Review

Development of In Vitro and In Vivo Evaluation Systems for Vitamin D Derivatives and Their Application to Drug Discovery

Kaori Yasuda [1], Miyu Nishikawa [2], Hiroki Mano [1], Masashi Takano [3], Atsushi Kittaka [3], Shinichi Ikushiro [2] and Toshiyuki Sakaki [1,*]

1   Department of Pharmaceutical Engineering, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu 939-0398, Toyama, Japan; kyasuda@pu-toyama.ac.jp (K.Y.); z16003@st.pu-toyama.ac.jp (H.M.)
2   Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu 939-0398, Toyama, Japan; m-nishikawa@pu-toyama.ac.jp (M.N.); ikushiro@pu-toyama.ac.jp (S.I.)
3   Faculty of Pharmaceutical Sciences, Teikyo University, Tokyo 173-8605, Japan; mtakano@pharm.teikyo-u.ac.jp (M.T.); akittaka@pharm.teikyo-u.ac.jp (A.K.)
*   Correspondence: tsakaki@pu-toyama.ac.jp

Abstract: We have developed an in vitro system to easily examine the affinity for vitamin D receptor (VDR) and CYP24A1-mediated metabolism as two methods of assessing vitamin D derivatives. Vitamin D derivatives with high VDR affinity and resistance to CYP24A1-mediated metabolism could be good therapeutic agents. This system can effectively select vitamin D derivatives with these useful properties. We have also developed an in vivo system including a *Cyp27b1*-gene-deficient rat (a type I rickets model), a *Vdr*-gene-deficient rat (a type II rickets model), and a rat with a mutant *Vdr* (R270L) (another type II rickets model) using a genome editing method. For *Cyp27b1*-gene-deficient and *Vdr* mutant (R270L) rats, amelioration of rickets symptoms can be used as an index of the efficacy of vitamin D derivatives. *Vdr*-gene-deficient rats can be used to assess the activities of vitamin D derivatives specialized for actions not mediated by VDR. One of our original vitamin D derivatives, which displays high affinity VDR binding and resistance to CYP24A1-dependent metabolism, has shown good therapeutic effects in *Vdr* (R270L) rats, although further analysis is needed.

Keywords: vitamin D; vitamin D receptor; split luciferase-based biosensor; CYP24A1-dependent metabolism; CYP27B1; rickets; genome editing

1. Introduction

The active form of vitamin D_3 ($1\alpha,25(OH)_2D_3$) plays essential roles in calcium and phosphate homeostasis, cellular proliferation and differentiation, and immune responses. Since it could cause hypercalcemia and hypercalciuria, its clinical utility is limited [1,2]. A huge number of vitamin D derivatives have been synthesized. Many of them have been studied in clinical trials for the treatment of type I rickets, osteoporosis, psoriasis, renal osteodystrophy, and also leukemia, pancreatic, prostate, and breast cancers [3–9]. A number of vitamin D derivatives have been approved by the FDA for clinical use in a variety of disorders, for example, 22-oxacalcitriol (Maxacalcitol) and calcipotriol (Dovonex) for treatment of psoriasis, 19-nor-$1\alpha,25(OH)_2D_2$ (Zemplar), 26,26,26,27,27,27-hexafluoro-$1\alpha,25(OH)_2D_3$ (Falecalcitriol), and doxercalciferol (Hectorol) for secondary hyperparathyroidism, and $1\alpha(OH)D_3$ (alfacalcidol) and eldecalcitol (Edirol) for osteoporosis. Although many vitamin D derivatives have antiproliferative activity, none have been approved for cancer treatment. So far, only a small number of clinical studies have taken place, such as EB1089 in a phase II study for pancreatic cancer [6,10], and Hectorol and Zemplar in phase I/II advanced androgen-insensitive prostate cancer trials [7,11,12]. Unfortunately, neither have produced any significant objective responses. However, a new $1\alpha,25(OH)_2D_3$ analog, 19-nor-14-epi-23-yne-$1\alpha,25(OH)_2D_3$ (inecalcitol), is being developed for prostate cancers and chronic leukemia [13,14].

The active form of vitamin D_3 ($1\alpha,25(OH)_2D_3$) plays essential roles in calcium and phosphate homeostasis, cellular proliferation and differentiation, and immune responses. Its clinical utility is limited because it can cause hypercalcemia and hypercalciuria. [1,2]. Several thousand vitamin D derivatives have been synthesized, and many have been studied in clinical trials to treat conditions, including type I rickets, osteoporosis, leukemia, psoriasis, renal osteodystrophy, and pancreatic, prostate, and breast cancers. [3–9]. A number of vitamin D derivatives have been approved by the FDA for clinical use in a variety of disorders. These derivatives include calcipotriol (Dovonex; Leo Pharmaceuticals) and 22-oxacalcitriol (Maxacalcitol; Chugai Pharmaceuticals) for treatment of psoriasis; 19-nor-$1\alpha,25(OH)_2D_2$ (Zemplar; Abbot Laboratories; Chicago, IL, USA), 26,26,26,27,27,27-hexafluoro- (Falecalcitriol; Sumitomo Pharmaceuticals and Taisho Pharmaceuticals), and doxercalciferol (Hectorol; Bone Care Int.; Middleton, WI, USA) for secondary hyperparathyroidism; and $1\alpha(OH)D_3$ (alfacalcidol; Chugai Pharmaceuticals Co., Ltd.; Tokyo, Japan) and eldecalcitol (Chugai Pharmaceuticals Co., Ltd.; Tokyo, Japan) for osteoporosis. Although many vitamin D derivatives, including those approved by the FDA for treating secondary hyperparathyroidism and renal osteodystrophy, have displayed antiproliferative activity, none have been approved for cancer treatment. To date, only a limited number of clinical studies have taken place, including a phase II study of EB1089 in pancreatic cancer. [6,10]. Hectorol and Zemplar have been studied in phase I/II advanced androgen-insensitive prostate cancer trials [7,11,12]. Unfortunately, neither produced any significant objective responses. Recently, a new $1\alpha,25(OH)_2D_3$ analog, inecalcitol, is being developed for prostate cancers and chronic leukemia [13,14].

In evaluating these vitamin D derivatives, (1) affinity for vitamin D receptor, (2) affinity for vitamin-D-binding protein (DBP), (3) resistance to metabolism by CYP24A1, and (4) ability to differentiate leukemia-derived HL-60 cells into macrophages are considered to be essential properties. In addition, they must show therapeutic efficacy in animal studies. In the case of derivatives under development for cancer treatment, therapeutic efficacy will be evaluated using tumor-bearing animals. Construction of appropriate evaluation models is indispensable for developing vitamin D derivatives for pharmaceutical use. We have developed in vitro systems that can easily measure vitamin D receptor (VDR) affinity [15–19] and CYP24A1-mediated metabolism [20–24]. We have also generated genetically modified rats using genome editing as follows: *Cyp27b1*-gene-deficient rats (a type 1 rickets model animal), vitamin D receptor-gene-deficient rats, and rats harboring a mutant vitamin D receptor (R270L) gene (type II rickets model animals) [25]. We have also generated *Cyp24a1*-gene-deficient rats to elucidate enzymes and metabolic pathways responsible for vitamin D derivative metabolism [26]. In this review, we describe the in vitro and in vivo systems we have developed for evaluation of vitamin D derivatives, and discuss the derivatives we have synthesized to date.

2. In Vitro System to Easily Examine the Affinity for VDR of Vitamin D Derivatives

2.1. Measurement of Binding Affinity of Vitamin D Derivatives for VDR

The widely used method for evaluating the binding ability of vitamin D derivatives for VDR in a cell-based assay system is a reporter assay that induces expression of luciferase (Luc) under the control of a promoter containing a vitamin D response element (VDRE) [27–29]. It is noted that it takes more than 12 h for the reporter protein to be expressed, and the direct binding between the receptor and the ligand cannot be evaluated. Although a competitive system using native VDR and tritium-labeled $1\alpha,25(OH)_2D_3$ was widely used, it is no longer commercially available. Thus, we tried to develop a new detection system that easily evaluates the affinity of vitamin D derivatives for VDR in a short time. We focused on the split-type luciferase technology [15–19,30–37]. This system can evaluate the affinity of the ligand by increasing or decreasing the luminescence of the split-type luciferase.

2.2. Development of a Novel Bioluminescent Sensor to Detect and Discriminate between Vitamin D Receptor Agonists and Antagonists in Living Cells (1st Generation)

