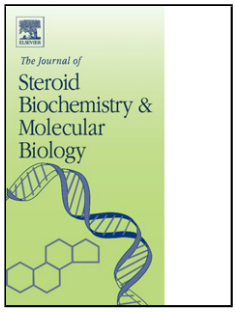

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Authors: Hiroki Mano, Shinichi Ikushiro, Nozomi Saito, Atsushi Kittaka, Toshiyuki Sakaki

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Development of a highly sensitive *in vitro* system to detect and discriminate between vitamin D receptor agonists and antagonists based on split-luciferase technique

Hiroki Mano [a], Shinichi Ikushiro [b], Nozomi Saito [c,d], Atsushi Kittaka [c] and Toshiyuki Sakaki [a]*

[a] Department of Pharmaceutical Engineering, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan:

[b] Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan:

[c] Faculty of Pharmaceutical Sciences, Teikyo University, Itabashi, Tokyo 173-8605 Japan:

[d] Present address: Meiji Pharmaceutical University, Kiyose, Tokyo 204-8588, Japan

*Corresponding author: Toshiyuki Sakaki
Department of Pharmaceutical Engineering,,
Faculty of Engineering, Toyama Prefectural University,
5180 Kurokawa, Imizu, Toyama 939-0398, Japan
Phone: +81-766-56-7500 Ext.913
Fax: +81-766-56-2498
E-mail: tsakaki@pu-toyama.ac.jp

ABSTRACT

Split-luciferase techniques are widely used to detect protein-protein interaction and bioactive small molecules including some hormones and vitamins. Previously, we successfully expressed chimeric proteins of luciferase and the ligand binding domain (LBD) of the vitamin D receptor (VDR), LucC-LBD-LucN in COS-7 cells. The LucC-LBD-LucN biosensor was named split-luciferase vitamin D biosensor (SLDB). This biosensor can detect and discriminate between VDR agonists and antagonists in mammalian cells. In this study, we established an *in vitro* screening system for VDR ligands using the SLDB proteins expressed in *Escherichia coli (E. coli)* cells. Our *in vitro* screening system using cell lysate of recombinant *E. coli* cells could be completed within 30 min, and its activity was unchanged after 10 freeze-thaw cycles. This highly sensitive and convenient system would be quite useful to screen VDR ligands with therapeutic potential for various bone-related diseases, age-related cognitive disorders, cancer, and immune disorders. In addition, our system might be applicable to diagnostic measurement of serum concentrations of 25-hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3.

Key words: vitamin D, vitamin D receptor, bioluminescent sensor, split-luciferase, screening system

Abbreviations : CYP27B1, 25-hydroxyvitamin D_3 1α-hydroxylase ; VDR, vitamin D receptor ; LBD, ligand binding domain ; LucN, N-terminal domain of luciferase ; LucC, C-terminal domain of luciferase ; SRC-1, steroid receptor coactivating factor 1 ; RXRα, retinoid X receptor α ; TIF2, transcriptional factor 2 ; DRIP205, vitamin D receptor interacting protein 205 ; ELISA, enzyme-linked immunosorbent assay

1. Introduction

In general, the active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$], is the ligand of the vitamin D receptor (VDR). $1\alpha,25(OH)_2D_3$ is converted from 25-hydroxyvitamin D_3 [$25(OH)D_3$] by 25-hydroxyvitamin D_3 1α-hydroxylase called CYP27B1 in the kidney to regulate calcium and bone homeostasis, cellular growth and differentiation and immune response through binding to the VDR [1-4]. Our previous study showed that $25(OH)D_3$ can also act as a ligand of VDR in human prostate PZ-HPV-7 cells [5]. When the natural ligands [$1\alpha,25(OH)_2D_3$ and $25(OH)D_3$] bind to the VDR, the $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$-VDR complex interacts with its heterodimeric partner retinoid X receptor α (RXRα) and forms a complex with coactivators such as steroid receptor coactivating factor 1 (SRC-1), vitamin D receptor interacting protein 205 (DRIP205) and transcriptional factor 2 (TIF2) [6-9]. This complex acts as a transcription factor that regulates the expression of multiple genes. Repression or disruption of vitamin D-related gene expression leads to some bone-related diseases such as rickets, osteoporosis, and osteomalacia [10]. Recently, Latimer et al. reported that vitamin D deficiency is associated with age-related cognitive decline and Alzheimer's disease [11]. Moreover, they reported that these diseases are mitigated or prevented by vitamin D supplementation [11-13]. Therefore, vitamin D and its analogs have the therapeutic potential, not only for bone-related diseases but also cognitive disorders and Alzheimer's disease.

