs at a level of 10^{-9} M. Culture medium was RPMI 1640 supplemented with 9% charcoal stripped fetal bovine serum, non-essential amino acids, HEPES buffer Glutamax, penicillin 100 μ g/mL, streptomycin 100 μ g/mL, fungizone 1 μ g/mL (GIBCO, Grand Island, NY) and 50 μ M β -mercaptoethanol (Sigma, St. Louis, MO)³⁵.

IL1 β production assay. Splenocytes were isolated from 7-week old C57BL/6 female mice and cultured at 2×10^6 /mL, 500 μ L/well, for 24 h at 37 °C in a humidified atmosphere. The vitamin D analogs or EtOH vehicle were added to the splenocyte cultures 2 h prior to addition of Lipopolysaccharide *W. coli* 055:B5 (LPS) (Difco Lab. Defrost MI) 100 ng/mL or PBS vehicle. Harvested supernatants were analyzed for levels of murine IL1 β by ELISA (R & D Systems Minneapolis, MN) according to the manufacturer's instructions. Results are expressed as mean IL1 β concentration \pm SEM of triplicate determinations (pg/mL) of culture supernatant. The amount of EtOH in the EtOH + PBS and EtOH + LPS culture was equal to that in the cultures of the vitamin D analogs at a level of 10^{-7} M. Culture medium was RPMI 1640 supplemented with 9% charcoal stripped fetal bovine serum, non-essential amino acids, HEPES buffer Glutamax, penicillin 100 μ g/mL, streptomycin 100 μ g/mL, fungizone 1 μ g/mL (GIBCO, Grand Island, NY) and 50 μ M β -mercaptoethanol (Sigma, St. Louis, MO). The difference in [IL1 β] between control and D₃ analog treatment was analyzed by one way ANOVA (Sigma Plot 13.0).

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Author Contributions

W.L. and Z.L. designed and coordinated the project. W.L., D.M., N.R. and Z.L. planned and designed the experiments. Z.L., H.C., E.T., Z.J., T.K., Z.W. and A.P. performed the experiments. A.B. and N.R. resolved VDR-compound co-crystal, J.B. resolved the crystal of the small molecule intermediate. D.M., A.S., A.P., R.T., N.R. and W.L. analyzed data. Z.L. drafted the manuscript together with N.R., all authors revised and reviewed the manuscript.

Additional Information

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