Two chimeric fusion proteins that contained both split-luciferase and the ligand binding domain (LBD) of the VDR were constructed. This fusion protein was labeled as LucN–LBD–LucC. It contained the N-terminal domain taken from luciferase (LucN), LBD, and C-terminal domain from luciferase (LucC) from N-terminus to C-terminus. LucC–LBD–LucN has the C-terminal domain of luciferase at the N-terminus of the fusion protein (Figure 1) [15]. Unexpectedly, the LucC–LBD–LucN worked better than LucN–LBD–LucC. Luciferase activity was significantly diminished by the addition of the VDR agonists to COS-7 cells that expressed LucC–LBD–LucN. On the other hand, the VDR antagonist notably enhanced the activity of the chimeric luciferase in a dose- and time-dependent manner. Our novel model for detecting and discriminating between VDR agonists and antagonists is very useful for testing synthetic analogs of vitamin D that show reasonable affinity for normal or mutant VDRs.
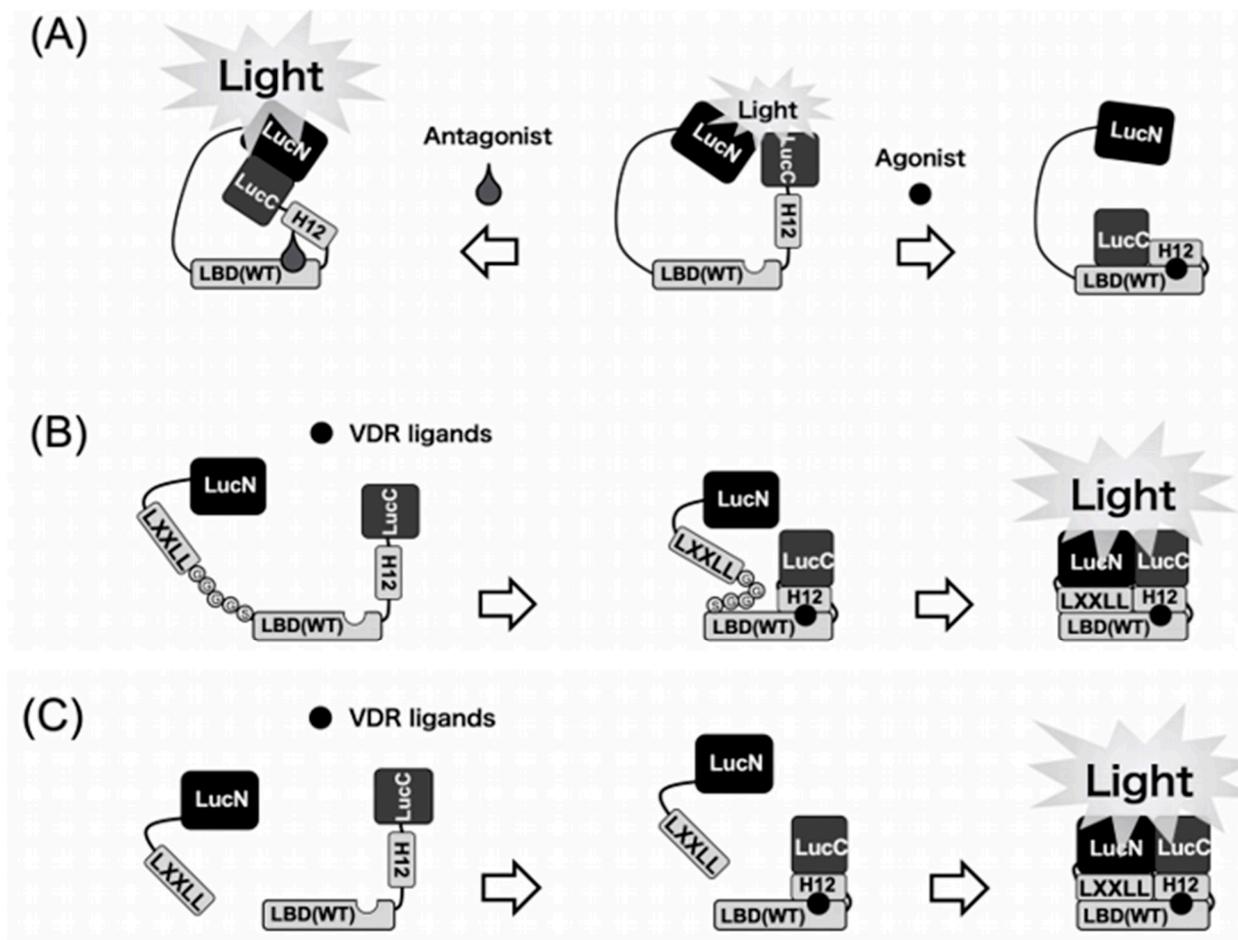
Figure 1. Schematic diagrams of the biosensors to detect VDR ligands. (**A**) 1st generation. Binding of the VDR agonists to the LBD may cause a conformational change of the LBD that leads to disruption of the functional complex between N-terminal and C-terminal domains of the luciferase. In contrast, binding of the antagonist leads to the reassembly of N-terminal and C-terminal domains of the luciferase to increase the activity. (**B**) 2nd generation. Binding of VDR ligands to the biosensor may cause a conformational change of helix12 (H12) in LBD. After conformational change of LBD, the LXXLL motif interacts with LBD in the biosensor. Then, this intramolecular dynamic change of the WT biosensor leads to reconstitution of the functional complex between LucN and LucC fragments of the split luciferase. (**C**) 3rd generation. Binding of the VDR ligands to the LBD–LucC may cause a positional change of helix12 in LBD. Then, the LucN–LXXLL and LBD–LucC forms a functional complex to exhibit the luciferase activity.

Patients with type II rickets showing the R274L mutation caused a 1000-fold reduction in the binding activity for $1\alpha,25(OH)_2D_3$ and remarkably lowered vitamin-D-related gene expression [38]. It is Arg274, located in LBD of VDR, that is responsible for attaching $1\alpha,25(OH)_2D_3$. This happens by a formation of an additional hydrogen bond with 1α-hydroxyl of $1\alpha,25(OH)_2D_3$. LucC–LBD (R274L)–LucN was constructed to investigate vitamin D ligands of high affinity for the mutant VDR (R274L). A total of 5 out of the 33 vitamin D analogs tested showed much higher binding for the mutant VDR (R274L) than the vitamin D hormone. The highest binding activity was shown by 2α-(2-(tetrazol-2-yl)ethyl)-(AH-1). These analogs might be considered as future drug candidates against HVDRR that is caused by the mutant VDR (R274L) [16].

2.3. Development of a Highly Sensitive In Vitro System to Detect and Discriminate between Vitamin D Receptor Agonists and Antagonists

We have established an in vitro screening system for VDR ligands using the LucC–LBD–LucN proteins expressed in *Escherichia coli (E. coli)* cells [17]. It should be noted that this system 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 osteoporosis, renal osteodystrophy, cancers, and immune disorders.

2.4. Design of a Biosensor Based on Split Luciferase for Detection of VDR Ligands (2nd Generation)

The model we developed is very useful for a fast investigation of VDR ligands. However, the sensitivity of our biosensor (LucC–LBD–LucN) is not as high as expected. LBD is known to interact via the LXXLL motif with transcription coactivators, such as SRC-1, TIF-2, or DRIP-205 to initiate vitamin-D-related gene expression, when binding natural VDR ligands. This is why we anticipated that it is the LXXLL motif that changes the enzymatic profile of luciferase–LBD biosensors. This is why LucN–LBD–LucC and not LucC–LBD–LucN was used as a basing fragment. We created a new biosensor consisting of the LBD (121–427 aa) of VDR, N- and C-terminal of firefly luciferase fragments (LucN (1–415 aa) and LucC (416-550 aa)), the LXXLL peptide sequence, and peptide sequence (Gly-Gly-Gly-Gly-Ser (GGGGS)) $\times$ 3 as the flexible linker [18]. This construct we labeled as LucN–LXXLL–(GGGGS) $\times$ 3–LBD–LucC WT biosensor and WT means the wild-type of LBD (Figure 1). Light intensity of luciferase is low when natural VDR ligands are absent. The luciferase light intensity is immediately and remarkably increased when the ligand is bound to the WT biosensor. To sum up, we have successfully created a very sensitive biosensor which shows the increase in light intensity when binding VDR agonists.

To this end, we developed a novel and WT biosensor of high sensitivity by examining three types of LXXLL peptides (NHPMLMNLLKDN, LTEMHPILTSLLQNGVDHV, and LSETHPLLWTLLSSTEGDSM) that interact with the LBD in response to $1\alpha,25(OH)_2D_3$ or synthetic VDR agonists. The COS-7 cells that expressed each type of biosensor were treated with $1\alpha,25(OH)_2D_3$ (100 nM) and then the luminescence was measured 90 min later. Among the 10 biosensors we constructed, one showed a reduction in intensity of light in response to $1\alpha,25(OH)_2D_3$. Seven biosensors showed an excellent increase in light intensity. Our best biosensor showed the light intensity ca. one-third of that of full-length native luciferase of firefly. Quite unexpectedly, $25(OH)D_3$, as the low-affinity VDR ligand, also enhanced the intensity of light in a concentration-dependent manner. The half maximal relative intensity of light was recorded at 1 nM of $1\alpha,25(OH)_2D_3$ and at 20 nM of $25(OH)D_3$, respectively. We then compared the binding activity of $1\alpha,25(OH)_2D_3$ and $25(OH)D_3$ for the mutant VDR (R274L). As previously mentioned, the substitution of Arg274 to Leu causes a 1000-fold decrease in affinity of $1\alpha,25(OH)_2D_3$. As expected, in the R274L biosensor the concentration–response curve of $1\alpha,25(OH)_2D_3$ was very similar to that of $25(OH)D_3$. Thus, the biosensor system we developed may be very useful in elucidating novel vitamin D analogs as drug candidates against type II rickets resulting from VDR mutation, such as R274L.

2.5. Development of a Novel Two-Molecule System with a Highly Sensitive Biosensor (3rd Generation)

In the next step, we developed a two-molecule system named LXXLL + LBD biosensor, as shown in Figure 1, with a combination of two components [19]. The two plasmids were co-transfected and two proteins were co-expressed in COS-7 cells. The LXXLL + LBD biosensor-expressing COS-7 cells were treated with 100 nM of $1\alpha,25(OH)_2D_3$, and luciferase light intensity was measured at 90 min after treatment. Among all combinations of LXXLL + LBD biosensor, relative light intensity of A1 + B1 [19] was the highest in all combinations. The relative light intensity of combination A1 + B1 was approximately a 90- to 100-fold increase in response to 100 nM of $1\alpha,25(OH)_2D_3$. It should be noted that the detection limit was 0.005 nM (5 pM) of $1\alpha,25(OH)_2D_3$, indicating that the sensitivity of LXXLL + LBD biosensor is higher than that of our previous biosensors. [15–18]. Our LXXLL + LBD biosensor might be used for the measurement of $1\alpha,25(OH)_2D_3$ and $25(OH)D_3$ in the plasma.

3. In Vitro Evaluation of CYP24A1-mediated Metabolism of Vitamin D Derivatives

3.1. Expression of Rat or Human CYP24A1 in E. coli Cells

The rat *Cyp24a1* cDNA was cloned from the rat kidney cDNA library [39], and the isolated cDNA clone contained the open reading frame consisting of 514 amino acids. Since the amino acid sequence showed less than 40 % homology with already known CYPs, the new CYP family name, CYP24, was given to this vitamin-D-24-hydroxylase.

The molecular mechanism of *CYP24A1* gene regulation is quite complicated, and many factors are tissue-specifically involved in the expression of *CYP24A1* [40–44]. These facts strongly suggest that CYP24A1 is a physiologically essential enzyme that regulates the level of the active form of vitamin D.

