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| Title | Synthetic Chemical Probes That Dissect Vitamin D Activities |
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| Author(s) | Nagata, Akiko; Akagi, Yusuke; Asano, Lisa; Kotake, Kenjiro; Kawagoe, Fumihiro; Mendoza, Aileen; Masoud, Sedghi, Shadi; Usuda, Kosuke; Yasui, Koji; Takemoto, Yasushi; Kittaka, Atsushi; Nagasawa, Kazuo; Uesugi, Motonari |
| Citation | ACS chemical biology (2019), 14(12): 2851-2858 |
| Issue Date | 2019-12-20 |
| URL | http://hdl.handle.net/2433/252796 |
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# Synthetic Chemical Probes That Dissect Vitamin D Activities

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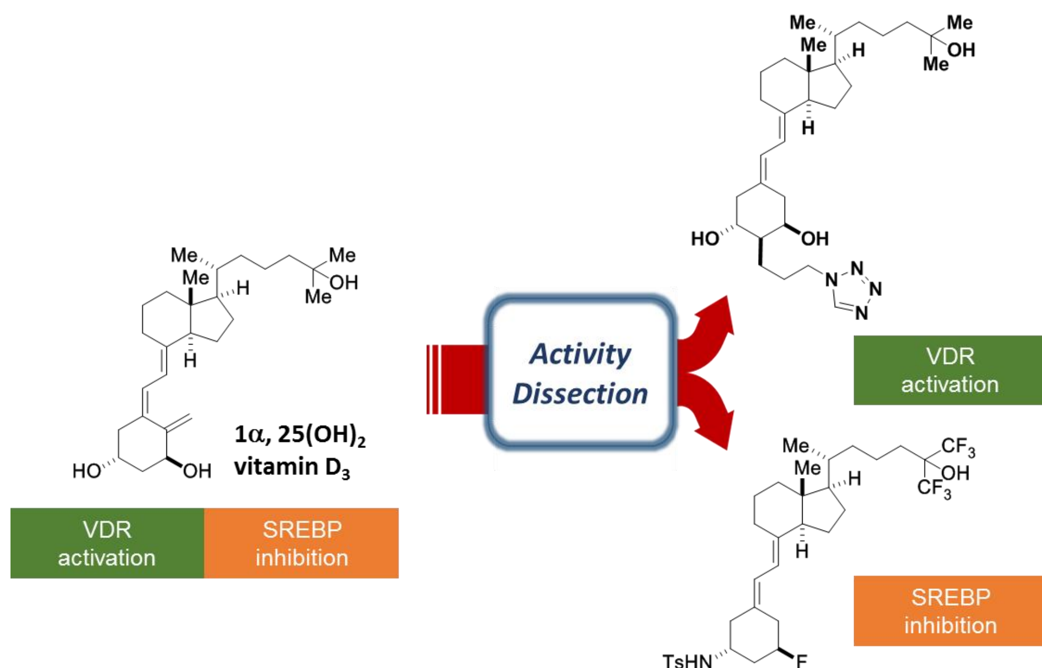
**Abstract:** Vitamin $D_3$ metabolites are capable of controlling gene expression in mammalian cells through two independent pathways: vitamin D receptor (VDR) and sterol regulatory element-binding protein (SREBP) pathways. In the present study, we dissect the complex biological activity of vitamin D by designing synthetic vitamin $D_3$ analogs specific for VDR or SREBP pathway, i.e., a VDR activator that lacks SREBP inhibitory activity, or an SREBP inhibitor devoid of VDR activity. These synthetic vitamin D probes permitted identification of one of the vitamin D-responsive genes, *Soat1*, as an SREBP-suppressed gene. The chemical probes developed in the present study may prove useful in dissecting the intricate interplay of vitamin D actions, thereby providing insights into how vitamin D target genes are regulated.