More than 3,000 vitamin D analogs have been synthesized and studied for potential clinical use to treat various diseases [14, 15]. Screening high-affinity VDR ligands from numerous vitamin D analogs is essential for developing novel medicines. Several *in*

vitro assay systems have been used to evaluate the affinity of VDR ligands. For example, a competitive assay using bovine thymus [16, 17], and an antibody system that specifically recognizes the ligand-bound VDR-SRC-1 complex have been developed [18]. However, to our knowledge, these systems are not commercially available now. Thus, development of a convenient *in vitro* system is of prime importance.

In this decade, some split-luciferase-based ligand detection systems have been established [19-22]. Split-luciferase has advantages of high sensitivity, high signal-to-background (S/B) ratio, reversibility, and quantitativity. Previously, we developed the LucC(416-550 aa)-LBD(human VDR LBD 121-427 aa)-LucN(1-415 aa) [LucC-LBD-LucN] biosensor using the split-luciferase technique to detect VDR ligands [23]. The LucC-LBD-LucN biosensor was named as split-luciferase vitamin D biosensor (SLDB) and can detect and discriminate between VDR agonists or antagonists in living mammalian cells. Moreover, we successfully screened and identified a high-affinity vitamin D analog for the R274L mutant type of VDR using the SLDB (R274L) [24]. Our living cell systems are quite useful because they could evaluate not only VDR affinity, but also cell permeability and compound toxicity. However, the cell-free system established in this study is highly attractive for specific and rapid evaluation of VDR affinity of the compounds.

Some reports showed the expression of split-luciferase-based biosensors in cell-free translation systems using rabbit reticulocyte lysate, wheat germ extract, or in *E. coli* cell lysate to detect ligand-protein or protein-protein interactions *in vitro* [25-27]. The detection time of *in vitro* assay systems is markedly shorter than mammalian cell-based assays. In this study, we successfully expressed the SLDB proteins using an *E. coli* expression system and utilized this system to analyze VDR ligand binding behavior.

2. Materials and methods

2.1. Materials

Ethanol (EtOH), 25(OH)D_3 were purchased from Wako Pure Chemicals (Osaka, Japan). 1α,25(OH)$_2D_3$ was purchased from Cayman Chemical. Co (Ann Arbor, MI, USA). NS-4 [28, 29], 73a, 78a and 83a [29] were synthesized by Kittaka's group (Teikyo University, Japan). Recombinant *firefly* luciferase protein was purchased from Promega, Madison, USA (Cat No. E1701, #Lot No. 0000178404).

2.2. Construction of the expression plasmid for bioluminescent sensor protein in E.coli cells

E. coli expression vector pET-11d-N-His was a kind gift from Dr. T. Yoshida (University of Toyama). An annealed oligonucleotide (5'-CATGCGCGGAAGCCATCACCATCACCATCACGGATCCCTCGAGAGGCCT-3' and 5'-GATCAGGCCTCTCGAGGGATCCGTGATGGTGATGGTGATGGCTTCCGCG-3') was ligated into *Nco*I/*Bam*HI-digested pET-11d (Novagen) to yield the N-terminal histidine (His)-tagged expression vector, pET-11d-N-His. To insert *Not*I site in multi-cloning site of this vector, an annealed oligonucleotide (5'-TCGAGGAATTCGCGGCCGCGTCGACG-3' with 5'-TCGACGTCGACGCGGCCGCGAATTCC-3') was ligated into *Xho*I site of pET-11d-N-His vector to yield the vector pET-11d-N-His-*Xho*I/*Not*I. Next, His tag in pET-11d-N-His-*Xho*I/*Not*I vector was replaced by the 6xHN tag using inverse PCR method to obtain the pET-11d-N-6xHN-*Xho*I/*Not*I vector. Inverse PCR was performed with KOD-plus DNA polymerase using the set of primers 5'-