When the deduced amino acid sequence from its cDNA was compared to that amino-terminal amino acid sequence of the CYP24A1 purified from rat kidney, it was found that the mature form of rat CYP24A1 lacks amino-terminal 32 amino acids. These results suggest that amino-terminal 32 amino acids function as a mitochondrial targeting signal, which is removed after translocation of CYP24A1 to mitochondria. We have successfully expressed the mature forms of rat and human CYP24A1 in *E. coli* cells to reveal their enzymatic properties [20–24].

3.2. Construction of a CYP24A1 Enzyme System Containing Adrenodoxin (ADX) and NADPH-Adrenodoxin Reductase (ADR)

The mitochondrial P450 system consists of three components: CYP, ADX, and ADR. Electrons are sequentially transferred from NADPH through ADR and ADX to CYP24A1 (Figure 2). Thus, CYP24A1-dependent activity was measured in an in vitro reconstituted system containing purified ADX and ADR proteins. On the other hand, in a whole-cell system, co-expression of mature forms of CYP24A1, ADX, and ADR in *E. coli* is required. We have demonstrated that the *E. coli* expression system is quite useful to investigate enzymatic properties of CYP24A1. Using this *E. coli* expression system, we have determined kinetic parameters of CYP24A1 in the metabolism of the native vitamin D and various vitamin D derivatives, and revealed their metabolic pathways [45–58].

3.3. CYP24A1-Dependent Multi-Step Reaction toward the Active form of Vitamin D_3

CYP24A1 plays central roles in vitamin D metabolism and produces a wide variety of metabolites. We revealed that rat or human CYP24A1 catalyzes a six-step reaction, starting with hydroxylation at the 24*R* position of $1\alpha,25(OH)_2D_3$ to produce the final metabolite, calcitroic acid (Figure 3). In addition, human CYP24A1 catalyzes a four-step reaction, starting with hydroxylation at the 23*S* position to produce the 26,23-lactone form (Figure 3). In the reaction of P450, it is often seen that the reaction product is not released from the substrate binding pocket and the reaction proceeds further. Thus, the two- or three-step reaction is not special in the P450 reaction; however, there is no other P450 that catalyzes

such a multi-step reaction for one substrate. Moreover, it is noted that the reaction by human CYP24A1 proceeds in a dual pathway, the C-24 pathway and the C-23 pathway. Interestingly, the ratio of the C-24 to C-23 pathways varies among animal species. In human CYP24A1, it is about 4:1, but, in rat CYP24A1, about 25:1; however, in animal species such as guinea pig and opossum, the C-23 pathway is major. In rat and human CYP24A1, the 326th amino acid residue from the N-terminus is Ala, whereas it is Gly in guinea pigs and opossum, and, when the Ala326 in rat and human CYP24A1 is replaced by Gly, it changes to the guinea pig type [59]. Given that inactivating the active form of vitamin D is the physiological role of CYP24A1, it may be less important whether the C-24 or C-23 pathway is predominant.
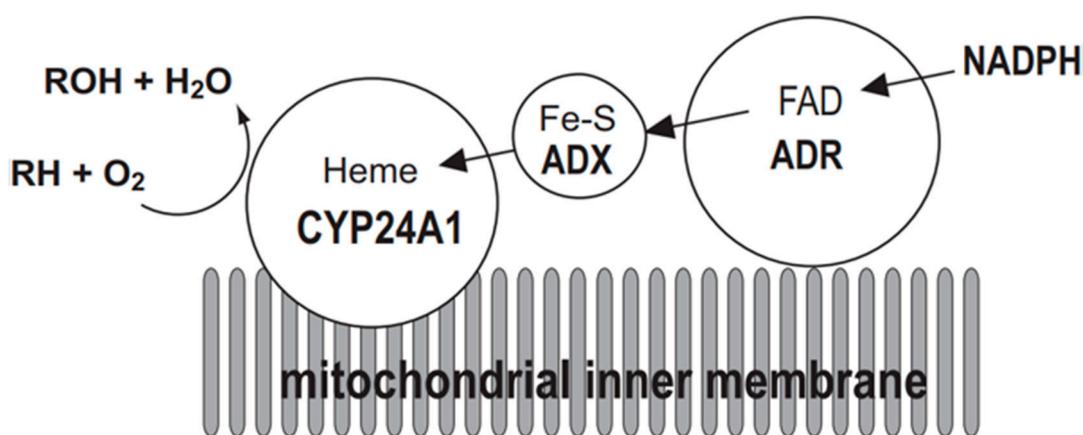
Figure 2. Mitochondrial electron transport chain of CYP24A1. CYP24A1-dependent mono-oxygenase activity requires the electron transfer from NADPH via NADPH-adrenodoxin oxidoreductase (ADR) and adrenodoxin (ADX) to the heme iron of CYP24A1 situated on the inner membrane of mitochondria. RH represents substrate of CYP24A1.
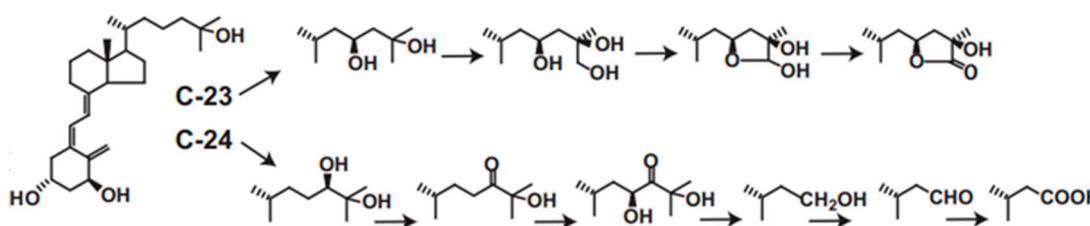

Figure 3. C-23 and C-24 oxidative pathways of $1\alpha,25(OH)_2D_3$ catalyzed by human CYP24A1. Human CYP24A1 catalyzes 6-step mono-oxygenation from C-24 hydroxylation to produce calcitroic acid, and 4-step mono-oxygenation from C-23 hydroxylation to the lactone formation.

3.4. Metabolism of Vitamin D Derivatives by CYP24A1

The CYP24A1 gene has two VDREs in the promoter region [40,41,60] and, when the active form of vitamin D binds to VDR, remarkable transcriptional induction of CYP24A1 occurs. When a large amount of CYP24A1 protein is expressed in the cell, the active form of vitamin D is inactivated via the metabolic pathways described above. This mechanism appears to be crucial for keeping the level of the active form of vitamin D. However, when a vitamin D derivative with a high affinity for VDR is developed as a drug, the drug binds to VDR to induce CYP24A1. Therefore, vitamin D derivatives that are not easily metabolized by CYP24A1 could be excellent drugs with long-lasting efficacy. Eldecalcitol, an osteoporosis treatment drug developed by Chugai Pharmaceutical Co., Ltd., has a 3-hydroxy-propyloxy group at the 2β position of $1\alpha,25(OH)_2D_3$ (Figure 4). We revealed that CYP24A1 hardly metabolizes Eldecalcitol [53] and suggest that the resistance to CYP24A1-dependent metabolism may be a key factor that keeps its efficacy for a long time [53,61–63]. We have investigated the metabolism of many vitamin D deriva-

tives by CYP24A1 and have clearly demonstrated the importance of CYP24A1-dependent metabolism. In addition, as mentioned above, the fact that there are animal species differences in the metabolic mode of $1\alpha,25(OH)_2D_3$ by CYP24A1 suggests that there are also animal species differences in the metabolism of vitamin D derivatives. Therefore, the development of vitamin D derivatives requires not only animal studies, but also metabolic studies using human CYP24A1 enzyme.
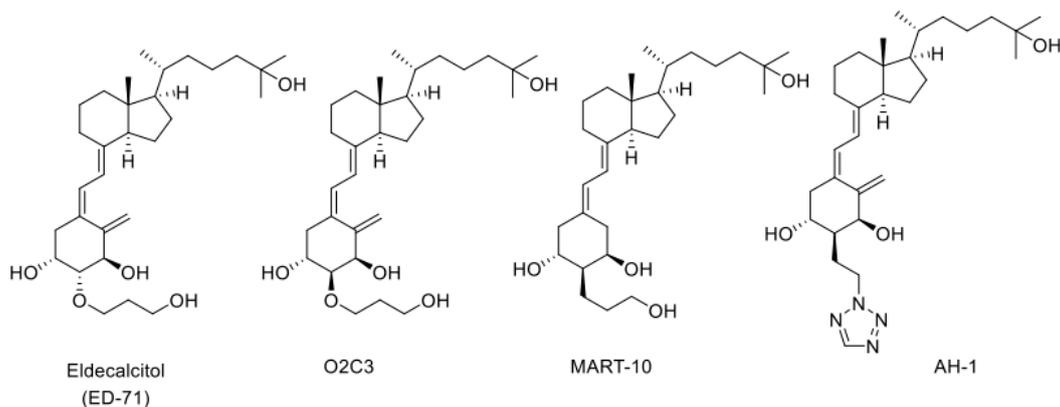
Figure 4. Structures of three CYP24A1-resistant VDR agonists: Eldecalcitol (ED-71), O2C3, MART-10, and AH-1.

3.5. CYP24A1-Resistant Vitamin D Derivatives with a Substituent at C2α Position

We have synthesized many of A-ring-modified derivatives with a substituent at the C2α position, which have unique biological activities [64–68]. Of these derivatives, 2α-(3-hydroxypropoxy)-$1\alpha,25(OH)_2D_3$ (O2C3), which is a C2-epimer of Eldecalcitol, was examined for the metabolism by CYP24A1. Five metabolites were detected in its metabolism by human CYP24A1, including both C-23 and C-24 oxidation pathways [48]. The K_m and k_{cat} values of human CYP24A1 for O2C3 were estimated to be approximately 16 times greater and 3 times lower than those for $1\alpha,25(OH)_2D_3$, respectively [48]. Accordingly, the catalytic efficiency (k_{cat}/K_m) of human CYP24A1 for O2C3 is only about 3% of $1\alpha,25(OH)_2D_3$. These results strongly suggest that O2C3 is much more resistant to CYP24A1-dependent metabolism than $1\alpha,25(OH)_2D_3$. It is noted that another C-2-substituted derivative, 19-nor-2α-(3-hydroxypropyl)-$1\alpha,25(OH)_2D_3$ (MART-10) (Figure 4), was more resistant to CYP24A1-dependent degradation than O2C3 [69–74]. The k_{cat}/K_m values of human CYP24A1 for MART-10 were about 0.3 % of those for $1\alpha,25(OH)_2D_3$.