## ■ Introduction

Vitamin $D_3$ [cholecalciferol, $VD_3$ (**1**)] is a lipophilic vitamin present in foods and also endogenously synthesized from 7-dehydrocholesterol following the exposure to sunlight. In humans, $VD_3$ is converted by CYP enzymes to 25-hydroxyvitamin $D_3$ [25(OH)$D_3$ (**2**)] and 1α,25-dihydroxyvitamin $D_3$ [1α,25$(OH)_2D_3$ (**3**)] (Fig. 1), exerting a range of physiological activities including calcium metabolism,[1] cellular differentiation,[2] and immune regulation.[3] Many of these physiological activities can be explained by the binding of 1α,25$(OH)_2D_3$ to the vitamin D receptor (VDR), a nuclear receptor that controls the gene expression.[4] This well-recognized mechanism of vitamin D action has been well investigated for many years since the cloning of chick VDR in 1987.[5]

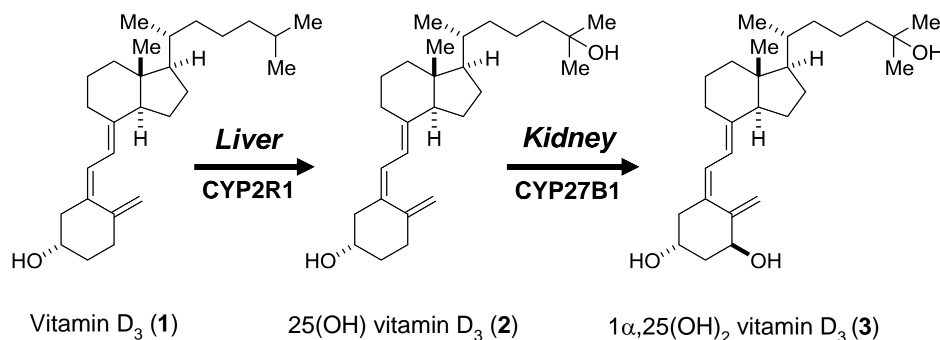



**Figure 1.** Structures of vitamin $D_3$ (**1**), 25(OH) vitamin $D_3$ (**2**) and 1α,25$(OH)_2$ vitamin $D_3$ (**3**).

On the other hand, vitamin D metabolites have also been reported to exert effects independent from VDR activation.[6-19] For example, growth inhibition and induction of apoptosis by 1α,25$(OH)_2D_3$ in breast cancer cells[7] persisted in VDR-knockout mice. 1α,25$(OH)_2D_3$ has been shown to interact with MARRS (membrane-associated rapid response steroid binding protein) for precise regulation of phosphate uptake.[8] Two minor vitamin D metabolites, 20-hydroxyvitamin $D_3$ and 20,23-dihydroxyvitamin $D_3$,[9-12] have also been reported as agonists or antagonists of non-VDR nuclear receptors.[13-16] However, detailed mechanisms or biological significance underlying these VDR-independent activities remain unclear.

Recently, we found that the most abundant $VD_3$ metabolite, 25(OH)$D_3$, down-regulates SREBP (sterol regulatory element-binding protein) independently of VDR.[19] SREBP is a non-nuclear receptor transcription factor that activates genes associated with cholesterol and fatty acid biosynthesis.[20-23] SREBP is expressed on the membrane of the endoplasmic reticulum (ER) where it forms a stable complex with its specific escort protein, SCAP (SREBP cleavage-activating protein).[21-23] When the amount of cholesterol decreases, the SREBP-SCAP complex translocates from ER to the Golgi, where the $NH_2$-terminal SREBP is proteolytically processed to generate the mature form of SREBP. This activated SREBP moves to the nucleus where it promotes the expression of lipogenic genes.[21-23] We previously found that direct association of 25(OH)$D_3$ (**2**) with SCAP suppresses the expression of SREBP-responsive genes by inducing proteolytic processing and ubiquitin-mediated degradation of SCAP.[19] Therefore, $VD_3$ metabolites are capable of influencing gene expression in mammalian cells through two independent pathways: VDR and SREBP pathways.

In the present study, we dissect the two independent activities by designing synthetic vitamin $D_3$ analogs specific for VDR or SREBP, *i.e.*, a VDR activator that lacks SREBP inhibitory activity, and an SREBP inhibitor devoid of VDR activity. Such chemical dissection permitted identification of one of the vitamin D-responsive genes as an SREBP-suppressed gene.