CACAACGCTGCAGGTGATGACGATGATAAGGGATCCCTCGAGAGGCCTGATC CGGC-3’ and 5’-ATTATGATTATGATTATGATTATGATTATGACCCATGGTATATCTCCTTCTTAAAG TTAAAC-3’. All the construction was confirmed by DNA sequencing using ABI3130 (Applied Biosystems, Tokyo, Japan). We previously reported the expressing plasmid pEBMulti-LucC-LBD-LucN vector [23]. The cDNA encoding LucC-LBD-LucN represented SLDB was inserted between *Xho*I and *Not*I site of pET-11-d-N-6xHN-*Xho*I/*Not*I vector. The resultant expression plasmid was named pET-11d-N-6xHN-SLDB vector.

2.3. Expression of the SLDB *proteins in E. coli cells*

The pET-11-d-N-6xHN-*Xho*I/*Not*I or pET-11d-N-6xHN-SLDB vector were transformed into *E. coli* strain BL21(DE3) (BioDynamics Laboratory Inc., Japan) in according to the manufacture’s instructions. A single colony from a fresh plate was picked and grown at 37 °C in 2 mL LB medium, containing 100 mg/mL ampicillin [LB(Amp)]. An overnight culture of these cells was used to inoculate in 500 mL culture of LB(Amp). Protein expression was induced at an OD_{600} of 0.4 with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside [IPTG (Nacalai Tesque, Inc., Japan)] followed by incubation for an additional 3 h at 15 °C. Cells were pelleted by centrifugation at 5000 x *g* at 4 °C for 10 min and resuspended in suspension buffer [25 mM Tris-HCl, pH 7.4, 10 mM DTT and protease inhibitor cocktail (Nacalai Tesque, Inc., Japan)] and were lysed by sonication for 15 s × 7. The lysate was centrifuged at 20000 × *g* for 30 min at 4 °C. The lysate containing the SLDB proteins were used for western blotting and *in vitro*

luciferase complementation assay. The lysate transformed pET-11-d-N-6xHN-*Xho*I/*Not*I vector was used as a diluent for the SLDB, named diluent solution. In this study, total 50 μl containing 0.068, 0.68 or 6.8 μl of the SLDB were called the SLDB 1, SLDB 10 or SLDB 100, respectively.

2.4. SDS-PAGE and Western blotting

Each of 0.1, 0.5, 1 and 5 μg of the SLDB, and Pre-stained protein markers (Nacalai Tesque, Inc., Japan) were separated by SDS-PAGE, and electrically transferred onto a PVDF membrane (GE Healthcare, Buckinghamshire, UK). The membranes were incubated with goat anti-luciferase antibody (1:1000, Promega, Madison, USA) in the Can Get Signal solution (Toyobo, Osaka, Japan) at room temperature for 1 h, and then reacted with secondary rabbit anti-goat IgG HRP-conjugated antibodies (1:5000, Santa Cruz, CA, U.S.A.) at room temperature for 1 h. The immobilized proteins were visualized using an ECL prime Western blotting reagent (GE Healthcare, Buckinghamshire, UK). Finally, the chemiluminescence signals were detected using the LAS-1000 image analyzer (Fujifilm, Tokyo, Japan).