Our in vivo studies using rats revealed that MART-10 had a potent anticancer effect, with a low calcemic effect, which is a suitable property as an anticancer drug. The resistance to CYP24A1 is also a suitable property of MART-10 as an anticancer drug.

4. In Vivo Evaluation System for Vitamin D Derivatives Using Genetically Modified Rats Generated by Genome Editing

4.1. Appearance and Growth of Genetically Modified (GM) Rats

Figure 5A shows WT, *Vdr* (R270L), and *Vdr*-KO rats fed an F-2 diet containing 0.75% Ca, and *Cyp27b1*-KO rats fed a diet containing 1.15% Ca at 15 weeks after birth. Although *Cyp27b1*-KO rats were much smaller than WT rats, body sizes of *Vdr* (R270L) and *Vdr*-KO rats were not so different from that of the WT rats. Figure 5A shows the *Vdr*- abnormal skin and alopecia of KO rats. Elasticity and softness of the skin of *Vdr*-KO rats were substantially lowered and the wavy skin was formed [25]. Keratinization was elevated and follicles decreased, and formation of cysts appeared in the dorsal skin of *Vdr*-KO rats [25].
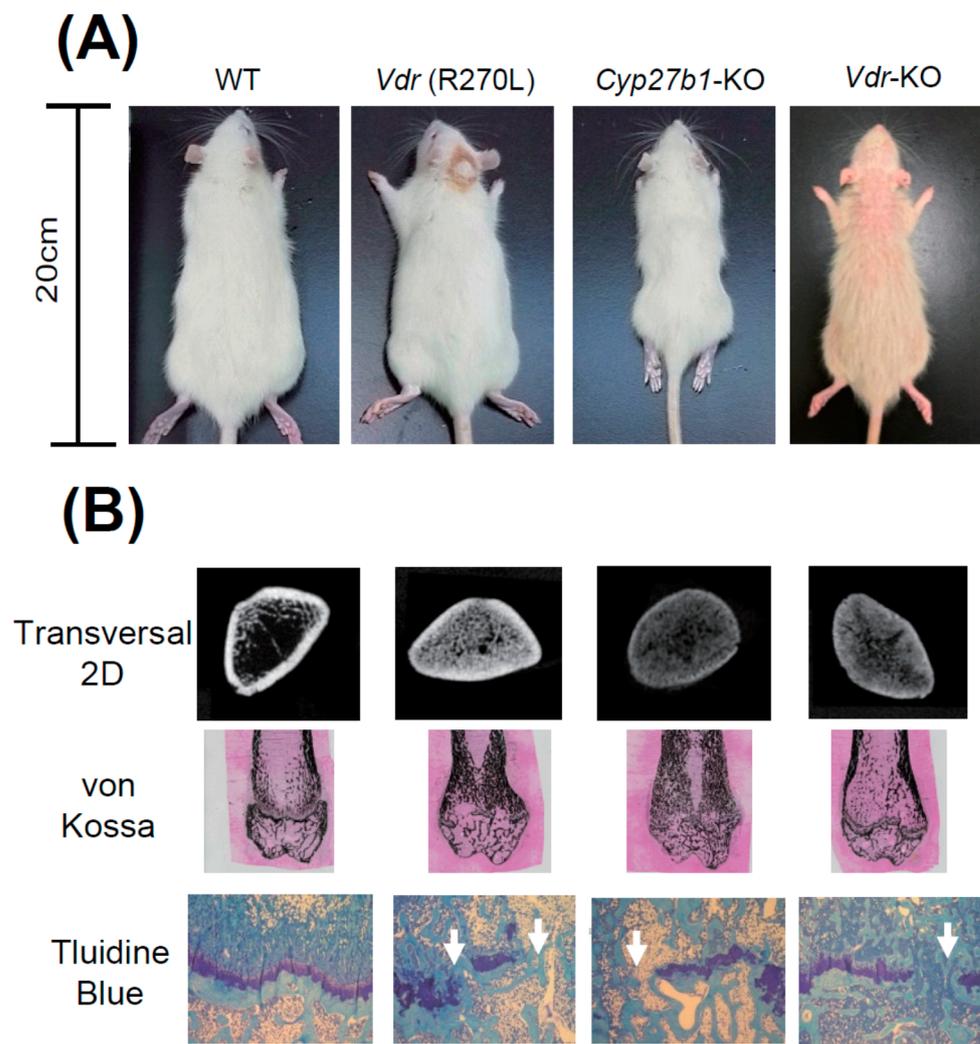
Figure 5. The appearance of GM rats and their abnormal bone formation [25]. (A) Comparison of body size and skin phenotype at 15 weeks of age. (B) First panels, 2D μ-CT images of horizontal section at distal femur; second panels, von Kossa staining of distal femur; bottom panels, toluidine blue staining of epiphyseal cartilage.

Figure 5A shows that growth was substantially diminished in *Cyp27b1*-KO rats compared to WT rats. However, only a slight decrease was observed in *Vdr* (R270L) and *Vdr*-KO rats. It was noted that approximately a half of male *Cyp27b1*-KO rats fed with the diet containing 0.75 % Ca died prior to 9 weeks of age, and none survived to 10 weeks of age (data not shown), whereas no animals had died at 15 weeks of age in the *Cyp27b1*-KO rats fed with the diet containing 1.15% Ca. Thus, the diet that contained 0.75% Ca was used for mutant *Vdr* (R270L) and *Vdr*-KO rats, while the diet containing 1.15 % Ca was used for *Cyp27b1*-KO rats.

4.2. Osteogenesis and Plasma Ca, PTH, and 1α,25(OH)₂D₃ Levels in the GM Rats

It is noted that *Cyp27b1*-KO rats are remarkably smaller than other rats. Figure 5B shows the middle region of the femur in 2D μCT scan images. The femur lengths of *Cyp27b1*-KO, *Vdr* (R270L), and *Vdr*-KO rats were found to be remarkably shorter than those of WT rats. The μCT scanning and von Kossa staining of femurs showed hyperplasia of calcified trabecular bones with a narrow medullary cavity in all the *Vdr* (R270L), *Cyp27b1*-KO, and *Vdr*-KO rats (Figure 5B). The *Vdr* (R270L) and *Vdr*-KO rats expressed no clear differences

in total bone mineral density (BMD). In contrast, the BMD of cortical bone in *Cyp27b1*-KO rats was substantially diminished [25].

Histological analysis of the epiphyseal cartilage demonstrated structural disorder of the growth plate in all the *Vdr* (R270L), *Cyp27b1*-KO, and *Vdr*-KO rats. Whereas WT growth plates contained aligned cartilage cells in the layered structure, growth plates in all three GM rats lost the sequential plate structure and cartilage cell alignment (Figure 5). Thus, the morphology of bone was abnormal in all three GM rats, and, in *Cyp27b1*-KO rats, the most significant disorders of bone were observed.

It is well known that rickets type I model *Cyp27b1*-KO mice, and rickets type II model *Vdr*-KO mice, have significantly lower plasma Ca levels than WT mice [75,76]. Expectedly, the plasma Ca level was substantially reduced, and the level of parathyroid hormone (PTH) in plasma was greatly increased in *Vdr* (R270L) rats and *Cyp27b1*-KO rats [25]. Unexpectedly, the plasma Ca level in *Vdr*-KO rats was normal at 15 weeks. In *Vdr*-KO rats, until 10 weeks, the plasma level of Ca was significantly lower than that in WT rats, and PTH level was substantially higher than that in WT rats [25]. Plasma PTH level in *Vdr*-KO rats was remarkably higher than that in WT rats; although, at 15 weeks, the level of Ca in plasma in *Vdr*-KO rats returns to normal. These findings might indicate that hyperparathyroidism occurred in *Vdr*-KO rats [25]. In addition, the putative incomplete formation of intercellular barriers in epithelial tissues, including the small intestine, in VDR-KO rats might cause the increased calcium permeability to result in the normal level of plasma Ca concentration [77].

Although plasma $1\alpha,25(OH)_2D_3$ level was significantly increased in *Vdr* (R270L) and *Vdr*-KO rats, it was significantly decreased in *Cyp27b1*-KO rats (8.0 ± 3.2 pg/mL (mean $\pm$ SEM, n = 7)) compared to WT rats (24.8 ± 5.2 pg/mL, (mean $\pm$ SEM, n = 7)) [25].

4.3. Effects of $25(OH)D_3$ Administration on Cyp27b1- KO Rats

As described previously [76], dietary administration of $25(OH)D_3$ recovered growth failure, skeletal disorders, and hypocalcemia of *Cyp27b1*-KO mice. Dietary administration of $25(OH)D_3$ to *Cyp27b1*-KO rats at 200 $\mu g\cdot kg^{-1}\cdot day^{-1}$ also significantly reversed growth failure [25]. The $25(OH)D_3$ administration normalized BMD of the cortex and trabecular bone of *Cyp27b1*-KO rats. Histological analysis of the femur clearly indicated a normal structure of the cortex and trabecular bone in *Cyp27b1*-KO rats [25]. The growth plate and chondrocytes were also normalized, and the plasma Ca and PTH levels of *Cyp27b1*-KO rats were fully normalized after $25(OH)D_3$ administration [25].

Plasma $1\alpha,25(OH)_2D_3$ level in *Cyp27b1*-KO rats was normalized by $25(OH)D_3$ administration. The 1α-hydroxylation activity toward $25(OH)D_3$ was observed in the liver mitochondrial fraction prepared from *Cyp27b1*-KO rats. It is noted that these results were similar to those obtained in our previous study using *Cyp27b1*-KO mice [76]. Because hepatic Cyp27a1 has a weak 1α-hydroxylation activity toward 25(OH)D3, Cyp27a1 is the most probable candidate to produce $1\alpha,25(OH)_2D_3$ from 25(OH)D3 in *Cyp27b1*-KO rats.