## ■ Results and Discussion

**Discovery of a VDR-selective activator.** In our effort to delineate $VD_3$ activity, we initially searched for $VD_3$ analogs that activate VDR but lack SREBP inhibitory activity. An in-house library of 250 vitamin D congeners[24-55] was screened for their ability to inhibit the activity of an SREBP-responsive luciferase reporter, in which expression of luciferase is controlled by three SREBP binding sites. We found that a series of vitamin D analogs, bearing alkyltriazole and alkyltetrazole substituents at C2 (**4**-**9**) (Fig. 2), displayed limited SREBP inhibitory activities (Fig. 3A). The ability of these six molecules[32-34] to activate VDR was next examined by a VDR-responsive reporter assay (Fig. 3B).[56] Comparison of the relative activities of the six compounds allowed us to select 2αC3Tet1 (**6**) as the VDR-selective activator.

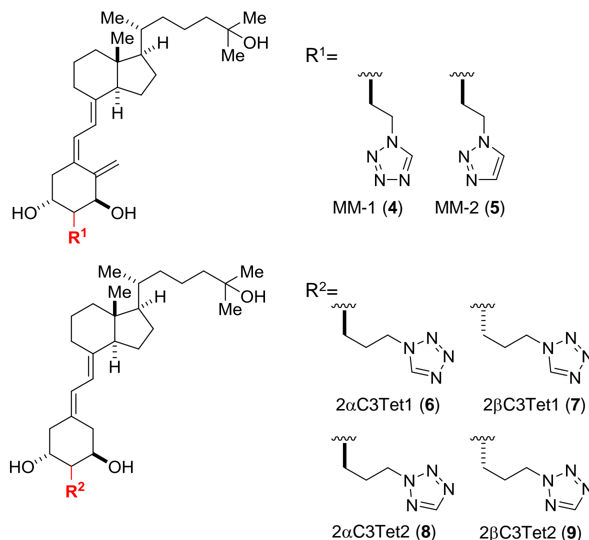



**Figure 2.** Structures of alkyltetrazole-substituted vitamin $D_3$ derivatives at C2 position.

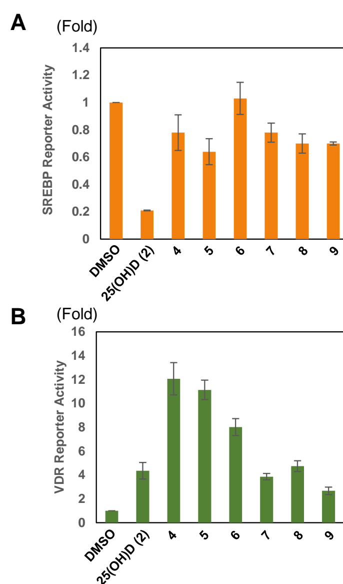



**Figure 3.** Effect of vitamin D analogs bearing alkyltriazole- and alkyltetrazole-substituents at C2 on SREBP or VDR activation. (A and B) Effect of $25(OH)D_3$ (**2**) and vitamin $D_3$ analogs (compounds **4**-**9**) on the ability of SREBPs (A) or VDR (B) to activate transcription of a luciferase reporter gene. CHO-K1 cells were transfected with the reporter gene in which the expression of luciferase was controlled by sterol-responsive element (SRE) (A) or vitamin D response element (VDRE) (B), and treated with 5 μM of compounds in a medium B. Values are mean ± SD.

**Discovery of an SREBP-selective inhibitor.** We next searched for vitamin D analogs that inhibit SREBP but do not activate VDR. It is well known that (i) the hydroxy groups at C1 and C3 in 1α,25$(OH)_2D_3$ (**3**) are critical for its interaction with VDR,[57] and that (ii) the analogs without exomethylene at C19 in A ring, so-called 19-norvitamin D derivatives, tend to exhibit weaker VDR-dependent calcium effects and better stability than naturally occurring $VD_3$.[58] In addition, our preliminary observations revealed that the 1α-NHBoc-25(OH)$D_3$ (**10**), bearing NHBoc group at C1α, maintained a similar SREBP inhibitory activity as those of 25(OH)$D_3$ (**2**) and 1α,25$(OH)_2D_3$ (**3**) (Fig. 4).[19] With these observations in mind, we designed and synthesized 26 compounds (compounds **11**-**36**) in which the C1 hydroxy group was replaced with nitrogen in the presence or absence of exomethylene at C19 (Fig. 4).[59] The ability of compounds **11-36** to modulate SREBP and VDR activities were evaluated in the corresponding luciferase reporter gene assay (Fig. 5A and B). The *N*-alkylated derivatives **12-16** exhibited significant SREBP inhibitory activity at 5 μM in CHO-K1 cells. Among the compounds we tested, the *N*-sulfonamide **32a** and the *N*-amide **26** and **29** substituted products inhibited SREBP as much as 25(OH)$D_3$ (**2**) did (Fig. 5A). Subsequent VDR reporter assays revealed that the derivative of **32a,** bearing *N*-Ts substituent with 19-nor type, showed the most potent inhibitory activity against SREBP with essentially no VDR agonistic activity. Discovery of **32a** encouraged us to synthesize the rest of the seven possible regio- and stereoisomers at C1 and C3 in **32a**, *i.e.*, **32b**-**h** (Fig. 4). [44,59] Upon subsequent evaluation, all of the isomers of **32a** exhibited potent SREBP inhibitory activity with limited VDR agonistic activity regardless of the regio- and stereochemistry at C1 and C3 (Fig. 5).