2.5. Definition of enzyme activity of the SLDB

Total 50 μl containing each of 1, 2.5, 5 and 10 ng of the recombinant *firefly* Luciferase proteins or SLDB 1, 10 and 100 were plated into 96 well plate for 10 min at room temperature. Then, 50 μl of the solution [25 mM Tris-HCl (pH 7.4), 20 mM $MgSO_4$, 2 mM D-Luciferin (Thermo Scientific, CA, U.S.A.) and 4 mM ATP] (hereafter, simply referred to as “Luciferin solution”) were injected into 96 well plate for 15 min at room temperature. The luminescence (photon counts) was measured using a

luminometer (Infinite 200 Pro 96-microplate luminometer, Tecan). Standard curve of recombinant *firefly* Luciferase and SLDB was shown in Sup Fig. 2. The recombinant *firefly* Luciferase activity; 89139 photon counts / 1 ng was defined as 1 U (light unit) (Sup Fig. 2A). The relative activity of the SLDB 1, 10 or 100 was calculated to be 3.63 $\times$ 10^{-3}, 3.63 $\times$ 10^{-2} and 3.63 $\times$ 10^{-1} U, respectively, based on the recombinant *firefly* Luciferase activity (Sup Fig. 2B).

2.6. In vitro luciferase complementation assay

The SLDB was diluted with the diluent solution and the total volumes were adjusted to the 50 μl (containing 10 μg of total proteins). Total 50 μl of the SLDB 1, 10 or 100 was plated into 96 well plate within 1-2 min. Next, EtOH or vitamin D analogs [1α,25$(OH)_2D_3$, NS-4, 73a, 78a or 83a] were added to the well at 0-1000 nM and incubated at room temperature (23-26 ^oC) (pre-incubation) for 1-120 min. As all vitamin D analogs were dissolved in EtOH at a final concentration of 1%, the wells containing 1% EtOH were used as controls. Then, 50 μl of detection solution was injected into 96 well plate. After addition of Luciferin solution, the light intensity of each well was measured by a 96-microplate luminometer (photon detection) at several time points (5-120 min) where the photons were counted for 0.4 sec. In this study, the relative light intensity in the presence of a vitamin D analog was calculated in comparison with the light intensity in the absence of a vitamin D analog (1% EtOH) as a negative control. Relative activity of the SLDB 1, 10, or 100 was shown in Sup Fig. 2B.

3. Results and discussion

3.1. Mechanism of ligand-induced conformational and functional changes of SLDB

In our previous report, we developed SLDB that can detect VDR ligands based on changes in luciferase-mediated light intensity [23]. When an agonist binds to SLDB, the luciferase light intensity was immediately and dramatically decreased, in contrast, luciferase light intensity was immediately and dramatically increased in response to antagonists in a dose-dependent manner. This phenomenon appears to depend on the position of helix 12 (H12) of the LBD. A schematic diagram of the SLDB is shown in Fig 1.

3.2. Expression of SLDB proteins in E.coli

To express SLDB proteins in *E.coli* cells, pET-11d-N-6xHN-SLDB vector was introduced into *E.coli* strain BL21(DE3). The SLDB was analyzed by Western blotting using an anti-luciferase antibody. The distinct band of the SLDB protein was shown in Sup Fig. 1. We observed a specific band with an apparent molecular M.W. of 95 kDa consistent with its calculated M.W. of 95 kDa.

3.3. Effect of 1α,25(OH)$_2$D$_3$ on light intensity of different amounts of SLDB

First, we examined the dose-dependent response of 1α,25(OH)$_2$D$_3$ using different amounts of SLDB. SLDB 1, 10, or 100 was plated onto 96-well plates. Various concentrations of 1α,25(OH)$_2$D$_3$ (0-1000 nM) were added to the wells and incubated for 10 min at room temperature. Light intensity was measured at 15 min after addition of Luciferin solution. As shown in Sup Fig. 4 and Fig. 2A, 1α,25(OH)$_2$D$_3$ decreased the luciferase activity and the relative light intensity in a dose-dependent manner. Detection