It is noted that $25(OH)D_3$ administration is highly effective in type I rickets model mice and rats. Because human CYP27A1 can convert $25(OH)D_3$ into $1\alpha,25(OH)_2D_3$, similar effects might be expected in humans.

4.4. Effects of $25(OH)D_3$ Administration on Vdr (R270L) Rats

The $25(OH)D_3$ administration also normalized bone disorders with increased cortical BMD of *Vdr* (R270L) rats [25]. The reduced plasma Ca level in *Vdr* (R270L) rats was normalized by $25(OH)D_3$ diet, and the elevated plasma PTH and $1\alpha,25(OH)_2D_3$ levels observed before $25(OH)D_3$ administration were reduced to the normal levels.

The plasma concentration of $25(OH)D_3$ in *Vdr* (R270L) rats fed a $25(OH)D_3$-containing diet was about 500 nM. This concentration was 20 times higher than that in WT rats. It is noted that the affinity of $1\alpha,25(OH)_2D_3$ for *Vdr* (R270L) is nearly the same as that of $25(OH)D_3$. Thus, $25(OH)D_3$ is thought to be a leading ligand of *Vdr* (R270L) in these rats, because plasma $1\alpha,25(OH)_2D_3$ level in the *Vdr* (R270L) rats after the $25(OH)D_3$ treatment

was much lower than that of $25(OH)D_3$ [25]. The remarkably higher levels of $24,25(OH)_2D_3$ and 24-oxo-$25(OH)D_3$ were consistent with the induction of *Cyp24a1* expression, which indicates the "*Vdr* (R270L)-dependent effects of $25(OH)D_3$". The remarkable effects of $25(OH)D_3$ administration on rickets symptoms in *Vdr* (R270L) rats indicate that $25(OH)D_3$ might be efficacious in the treatment of patients with type II rickets caused by the human VDR mutant (R274L).

4.5. Predicted Effects of the Vitamin D Derivative AH-1 towards Patients with Type II Rickets Harboring VDR (R274L) cDNA

As described in the previous sections, AH-1 showed a high binding ability to VDR (R274L) and a high resistance to CYP24A1-dependent metabolism. These results suggest that AH-1 could demonstrate therapeutic effects on type II rickets caused by VDR (R274L). Currently, we administered AH-1 to VDR (R270L) rats, and the expected results have been obtained (data not shown).

4.6. Elucidation of Molecular Mechanism Vitamin D Actions by Comparison among the GM Rats

Various vitamin D actions could be elucidated by comparing physiological conditions, such as bone and skin formation, and multiple serum parameters, such as Ca, P, $25(OH)D_3$, and PTH, in the GM rats generated in this study (Figure 6). Previous studies have showed genomic and nongenomic actions of vitamin D mediated by VDR [78,79], and VDR-independent actions of vitamin D [80]. The VDR-independent effect of $25(OH)D_3$ on lipid metabolism by inducing degradation of SREBP/SCAP was recently reported. In addition, ligand-independent effects of VDR have been reported [81]. Thus, at least five types of effects of vitamin D and/or the VDR should be considered, namely: (1) VDR-dependent effects of $1\alpha,25(OH)_2D_3$ [19,20], (2) VDR-independent effects of $1\alpha,25(OH)_2D_3$ [80], (3) VDR-dependent effects of $25(OH)D_3$ (VDR-$25(OH)D_3$) [82], (4) VDR-independent effects of $25(OH)D_3$ [83], and (5) ligand-independent effects of VDR (Table 1) [81].
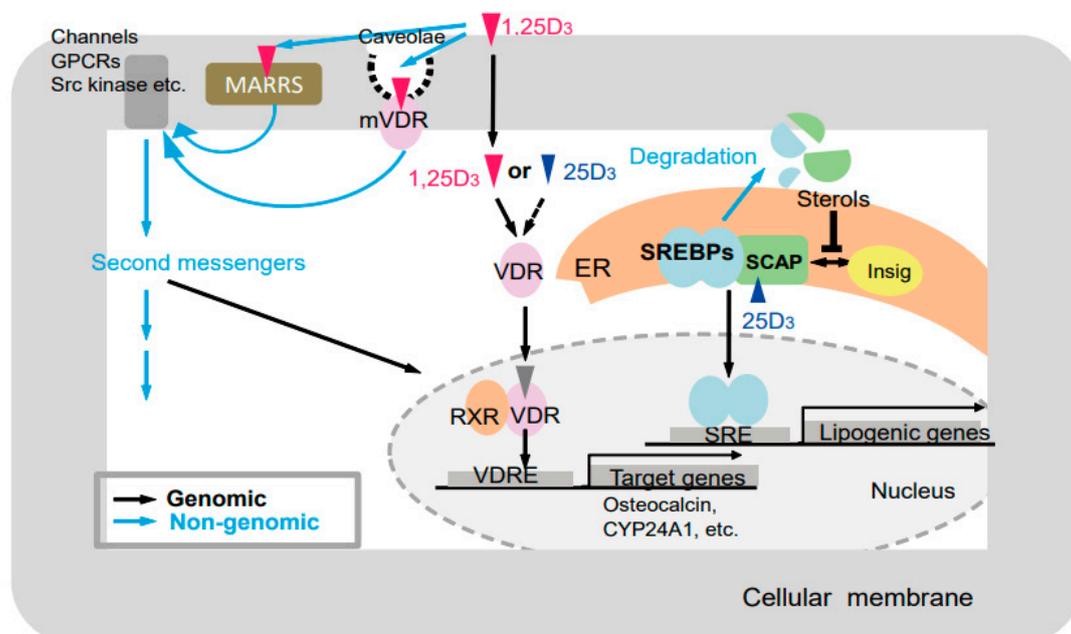
Figure 6. Putative modes of action of vitamin D [25]. Black and blue arrows indicate genomic and nongenomic pathways, respectively. GPCRs, G-protein-coupled receptor; MARRS, (membrane-associated, rapid response steroid-binding) receptor; VDR, vitamin D receptor; mVDR, membrane-bound vitamin D receptor; RXR, retinoid X receptor; VDRE, vitamin D response element; ER, endoplasmic reticulum; SREBPs, sterol regulatory-element–binding proteins; SCAP, SREBP cleavage-activating protein; SRE, sterol regulatory element.

Table 1. Vitamin D and/or VDR actions observed in WT and GM rats [25].

Rat Strain	Mode of Action of Vitamin D				
	(1) Vdr-1,25D3	**(2)** non Vdr-1,25D3	**(3)** Vdr-25D3	**(4)** non Vdr-25D3	**(5)** Vdr-no ligand
WT	+	+	+	+	+
Vdr (R270L)	−	+	+	+	+
Cyp27b1-KO	−	−	+	+	+
Vdr-KO	−	+	−	+	−

Vdr-1,25D3; Vdr-dependent action of: non-Vdr-1,25D3; Vdr-independent action of: Vdr-25D3; Vdr-dependent action of 25(OH)D3: non-Vdr-25D3; Vdr-independent action of 25(OH)D3: Vdr-no ligand; ligand-independent action of Vdr.

Comparison between wild-type and *Vdr* (R270L) rats could reveal (1) VDR-dependent $1\alpha,25(OH)_2D_3$ effects (Table 1). Comparison between *Vdr* (R270L) and *Cyp27b1*-KO rats may reveal (2) VDR-independent effects of $1\alpha,25(OH)_2D_3$. In addition, comparison between *Vdr* (R270L) and *Vdr*-KO rats may reveal (3) VDR-dependent effects of $25(OH)D_3$ or (5) ligand-independent effects of the VDR. Thus, our GM rats appear to be useful for the elucidation of molecular mechanism vitamin D actions and the development of vitamin D derivatives for clinical treatment.

5. Conclusions

The vitamin D derivative evaluation systems we have developed in this study are quite useful. They can readily measure VDR affinity and CYP24A1-mediated metabolism. In addition, the GM rats we have generated by genome editing are highly useful for evaluating the efficacy, safety, and pharmacokinetics of vitamin D derivatives. The reasons rats were used in this study instead of mice include their much larger body size and greater blood volume relative to mice, rendering rats more suitable for pharmacokinetic studies. We hope these evaluation systems will contribute to the near-future development of drugs with excellent therapeutic potential.

References

1. Osborn, J.L.; Schwartz, G.G.; Smith, D.; Bahnson, R.; Day, R.; Trump, D.L. Phase II trial of oral 1,25-dihydroxyvitamin D (calcitriol) in hormone refractory prostate cancer. *Urol. Oncol. Semin. Orig. Investig.* **1995**, *1*, 195–198. [CrossRef]
2. Gross, C.; Stamey, T.; Hancock, S.; Feldman, D. Treatment of early recurrent prostate cancer with 1,25-dihydroxyvitamin D3 (calcitriol). *J. Urol.* **1998**, *15*, 2035–2039. [CrossRef]
3. Binderup, L.; Latini, S.; Bretting, C.; Calverley, M.; Hansen, K. 20-EPI-vitamin D3 analogues: A novel class of potent regulators of cell growth and immune responses. *Biochem. Pharmacol.* **1991**, *42*, 1569–1575. [CrossRef]
4. Bishop, J.E.; Collins, E.D.; Okamura, W.H.; Norman, A.W. Profile of ligand specificity of the vitamin D binding protein for 1α,25-dihydroxyvitamin d3 and its analogs. *J. Bone Miner. Res.* **2009**, *9*, 1277–1288. [CrossRef] [PubMed]
5. Bouillon, R.; Okamura, W.H.; Norman, A.W. Structure-function relationships in the vitamin D endocrine system. *Endocr. Rev.* **1995**, *16*, 200–257. [PubMed]
6. Evans, T.R.; Colston, K.W.; Lofts, F.J.; Cunningham, D.; Anthoney, D.A.; Gogas, H.; de Bono, J.S.; Hamberg, K.J.; Skov, T.; Mansi, J.L. A phase II trial of the vitamin D analogue Seocalcitol (EB1089) in patients with inoperable pancreatic cancer. *Br. J. Cancer* **2002**, *86*, 680–685. [CrossRef] [PubMed]