**Optimization of compound 32.** Further optimization of **32** was carried out to minimize its effect on VDR activity. Specifically, we prepared compounds **37c**-**f** (Fig. 4), which were replaced with either a sulfonamide group or a fluorine atom C1 and C3 hydroxy group of 1α,25$(OH)_2D_3$ (**3**).[59] The biological activity of these four derivatives **37c**-**f** was evaluated in the luciferase reporter gene assays. The results revealed that all four derivatives exhibited an improved selectivity compared with that of **32a**. There was no detectable VDR activation at 5 μM, while potent SREBP inhibitory activity was maintained at the same concentration (Fig. 5). Their structures were further optimized by modifying the C17 side chain in CD ring. In a number of vitamin D-based pharmaceuticals, the side chain is fluorinated to improve metabolic stability.[60,61] Thus, the side chain of **37c**-**f** was replaced with a 26,27-ditrifluoromethyl, similar to the clinically-used drug falecalcitriol, to yield compounds **38c**-**f** (Fig. 4).[59] Luciferase reporter gene assays revealed that all the isomers potently inhibit SREBP with limited VDR agonist activity regardless of the regio- and stereochemistry at C1 and C3 (Fig. 5). As mentioned, treatment with 25(OH)$D_3$ induces the degradation of both SREBP and SCAP. Western blot analysis showed that compounds **38c**, **38d** and **38e** induced the degradation similarly to 25(OH)$D_3$ (**2**). Remarkably, **38d** induced more degradation of SREBP and SCAP than 25(OH)$D_3$, while the actin level was unchanged (Fig. 5C). From these findings, **38d** was chosen as an SREBP-selective vitamin D analog.