limits for 1α,25(OH)$_2$D$_3$ were 0.05-0.1 nM, 0.1-0.5 nM, or 1-5 nM using the SLDB 1, 10, and 100, respectively (Sup Fig. 4A-C). To determine the K_d values of agonists, the concentration of SLDB should be much lower than those of agonists. However, low concentrations of SLDB causes low luminescence intensity. Because SLDB 10 showed high sensitivity and enough luminescence intensity, we used SLDB 10 in the following experiments. Next, we examined the dose-dependent response of 25(OH)D$_3$, which is a natural low-affinity VDR ligand. 25(OH)D$_3$ decreased the relative light intensity in a dose-dependent manner. Detection limits for 25(OH)D$_3$ were over 10 nM using SLDB 10 (Fig. 2B). The addition of over 1 nM of 1α,25(OH)$_2$D$_3$ decreased the light intensity of SLDB 10 to approximately 20% (Fig. 2A). In contrast, over 1000 nM of 25(OH)D$_3$ was required to decrease the light intensity of SLDB 10 (Fig. 2B). These results indicated that our system could evaluate VDR ligand affinity.

3.4. Effect of antagonists on the light intensity of SLDB

We examined and compared the dose-dependent response of four antagonists (NS-4, 73a, 78a and 83a) using SLDB 10 (Fig. 3). These compounds have antagonistic activity towards 1α,25(OH)$_2$D$_3$-induced differentiation of HL-60 cells [29]. Each antagonist increased the relative light intensity in a dose-dependent manner (Fig. 3E). However, the maximum of the relative light intensity differed among these compounds. The differences in maximal light intensity likely represents subtle differences of the position of H12 of the LBD and conformational changes after antagonist binding. Previously, we explained the relationship between antagonist potency and α,β-unsaturated lactone structure on the side chain using more than 60 antagonists [28, 29], and a Cys403 or Cys410 residue of the LBD of VDR may cause nucleophilic attack on the sp^2 β-carbon

of the lactone ring with a Michael-type addition. This phenomenon would affect the position of H12.

3.5. Determination of the detection time after Luciferin solution addition and pre-incubation time

As shown in Sup Fig. 3, maximal luciferase activity was observed at 15-20 min after adding Luciferin solution (Sup Fig. 3A). However, the relative light intensity [100 nM 1α,25$(OH)_2D_3$ (+) / 1% EtOH (-)] was unchanged for 5-120 min (Sup Fig. 3B). Thus, we determined the timing for photon counting to be 15 min after adding the Luciferin solution.

Next, we investigated the pre-incubation time before adding Luciferin solution. The SLDB 10 was pre-incubated with 1% EtOH (-) or 100 nM 1α,25$(OH)_2D_3$ (+) for 1-120 min at room temperature. Then, we measured the luciferase activity (photon counting for 0.4 sec) of SLDB (Fig. 4A) at 15 min after adding Luciferin solution. Although maximal luciferase activity was observed at pre-incubation time of 5-10 min, the relative light intensity [100 nM 1α,25$(OH)_2D_3$ (+) / 1% EtOH (-)] was unchanged even at pre-incubation time of 120 min (Fig. 4B). Therefore, we determined the pre-incubation time to be 10 min in this study. It is noted that our *in vitro* system could be completed within about 30 min.

3.6. Effect of multiple freeze-thaw cycles on SLDB activity

Finally, we examined the effect of the multiple freeze-thaw cycles on the luciferase activity of SLDB. Surprisingly, luciferase activity (Sup Fig. 5A) and the relative light intensity [100 nM 1α,25$(OH)_2D_3$ (+) / 1% EtOH (-)] of the SLDB was not changed

after each freeze-thaw cycles (Sup Fig. 5B). These results indicate that the SLDB was highly resistant to multiple freeze-thaw cycles. Thus, this system may be practically useful as a screening system for VDR ligands that preliminarily distinguished potential agonists or antagonists in a short time.