7. Liu, G.; Oettel, K.; Ripple, G.; Staab, M.J.; Horvath, D.; Alberti, D.; Arzoomanian, R.; Marnocha, R.; Bruskewitz, R.; Mazess, R.; et al. Phase I trial of 1alpha-hydroxyvitamin D(2) in patients with hormone refractory prostate cancer. *Clin. Cancer Res.* **2002**, *8*, 2820–2827. [PubMed]

8. Yamada, S.; Shimizu, M.; Yamamoto, K. Structure-function relationships of vitamin D including ligand recognition by the vitamin D receptor. *Med. Res. Rev.* **2003**, *23*, 89–115. [CrossRef]

9. Masuda, S.; Jones, G. Promise of vitamin D analogues in the treatment of hyperproliferative conditions. *Mol. Cancer Ther.* **2006**, *5*, 797–808. [CrossRef]

10. Lin, R.; Nagai, Y.; Sladek, R.; Bastien, Y.; Ho, J.; Petrecca, K.; Sotiropoulou, G.; Diamandis, E.P.; Hudson, T.J.; White, J.H. Expression profiling in squamous carcinoma cells reveals pleiotropic effects of vitamin D3 analog EB1089 signaling on cell proliferation, differentiation, and immune system regulation. *Mol. Endocrinol.* **2002**, *16*, 1243–1256. [CrossRef]

11. Liu, G.; Wilding, G.; Staab, M.J.; Horvath, D.; Miller, K.; Dresen, A.; Alberti, N.; Arzoomanian, R.; Chappell, R.; Bailey, H.H. Phase II study of 1alpha-hydroxyvitamin D(2) in the treatment of advanced androgen-independent prostate cancer. *Clin. Cancer Res.* **2003**, *9*, 4077–4083.

12. Schwartz, G.G.; Hall, M.C.; Stindt, D.; Patton, S.; Lovato, J.; Torti, F.M. Phase I/II study of 19-nor-1alpha-25-dihydroxyvitamin D2 (paricalcitol) in advanced, androgen-insensitive prostate cancer. *Clin. Cancer Res.* **2005**, *11*, 8680–8685. [CrossRef] [PubMed]

13. Medioni, J.; Deplanque, G.; Ferrero, J.M.; Maurina, T.; Rodier, J.M.; Raymond, E.; Allyon, J.; Maruani, G.; Houillier, P.; Mackenzie, S.; et al. Phase I safety and pharmacodynamic of inecalcitol, a novel VDR agonist with docetaxel in metastatic castration-resistant prostate cancer patients. *Clin. Cancer Res.* **2014**, *20*, 4471–4477. [CrossRef] [PubMed]

14. Studzinski, G.P.; Gocek, E.; Coffman, F.; Danilenko, M. Effects of Vitamin D Derivatives on Differentiation, Cell Cycle, and Apoptosis in Hematological Malignancies. *Vitamin. D (Forth Edition)* **2018**, *2*, 761–799.

15. Mano, H.; Nishikawa, M.; Yasuda, K.; Ikushiro, S.; Saito, N.; Takano, M.; Kittaka, A.; Sakaki, T. Development of Novel Bioluminescent Sensor to Detect and Discriminate between Vitamin D Receptor Agonists and Antagonists in Living Cells. *Bioconjug. Chem.* **2015**, *26*, 2038–2045. [CrossRef] [PubMed]

16. Mano, H.; Nishikawa, M.; Yasuda, K.; Ikushiro, S.; Saito, N.; Sawada, D.; Honzawa, S.; Takano, M.; Kittaka, A.; Sakaki, T. Novel screening system for high-affinity ligand of heredity vitamin D-resistant rickets-associated vitamin D receptor mutant R274L using bioluminescent sensor. *J. Steroid Biochem. Mol. Biol.* **2017**, *167*, 61–66. [CrossRef]

17. Mano, H.; Ikushiro, S.; Saito, N.; Kittaka, A.; Sakaki, T. Development of a highly sensitive in vitro system to detect and discriminate between vitamin D receptor agonists and antagonists based on split-luciferase technique. *J. Steroid Biochem. Mol. Biol.* **2018**, *178*, 55–59. [CrossRef]

18. Mano, H.; Ikushiro, S.; Sakaki, T. Novel split luciferase-based biosensors for evaluation of vitamin D receptor ligands and their application to estimate CYP27B1 activity in living cells. *J. Steroid Biochem. Mol. Biol.* **2018**, *183*, 221–227. [CrossRef]

19. Mano, H.; Takano, M.; Ikushiro, S.; Kittaka, A.; Sakaki, T. Novel biosensor using split-luciferase for detecting vitamin D receptor ligands based on the interaction between vitamin D receptor and coactivator. *Biochem. Biophys. Res. Commun.* **2018**, *505*, 460–465. [CrossRef] [PubMed]

20. Akiyoshi-Shibata, M.; Sakaki, T.; Ohyama, Y.; Noshiro, M.; Okuda, K.; Yabusaki, Y. Further Oxidation of 24,25-Dihydroxyvitamin D_3 by 25-Hydroxyvitamin D_3 24- Hydroxylase Itself. *Eur. J. Biochem.* **1994**, *224*, 335–343. [CrossRef]

21. Sakaki, T.; Sawada, N.; Nonaka, Y.; Ohyama, Y.; Inouye, K. Metabolic studies using recombinant Escherichia coli cells producing rat mitochondrial CYP24: CYP24 can convert 1α,25-dihydroxyvitamin D3 to calcitroic acid. *JBIC J. Biol. Inorg. Chem.* **1999**, *262*, 43–48. [CrossRef]

22. Sakaki, T.; Sawada, N.; Komai, K.; Shiozawa, S.; Yamada, S.; Yamamoto, K.; Ohyama, Y.; Inouye, K. Dual metabolic pathway of 25-hydroxyvitamin D_3 catalyzed by human CYP24. *Eur. J. Biochem.* **2000**, *267*, 6158–6165. [CrossRef]

23. Sawada, N.; Kusudo, T.; Sakaki, T.; Hatakeyama, S.; Hanada, M.; Abe, D.; Kamao, M.; Okano, T.; Ohta, M.; Inouye, K. Novel metabolism of 1α,25-dihydroxyvitamin D_3 with C_{24}–C_{25} bond cleavage catalyzed by human CYP24A1. *Biochemistry* **2004**, *43*, 4530–4537. [CrossRef]

24. Hamamoto, H.; Kusudo, T.; Urushino, N.; Masuno, H.; Yamamoto, K.; Yamada, S.; Kamakura, M.; Ohta, M.; Inouye, K.; Sakaki, T. Structure-function analysis of vitamin D 24-hydroxylase (CYP24A1) by site-directed mutagenesis: Amino acid resides responsible for species-based difference of CYP24A1 between humans and rats. *Mol. Pharmacol.* **2006**, *70*, 120–128. [CrossRef] [PubMed]

25. Nishikawa, M.; Yasuda, K.; Takamatsu, M.; Abe, K.; Okamoto, K.; Horibe, K.; Mano, H.; Nakagawa, K.; Tsugawa, N.; Hirota, Y.; et al. Generation of novel genetically modified rats to reveal the molecular mechanisms of vitamin D actions. *Sci. Rep.* **2020**, *10*, 5677. [CrossRef]

26. Yasuda, K.; Nishikawa, M.; Okamoto, K.; Horibe, K.; Mano, H.; Yamaguchi, M.; Okon, R.; Nakagawa, K.; Tsugawa Okano, T.; Kawagoe, F.; et al. Elucidation of metabolic pathways of 25-hydroxyvitamin D3 mediated by CYP24A1 and Cyp3A using *Cyp24a1* knockout rats generated by CRISPR/Cas9 System. *J. Biol. Chem.* **2021**, *296*, 100668. [CrossRef] [PubMed]

27. Nakabayashi, M.; Tsukahara, Y.; Iwasaki-Miyamoto, Y.; MihoriShimazaki, M.; Yamada, S.; Inaba, S.; Oda, M.; Shimizu, M.; Makishima, M.; Tokiwa, H.; et al. Crystal structures of hereditary vitamin D-resistant rickets-associated vitamin D receptor mutants R270L and W282R bound to 1,25-dihydroxyvitamin D3 and synthetic ligands. *J. Med. Chem.* **2013**, *56*, 6745–6760. [CrossRef] [PubMed]

28. Tashiro, K.; Ishii, C.; Ryoji, M. Role of distal upstream sequence in vitamin D-induced expression of human CYP24 gene. *Biochem. Biophys. Res. Commun.* **2007**, *358*, 259–265. [CrossRef] [PubMed]

29. Yamamoto, K.; Masuno, H.; Choi, M.; Nakashima, K.; Taga, T.; Ooizumi, H.; Umesono, K.; Sicinska, W.; VanHooke, J.; DeLuca, H.F.; et al. Three-dimensional modeling of and ligand docking to vitamin D receptor ligand binding domain. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 1467–1472. [CrossRef]

30. Kim, H.K.; Cho, E.J.; Jo, S.m.; Sung, B.R.; Lee, S.; Yun, S.H. A split luciferase complementation assay for studying in vivo protein-protein interactions in filamentous ascomycetes. *Curr. Genet.* **2012**, *58*, 179–189. [CrossRef] [PubMed]

31. Azad, T.; Tashakor, A.; Hosseinkhani, S. Split-luciferase complementary assay: Applications, recent developments, and future perspectives. *Anal. Bioanal. Chem.* **2014**, *406*, 5541–5560. [CrossRef]

32. Shavkunov, A.S.; Ali, S.R.; Panova-Elektronova, N.I.; Laezza, F. Split-luciferase complementation assay to detect channel-protein interactions in live cells. *Methods Mol. Biol.* **2015**, *1278*, 497–514. [PubMed]