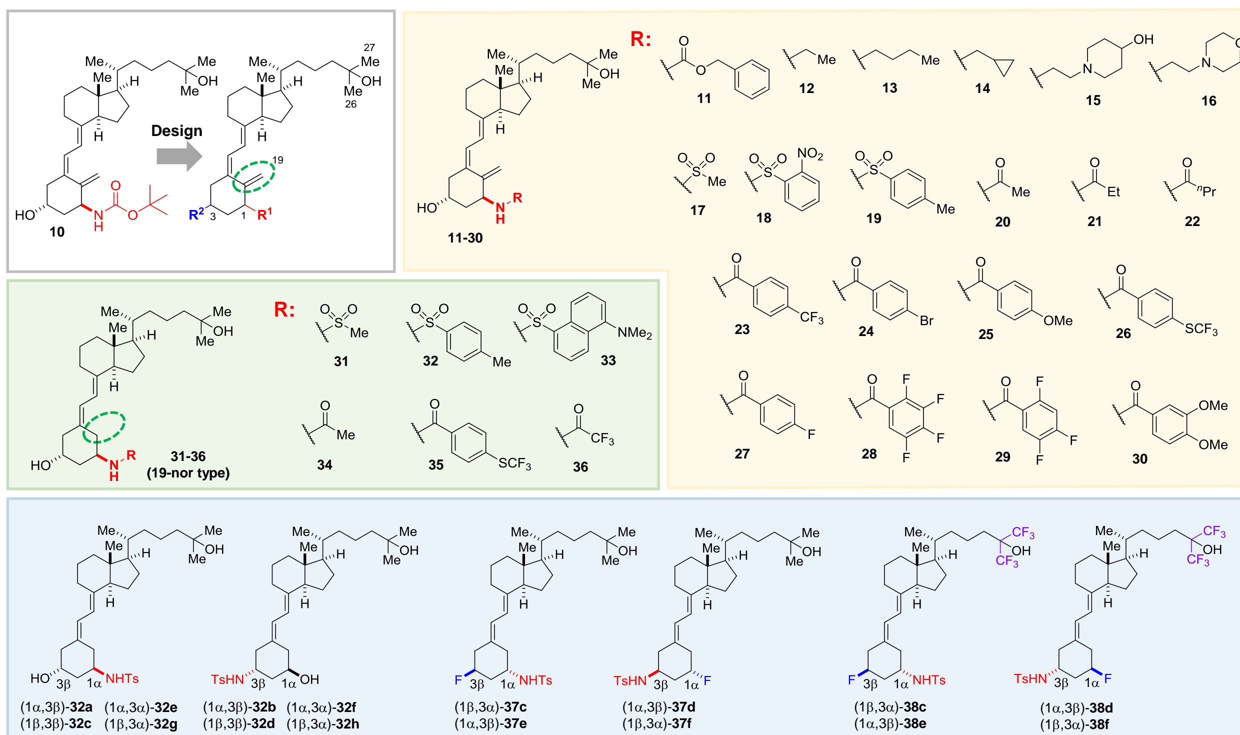


**Figure 4 .** Structures of 1$N$-substituted vitamin $D_3$ and 19-norvitamin $D_3$ derivatives.

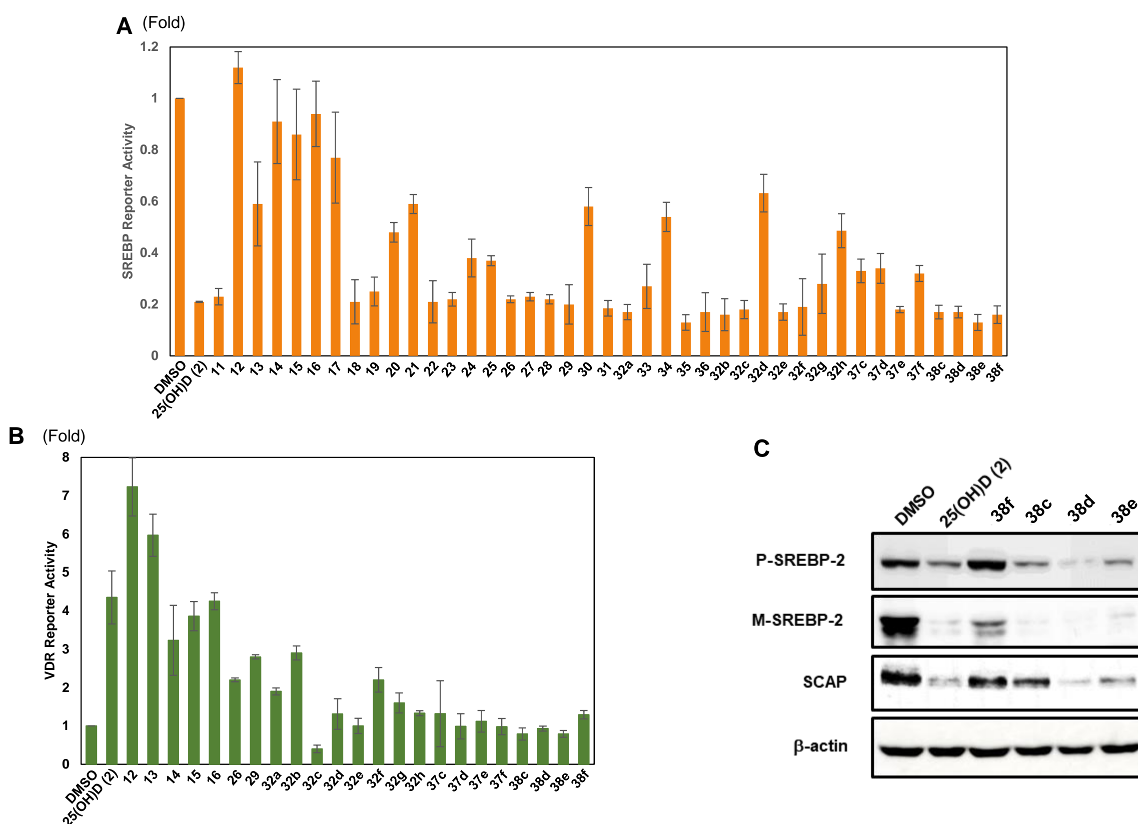



**Figure 5.** Effect of vitamin D analogs (compounds **11-36, 32b-h, 37c-f, 38c-f**) on SREBP or VDR activation. (A and B) Effect of vitamin D analogs (compounds **12-16, 26, 29, 32a-h, 37c-f, 38c-f**) on the ability of SREBPs (A) or VDR (B) to activate transcription of a luciferase reporter gene. CHO-K1 cells were transfected with each reporter gene, and treated with 5 μM of compounds in a medium B. Values are mean ± SD. (C) Western blotting analysis of SREBP-2 and SCAP. CHO-K1 cells were treated with 5 μM of compounds **38c-f** in a medium B. The levels of precursor (P) and mature (M) forms of SREBP-2 and SCAP were analyzed by western blotting.

**Dissection of vitamin D-responsive genes.** 1α,25$(OH)_2D_3$ promotes the expression of numerous genes in mammalian cells. Most of these genes are considered to be induced primarily through the VDR pathway.[4,62-65] However, among those $VD_3$-responsive genes, there may exist genes whose expression is down-regulated by SREBP independently of VDR activity. It is challenging to distinguish between these two types of genes using natural $VD_3$ metabolites. Discrimination of these genes may be permitted by using our two vitamin D analogs, **6** and **38d**. We comprehensively analyzed genes whose expression was promoted by 25(OH)$D_3$ (**2**) in CHO-K1 cells. RNA sequencing (RNA-seq) analysis indicated that 25(OH)$D_3$ induced the expression of 1,277 genes more than two-fold, including *Cyp24a1*, a well-known VDR-responsive gene.[66] Similar analysis was performed using compounds **6** and **38d**, respectively. Many of the 1,277 genes were likewise induced by **6**, indicating that these genes are controlled by VDR.