In conclusion, we successfully established a highly sensitive, rapid, *in vitro* detection system for VDR ligands. Furthermore, our *in vitro* assay system appears to be more convenient, rapid, and low-cost compared to other methods. Natural VDR ligands including $1\alpha,25(OH)_2D_3$ and $25(OH)D_3$, and vitamin D analogs are metabolized in the body by various metabolic enzymes such as CYP24A1. Moreover, recently, Slominski et al., reported novel metabolic pathways of vitamin D_3 initiated by CYP11A1 [30, 31]. Thus, measuring VDR affinity of those metabolites is important to predict *in vivo* efficacy of VDR analogs. It is well-known that low levels of serum $25(OH)D_3$ are closely linked to bone diseases such as rickets, osteomalacia, and osteoporosis. In addition, an epidermal study has demonstrated a significant correlation between the morbidity of prostate cancer and serum $25(OH)D_3$ levels [32, 33]. Thus, measuring serum $25(OH)D_3$ levels is quite important to determine an individual's vitamin D status. Although an enzyme-linked immunosorbent assay (ELISA)-based detection system is generally used to quantify concentrations of $1\alpha,25(OH)_2D_3$ and $25(OH)D_3$ [34], the ELISA system is inconvenient and very expensive. In contrast, our *in vitro* assay system using SLDB is a convenient and low-cost system. Therefore, our system could be used for the diagnostic measurement of serum concentrations of $1\alpha,25(OH)_2D_3$ and $25(OH)D_3$.

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Figure Legends

Fig. 1. Schematic diagram of SLDB to detect and discriminate between VDR agonists and antagonists. Binding of the agonists to SLDB may cause a conformational change of helix12 (H12) in LBD that leads to disruption of the functional complex between LucN and LucC fragments of the split-luciferase. In contrast, binding of the antagonist to the SLDB may cause a different conformational change of H12 to enhance SLDB activity.

Fig. 2. 1α,25(OH)$_2$D$_3$ or 25(OH)D$_3$ dose-dependent relative light intensity of SLDB. SLDB 10 was pre-incubated with various concentrations (0-100 nM) of 1α,25(OH)$_2$D$_3$ (A) or (0-1000 nM) of 25(OH)D$_3$ (B) for 10 min at room temperature. The luminescence was measured at 15 min after addition of Luciferin solution. The relative light intensity compared to the control (0 nM=1% EtOH) was shown. Data are represented as mean ± SD, n = 4 (A) and mean ± SEM, n = 4 (B).

Fig. 3. Effects of various VDR antagonists on the light intensity of SLDB. Chemical structures of VDR antagonists (A) NS-4, (B) 73a, (C) 78a and (D) 83a. (E) SLDB 10 was pre-incubated with various concentrations (0-100 nM) of VDR antagonists (NS-4, 73a, 78a or 83a) for 10 min at room temperature. The luminescence was measured at 15 min after addition of Luciferin solution. The relative light intensity compared to the control (0 nM=1% EtOH) was shown. Data are represented as mean ± SEM, n = 6.

Fig. 4. Effects of pre-incubation time with 100 nM 1α,25(OH)$_2$D$_3$ on SLDB activity.

(A) Photons were counted for 0.4 sec at 15 min after pre-incubation for the period shown in the figure, and normalized values per second were shown. (B) Relative light intensity of SLDB 10 in the presence (+) or absence (-) of 100 nM 1α,25$(OH)_2D_3$ after pre-incubation for 1-120 min at room temperature. The relative light intensity compared to the control (-) was shown. Data are represented as mean ± SD, n = 4 (A) and mean ± SEM, n = 4 (B).

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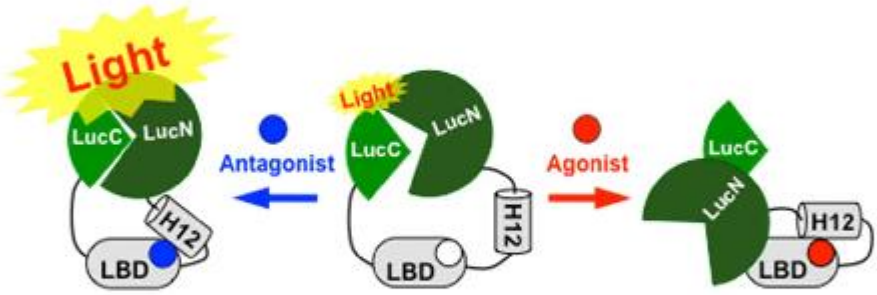