33. Varnum, M.M.; Clayton, K.A.; Yoshii-Kitahara, A.; Yonemoto, G.; Koro, L.; Ikezu, S.; Ikezu, T. A split-luciferase complementation, real-time reporting assay enables monitoring of the disease-associated transmembrane protein TREM2 in live cells. *J. Biol. Chem.* **2017**, *292*, 10651–10663. [CrossRef] [PubMed]

34. Moustaqil, M.; Bhumkar, A.; Gonzalez, L.; Raoul, L.; Hunter, D.J.B.; Carrive, P.; Sierecki, E.; Gambin, Y. A Split-Luciferase Reporter Recognizing GFP and mCherry Tags to Facilitate Studies of Protein-Protein Interactions. *Int. J. Mol. Sci.* **2017**, *18*, 2681. [CrossRef]

35. Forster, L.; Grätz, L.; Mönnich, D.; Bernhardt, G.; Pockes, S. A Split Luciferase Complementation Assay for the Quantification of beta-Arrestin2 Recruitment to Dopamine D(2)-Like Receptors. *Int. J. Mol. Sci.* **2020**, *21*, 6103. [CrossRef] [PubMed]

36. Spillmann, M.; Thurner, L.; Romantini, N.; Zimmermann, M.; Meger, B.; Behe, M.; Waldhoer, M.; Schertler, G.F.X.; Berger, P. New Insights into Arrestin Recruitment to GPCRs. *Int. J. Mol. Sci.* **2020**, *21*, 4949. [CrossRef] [PubMed]

37. Li, Y.C.; Lytle, N.K.; Gammon, S.T.; Wang, L.; Hayes, T.K.; Sutton, M.N.; Bast, R.C., Jr.; Der, C.J.; Piwnica-Worms, D.; McCormick, F.; et al. Analysis of RAS protein interactions in living cells reveals a mechanism for pan-RAS depletion by membrane-targeted RAS binders. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 12121–12130. [CrossRef] [PubMed]

38. Malloy, P.J.; Pike, J.W.; Feldman, D. The vitamin D receptor and the syndrome of hereditary 1,25-dihydroxyvitamin D-resistant rickets. *Endocr. Rev.* **1999**, *20*, 156–188. [PubMed]

39. Ohyama, Y.; Noshiro, M.; Okuda, K. Cloning and expression of cDNA encoding 25-hydroxyvitamin D3 24-hydroxylase. *FEBS Lett.* **1991**, *278*, 195–198. [CrossRef]

40. Ohyama, Y.; Ozono, K.; Uchida, M.; Shinki, T.; Kato, S.; Suda, T.; Yamamoto, O.; Noshiro, M.; Kato, Y. Identification of a vitamin D-responsive element in the 5′-flanking region of the rat 25-hydroxyvitamin D3 24-hydroxylase gene. *J. Biol. Chem.* **1994**, *269*, 10545–10550. [CrossRef]

41. Zierold, C.; Mings, J.A.; DeLuca, H.F. Parathyroid hormone regulates 25-hydroxyvitamin D3-24-hydroxylase mRNA by altering its stability. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 13572–13576. [CrossRef] [PubMed]

42. Dhawan, P.; Peng, X.; Sutton, A.L.; MacDonald, P.N.; Croniger, C.M.; Trautwein, C.; Centrella, M.; McCarthy, T.L.; Christakos, S. Functional cooperation between CCAAT/enhancer-binding proteins and the vitamin D receptor in regulation of 25-hydroxyvitamin D3 24-hydroxylase. *Mol. Cell Biol.* **2005**, *25*, 472–487. [CrossRef] [PubMed]

43. Meyer, M.B.; Goetsch, P.D.; Pike, J.W. A downstream intergenic cluster of regulatory enhancers contributes to the induction of CYP24A1 expression by 1α,25-dihydroxyvitamin D3. *J. Biol. Chem.* **2010**, *285*, 15599–15610. [CrossRef] [PubMed]

44. Meyer, M.B.; Lee, S.M.; Carlson, A.H.; Benkusky, N.A.; Kaufmann, M.; Jones, G.; Pike, J.W. A chromatin-based mechanism controls differential regulation of the cytochrome P450 gene Cyp24a1 in renal and non-renal tissues. *J. Biol. Chem.* **2019**, *294*, 14467–14481. [CrossRef]

45. Sakaki, T.; Sawada, N.; Abe, D.; Komai, K.; Shiozawa, S.; Nonaka, Y.; Nakagawa, K.; Okano, T.; Ohta, M.; Inouye, K. Metabolism of 26,26,26,27,27,27-F6-1α,25-dihydroxyvitamin D3 by CYP24: Species-based difference between humans and rats. *Biochem. Pharmacol.* **2003**, *65*, 1957–1965. [CrossRef]

46. Kusudo, T.; Sakaki, T.; Abe, D.; Fujishima, T.; Kittaka, A.; Takayama, H.; Ohta, M.; Inouye, K. Metabolism of 20-epimer of 1α,25-dihydroxyvitamin D3 by CYP24: Species-based difference between humans and rats. *Biochem. Biophys. Res. Commun.* **2003**, *309*, 885–892. [CrossRef]

47. Kusudo, T.; Sakaki, T.; Abe, D.; Fujishima, T.; Kittaka, A.; Takayama, H.; Hatakeyama, S.; Ohta, M.; Inouye, K. Metabolism of A-ring diastereomers of 1α,25-dihydroxyvitamin D$_3$ by CYP24A1. *Biochem. Biophys. Res. Commun.* **2004**, *321*, 774–782. [CrossRef] [PubMed]

48. Abe, D.; Sakaki, T.; Kusudo, T.; Kittaka, A.; Saito, N.; Suhara, Y.; Fujishima, T.; Takayama, H.; Hamamoto, H.; Kamakura, M.; et al. Metabolism of 2α-propoxy-1α,25-dihydroxyvitamin D$_3$ and 2α-(3-hydroxypropoxy)- 1α,25-dihydroxyvitamin D$_3$ by human CYP27A1 and CYP24A1. *Drug Metab. Dispos.* **2005**, *33*, 778–784. [CrossRef]

49. Urushino, N.; Nakabayashi, S.; Arai, M.A.; Kittaka, A.; Chen, T.C.; Yamamoto, K.; Hayashi, K.; Kato, S.; Ohta, M.; Kamakura, M.; et al. Kinetic Studies of 25-Hydroxy-19-Nor-Vitamin D$_3$ and 1α,25-Dihydroxy-19-Nor-Vitamin D$_3$ Hydroxylation by CYP27B1 and CYP24A1. *Drug Metab. Dispos.* **2007**, *35*, 1482–1488. [CrossRef]

50. Saito, N.; Suhara, Y.; Abe, D.; Kusudo, T.; Ohta, M.; Yasuda, K.; Sakaki, T.; Honzawa, S.; Fujishima, T.; Kittaka, A. Synthesis of 2α-propoxy-1α,25-dihydroxyvitamin D3 and comparison of its metabolism by human CYP24A1 and rat CYP24A1. *Bioorg. Med. Chem.* **2009**, *17*, 4296–4301. [CrossRef]

51. Sawada, D.; Tsukuda, Y.; Yasuda, K.; Sakaki, T.; Saito, H.; Takagi, K.-I.; Takenouchi, K.; Chen, T.; Reddy, G.S.; Kittaka, A. Synthesis and Biological Activities of 1α,4α,25- and 1α,4β,25-Trihydroxyvitamin D3 and Their Metabolism by Human CYP24A1 and UDP-Glucuronosyltransferase. *Chem. Pharm. Bull.* **2012**, *60*, 1343–1346. [CrossRef]

52. Yasuda, K.; Ikushiro, S.; Kamakura, M.; Takano, M.; Saito, N.; Kittaka, A.; Chen, T.; Ohta, M.; Sakaki, T. Human cytochrome P450-dependent differential metabolism among three 2α-substituted-1α,25-dihydroxyvitamin D3 analogs. *J. Steroid Biochem. Mol. Biol.* **2013**, *133*, 84–92. [CrossRef]

53. Yasuda, K.; Iwanaga, Y.; Ogawa, K.; Mano, H.; Ueno, S.; Kimoto, S.; Ohta, M.; Kamakura, M.; Ikushiro, S.; Sakaki, T. Human hepatic metabolism of the anti-osteoporosis drug eldecalcitol (ED-71) involves sterol C4-methyl oxidase. *Pharmacol. Res. Prospect.* **2015**, *3*, e00120.

54. Takano, M.; Yasuda, K.; Higuchi, E.; Tohyama, E.; Takeuchi, A.; Sakaki, T.; Kittaka, A. Synthesis, metabolism, and biological activity of 2-[3-(tetrazolyl) propyl]-1α,25-dihydroxy-19-norvitamin D3. *J. Steroid Biochem. Mol. Biol.* **2016**, *164*, 40–44. [CrossRef] [PubMed]

55. Kawagoe, F.; Sugiyama, T.; Yasuda, K.; Uesugi, M.; Sakaki, T.; Kittaka, A. Concise synthesis of 23-hydroxylated vitamin D3 metabolites. *J. Steroid Biochem. Mol. Biol.* **2018**, *186*, 161–168. [CrossRef] [PubMed]

56. Kawagoe, F.; Yasuda, K.; Mototani, S.; Sugiyama, T.; Uesugi, M.; Sakaki, T.; Kittaka, A. Synthesis and CYP24A1-Dependent Metabolism of 23-Fluorinated Vitamin D3 Analogues. *ACS Omega* **2019**, *4*, 11332–11337. [CrossRef] [PubMed]

57. Kawagoe, F.; Mototani, S.; Yasuda, K.; Nagasawa, K.; Uesugi, M.; Sakaki, T.; Kittaka, A. Introduction of Fluorine Atoms to Vitamin D3 Side-chain and Synthesis of 24,24- Difluoro-25-hydroxyvitamin D3. *J. Steroid Biochem. Mol. Biol.* **2019**, *195*, 105477. [CrossRef]

58. Milczarek, M.; Chodyński, M.; Pietraszek, A.; Stachowicz-Suhs, M.; Yasuda, K.; Sakaki, T.; Wietrzyk, J.; Kutner, A. Synthesis, CYP24A1-Dependent Metabolism and Antiproliferative Potential against Colorectal Cancer Cells of 1,25-Dihydroxyvitamin D2 Derivatives Modified at the Side Chain and the A-Ring. *Int. J. Mol. Sci.* **2020**, *21*, 642. [CrossRef]