From the 1,277 vitamin D-responsive genes, we further selected the genes whose expression was induced more than two-fold by **38d** but stayed within 0.5-1.5 fold with **6**. A total of 209 genes were extracted as candidate genes that are controlled by SREBP rather than VDR (listed in a supplemental Excel file). Gene ontology (GO) analysis of the 209 genes indicate that the list of the candidate genes was enriched in those related to RNA binding proteins, including RNA polymerase III and aminoacyl-tRNA synthetases (Figures S1 and S2). RNA polymerase III, which transcribes DNA to synthesize ribosomal 5S rRNA and tRNA, and aminoacyl-tRNA synthetases are both essential for protein biosynthesis, raising the possibility that 25(OH)$D_3$ upregulates protein biosynthesis through inhibition of SREBP.

Considering the lipogenic role of SREBP, we focused on six lipid metabolism-related genes (Table S3). Repeated qPCR experiments confirmed that *Abca1* (ATP Binding Cassette, Subfamily A, Member 1) and

*Soat1* (sterol *O*-acyltransferase 1) genes were induced by both **2** and **38d**, but not by **6** (Fig. S3 and Fig. 6A). *ABCA1*, a membrane transporter that excretes cholesterol out of the cell, is already known to be negatively regulated by SREBP.[67] Hence, we selected *Soat1* gene for further investigation.

SOAT1 is an enzyme in the endoplasmic reticulum (ER) that converts cholesterol into cholesterol ester and stores it in oil droplets.[68] To examine the role of SREBPs in the expression of SOAT1, their respective mRNA and protein levels were measured following the knock down of both *Srebp-1* and *Srebp-2* genes in CHO-K1 cells. Treatment with siRNA of SREBPs (siSREBP) decreased the mRNA levels of both SREBP-1 and SREBP-2, and increased that of *Soat1* by about 3-fold; whereas the expression of SREBP-responsive genes, such as *Hmgcr* (HMGCoA reductase) and *Fasn* (fatty acid synthetase), were reduced (Fig. 6B). Western blot analysis also showed that siSREBP treatment increased the protein levels of SOAT1 (Fig. 6C). These results suggest that SREBPs suppress the expression of SOAT1.