Fig. 1

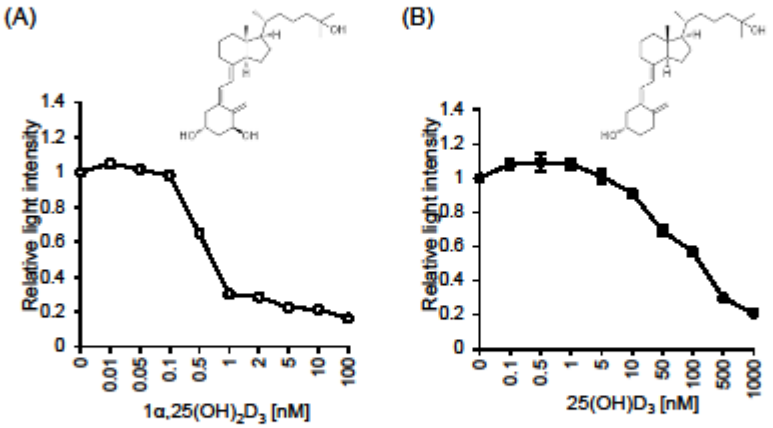

Fig. 2

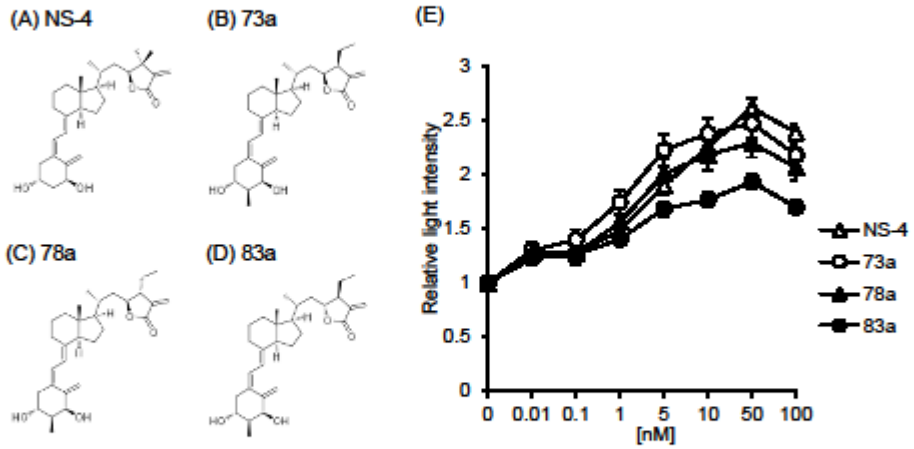

Fig. 3

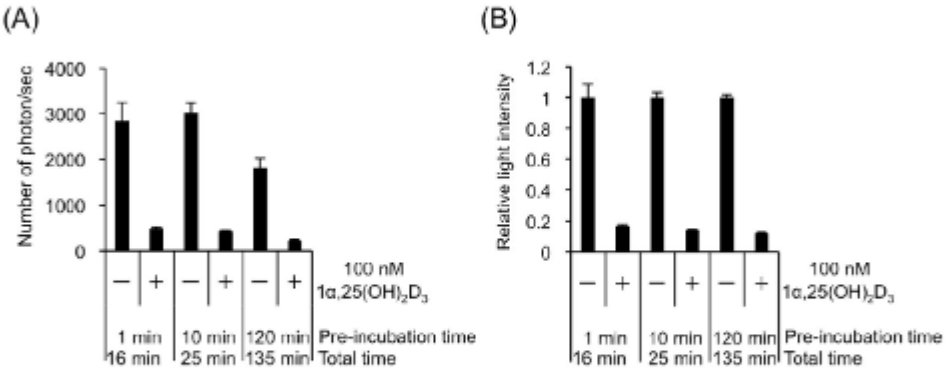

Fig. 4