59. Prosser, D.E.; Kaufmann, M.; O'Leary, B.; Byford, V.; Jones, G. Single A326G mutation converts human CYP24A1 from 25-OH-D3-24-hydroxylase into -23-hydroxylase, generating 1,25-(OH)2D3-26,23-lactone. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 12673–12678. [CrossRef]

60. Ohyama, Y.; Ozono, K.; Uchida, M.; Yoshimura, M.; Shinki, T.; Suda, T.; Yamamoto, O. Functional assessment of two vitamin D-responsive elements in the rat 25-hydroxyvitamin D3 24-hydroxylase gene. *J. Biol. Chem.* **1996**, *271*, 30381–30385. [CrossRef]

61. Shankar, V.N.; Dilworth, F.J.; Makin, H.L.; Schroeder, N.J.; Trafford, D.J.; Kissmeyer, A.M.; Calverley, M.J.; Binderup, E.; Jones, G. Metabolism of the vitamin D analog EB1089 by cultured human cells: Redirection of hydroxylation site to distal carbons of the side-chain. *Biochem. Pharmacol.* **1997**, *53*, 783–793. [CrossRef]

62. Lechner, D.; Manhardt, T.; Bajna, E.; Posner, G.H.; Cross, H.S. A 24-phenylsulfone analog of vitamin D inhibits 1alpha,25-dihydroxyvitamin D(3) degradation in vitamin D metabolism-competent cells. *J. Pharmacol. Exp. Ther.* **2007**, *320*, 1119–1126. [CrossRef] [PubMed]

63. Schuster, I.; Egger, H.; Astecker, N.; Herzig, G.; Schüssler, M.; Vorisek, G. Selective inhibitors of CYP24: Mechanistic tools to explore vitamin D metabolism in human keratinocytes. *Steroids* **2001**, *66*, 451–462. [CrossRef]

64. Kittaka, A.; Suhara, Y.; Takayanagi, H.; Fujishima, T.; Kurihara, M.; Takayama, H. A concise and efficient route to 2alpha-(omega-hydroxyalkoxy)-1alpha,25-dihydroxy vitamin D3: Remarkably high affinity to vitamin D receptor. *Org. Lett.* **2000**, *2*, 2619–2622. [CrossRef] [PubMed]

65. Suhara, Y.; Nihei, K.I.; Tanigawa, H.; Fujishima, T.; Konno, K.; Nakagawa, K.; Okano, T.; Takayama, H. Syntheses and biological evaluation of novel 2alpha-substituted 1alpha,25-dihydroxyvitamin D3 analogues. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1129–1132. [CrossRef]

66. Suhara, Y.; Nihei, K.I.; Kurihara, M.; Kittaka, A.; Yamaguchi, K.; Fujishima, T.; Konno, K.; Miyata, N.; Takayama, H. Efficient and versatile synthesis of novel 2alpha-substituted 1alpha,25-dihydroxyvitamin D(3) analogues and their docking to vitamin D receptors. *J. Org. Chem.* **2001**, *66*, 8760–8771. [CrossRef] [PubMed]

67. Saito, N.; Suhara, Y.; Kurihara, M.; Fujishima, T.; Honzawa, S.; Takayanagi, H.; Kozono, T.; Matsumoto, M.; Ohmori, M.; Miyata, N.; et al. Design and efficient synthesis of 2 alpha-(omega-hydroxyalkoxy)-1 alpha,25-dihydroxyvitamin D3 Analogues, including 2-epi-ED-71 and their 20-epimers with HL-60 cell differentiation activity. *J. Org. Chem.* **2004**, *69*, 7463–7471. [CrossRef] [PubMed]

68. Takahashi, E.; Nakagawa, K.; Suhara, Y.; Kittaka, A.; Nihei, K.; Konno, K.; Takayama, H.; Ozono, K.; Okano, T. Biological activities of 2alpha-substituted analogues of 1alpha,25-dihydroxyvitamin D3 in transcriptional regulation and human promyelocytic leukemia (HL-60) cell proliferation and differentiation. *Biol. Pharm. Bull.* **2006**, *29*, 2246–2250. [CrossRef]

69. Ono, K.; Yoshida, A.; Saito, N.; Fujishima, T.; Honzawa, S.; Suhara, Y.; Kishimoto, S.; Sugiura, T.; Waku, K.; Takayama, H.; et al. Efficient synthesis of 2-modified 1alpha,25-dihydroxy-19-norvitamin D3 with Julia olefination: High potency in induction of differentiation on HL-60 cells. *J. Org. Chem.* **2003**, *68*, 7407–7415. [CrossRef]

70. Arai, M.A.; Takeyama, K.; Ito, S.; Kato, S.; Chen, T.C.; Kittaka, A. High-throughput system for analyzing ligand-induced cofactor recruitment by vitamin D receptor. *Bioconjug. Chem.* **2007**, *18*, 614–620. [CrossRef]

71. Chen, T.; Persons, K.; Zheng, S.; Mathieu, J.; Holick, M.; Lee, Y.; Bao, B.; Arai, M.; Kittaka, A. Evaluation of C-2-substituted 19-nor-1α,25-dihydroxyvitamin D3 analogs as therapeutic agents for prostate cancer. *J. Steroid Biochem. Mol. Biol.* **2007**, *103*, 717–720. [CrossRef]

72. Flanagan, J.N.; Zheng, S.; Chiang, K.C.; Kittaka, A.; Sakaki, T.; Nakabayashi, S.; Zhao, X.; Spanjaard, R.A.; Persons, K.S.; Mathieu, J.S.; et al. Evaluation of 19-nor-2alpha-(3-hydroxypropyl)-1alpha,25- dihydroxyvitamin D3 as a therapeutic agent for androgen-dependent prostate cancer. *Anticancer Res.* **2009**, *29*, 3547–3553.

73. Chen, T.C.; Kittaka, A. Novel vitamin d analogs for prostate cancer therapy. *Int. Sch. Res. Not.* **2011**, *2011*, 301490. [CrossRef]

74. Chiang, K.C.; Yeh, C.N.; Chen, H.Y.; Lee, J.M.; Juang, H.H.; Chen, M.F.; Takano, M.; Kittaka, A.; Chen, T.C. 19-Nor-2α-(3-hydroxypropyl)-1α,25- dihydroxyvitamin D3 (MART-10) is a potent cell growth regulator with enhanced chemotherapeutic potency in liver cancer cells. *Steroids* **2011**, *76*, 1513–1519. [CrossRef] [PubMed]

75. Hirota, Y.; Nakagawa, K.; Mimatsu, S.; Sawada, N.; Sakaki, T.; Kubodera, N.; Kamao, M.; Tsugawa, N.; Suhara, Y.; Okano, T. Nongenomic effects of 1α,25-dihydroxyvitamin D3 on cartilage formation deduced from comparisons between Cyp27b1 and Vdr knockout mice. *Biochem. Biophys. Res. Commun.* **2017**, *483*, 359–365. [CrossRef]

76. Nishikawa, M.; Kaori Yasuda, K.; Takamatsu, M.; Abe, K.; Nakagawa, K.; Tsugawa, N.; Hirota, Y.; Tanaka, K.; Yamashita, S.; Ikushiro, S.; et al. Generation of 1,25-dihydroxyvitamin D$_3$ in *Cyp27b1* knockout mice by treatment with 25-hydroxyvitamin D$_3$ rescued their rachitic phenotypes. *J. Steroid Biochem. Mol. Biol.* **2019**, *185*, 71–79. [CrossRef] [PubMed]

77. Zhang, Y.G.; Wu, S.; Lu, R.; Zhou, D.; Zhou, J.; Carmeliet, G.; Petrof, E.; Claud, E.C.; Sun, J. Tight junction CLDN2 gene is a direct target of the vitamin D receptor. *Sci. Rep.* **2015**, *5*, 10642. [CrossRef]

78. Norman, A.W.; Bishop, J.E.; Collins, E.D.; Seo, E.G.; Satchell, D.P.; Dormanen, M.C.; Zanello, S.B.; Farach-Carson, M.C.; Bouillon, R.; Okamura, W.H. Differing shapes of 1 alpha,25-dihydroxyvitamin D3 function as ligands for the D-binding protein, nuclear receptor and membrane receptor: A status report. *J. Steroid Biochem. Mol. Biol.* **1996**, *56*, 13–22. [CrossRef]

79. Mizwicki, M.T.; Norman, A.W. The vitamin D sterol-vitamin D receptor ensemble model offers unique insights into both genomic and rapid-response signaling. *Sci. Signal.* **2009**, *2*, re4. [CrossRef]

80. Hii, C.S.; Ferrante, A. The Non-Genomic Actions of Vitamin D. *Nutrients* **2016**, *8*, 135. [CrossRef] [PubMed]

81. Skorija, K.; Cox, M.; Sisk, J.M.; Dowd, D.R.; MacDonald, P.N.; Thompson, C.C.; Demay, M.B. Ligand-independent actions of the vitamin D receptor maintain hair follicle homeostasis. *Mol. Endocrinol.* **2005**, *19*, 855–862. [CrossRef] [PubMed]

82. Munetsuna, E.; Kawanami, R.; Nishikawa, M.; Ikeda, S.; Nakabayashi, S.; Ohta, M.; Kamakura, M.; Ikushiro, S.; Sakaki, T. Anti-proliferative activity of 25-hydroxyvitamin D3 in human prostate cells. *Mol. Cell Endocrinol.* **2014**, *382*, 960–970. [CrossRef] [PubMed]

83. Asano, L.; Watanabe, M.; Ryoden, Y.; Usuda, K.; Yamaguchi, T.; Khambu, B.; Takashima, M.; Sato, S.I.; Sakai, J.; Nagasawa, K.; et al. Vitamin D Metabolite, 25-Hydroxyvitamin D, Regulates Lipid Metabolism by Inducing Degradation of SREBP/SCAP. *Cell Chem. Biol.* **2017**, *24*, 207–217. [CrossRef] [PubMed]