The intracellular cholesterol level is maintained by the balance between biosynthesis and extracellular transport. It is known that SREBP tightly regulates these two processes by controlling relevant biosynthetic genes and transporter genes, including *Abca1*.[21,22,69-71] Our findings suggested that SREBP also represses the intracellular storage of cholesterol by down-regulating the expression of SOAT1, an enzyme that converts cholesterol to cholesterol ester (Fig. 6D). The down-regulation of SOAT1 is thought to contribute in turn to the inactivation of SREBP through increased levels of free cholesterol. In fact, it has been reported that SREBP is inactivated when SOAT1 is pharmacologically or genetically suppressed in glioma cells.[72] The SREBP-mediated suppression of SOAT1 would serve as a negative feedback mechanism for preventing excessive activation of SREBP, enabling SREBP to precisely regulate intracellular cholesterol level.

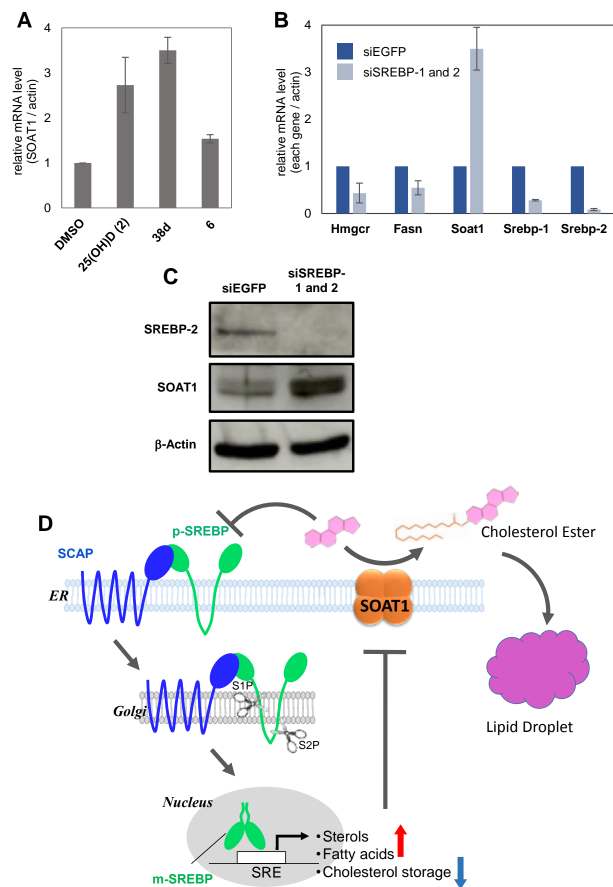



**Figure 6.** SOAT1 gene expression is negatively regulated by SREBPs. (A) CHO-K1 cells were incubated in medium B containing each compound for 24 hr. The mRNA level of SOAT1 was assessed by qPCR analysis. Data were analyzed by ΔΔ-Ct method with actin as reference control. (B and C) CHO-K1 cells were transfected with siRNAs targeting hamster *Srebp-1* and *Srebp-2* in medium A. After 24 hr incubation, the medium was replaced with medium B and the cells were further incubated for 24 hr. The mRNA level of *Hmgcr*, *Fasn*, *Soat1*, *Srebp-1* or *Srebp-2* was assessed by qPCR analysis. Data were analyzed by ΔΔ-Ct method with actin as reference control. (B). The protein level of SREBP-2, SOAT1 and β-Actin was analyzed by western blots (C). (D) A proposed model for SREBP's role in regulating cellular cholesterol storage.

## ■ Conclusions

Vitamin D is a popular supplement highly regarded for its role and impact on various bodily health issues and diseases, including bone metabolism, immune response, central nervous system, cardiovascular function, metabolic diseases, and cancers. However, these benefits are likely to be the results of the intricate interplay of multiple vitamin D actions. The new set of pharmacological tools developed in the present study provides means for interrogating those vitamin D benefits and may serve as a starting point of designing vitamin D-based pharmaceuticals selective for each indication.

## ■ Associated Content

### Supporting Information

The Supporting Information is available.

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## ■ Acknowledgments

This work was supported by AMED-CREST, Japan Agency for Medical Research and Development, the International Collaborative Research Program of Institute for Chemical Research (2018-57 and 2017-53), and JSPS A3 Foresight Program. This work was inspired by the international and interdisciplinary environments of JSPS Core-to-Core Program of ACBI (Asian Chemical Biology Initiative).

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