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Further Developments of the Phenyl-Pyrrolyl Pentane Series of Non-steroidal Vitamin D Receptor Modulators as Anticancer Agents

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ABSTRACT

Vitamin D_3 receptor (VDR), belonging to the nuclear receptor superfamily, is a potential molecular target for anticancer drug discovery. In this study, a series of non-steroidal vitamin D mimics with a phenyl-pyrrolyl pentane skeleton were synthesized with therapeutic potential for cancer treatment. Among them, **11b** and **11g** were identified as the most effective agents in reducing the viability of four cancer cell lines, particularly breast cancer cells, with IC_{50} values in the submicromolar concentration range. In addition, **11b** and **11g** possessed VDR binding affinity and displayed significant partial VDR agonistic activity determined by dual luciferase reporter assay and human leukemia cell line (HL-60 cells) differentiation assay. Furthermore, **11b** and **11g** inhibited tumor growth in the orthotopic breast tumor model due to the inhibition of cell proliferation and induction of cell apoptosis. More importantly, **11b** and **11g**, exhibiting favorable pharmacokinetic behavior *in vivo*, did not increase serum calcium levels or cause any other apparent side effects. In summary, **11b** and **11g** act as novel VDR modulators and may be promising candidates for cancer chemotherapy.

Keywords: vitamin D_3 receptor (VDR) modulator, non-steroidal vitamin D analog, phenyl-pyrrolyl pentane skeleton, antitumor agent, calcitriol

INTRODUCTION

The active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 (calcitriol, 1,25-$(OH)_2D_3$), regulates multiple signaling pathways through binding to its intracellular receptor, vitamin D_3 receptor (VDR), which belongs to the nuclear receptor superfamily of steroid hormone. Calcitriol and its synthetic analogs play important roles in calcium homeostasis, bone mineralization and immune regulation.[1-3] However, over the past two decades it has become clear that calcitriol and its analogs might have therapeutic potential as anticancer agents by modulating proliferation, apoptosis and differentiation of a variety of cancer cell types through the VDR signaling pathway.[4, 5]

Preclinical research indicates that vitamin D deficiency raises the risk of developing cancers, such as colon cancer,[6, 7] breast cancer,[8, 9] and prostate cancer[10, 11] while dietary calcitriol and its analogs result in a significant inhibition of tumor growth and eventual tumor burden.[12,13] Additionally, *Vdr*-null mice are more sensitive to carcinogen-induced tumorigenesis.[14] More importantly, clinical studies further support the development of vitamin D modulators as preventative and therapeutic anticancer agents. Garland, et al. discovered that the exposure to sufficient sunlight could induce enough vitamin D_3 and reduce the mortality risk of patients with colon cancer.[15] Lappe, et al. also discovered that improving calcium and vitamin D nutritional status substantially reduces cancer risk in postmenopausal women.[16] Moreover, seocalcitol, a vitamin D_3 analog, displayed antitumour activity in the treatment of the hepatocellular carcinoma (HCC) in the phase II trial study.[17] Combination therapy with calcitriol and other anticancer agents improved survival of prostate

cancer patients in the phase II trials.[18, 19] Therefore, it is essential to develop vitamin D modulators as preventative and therapeutic anticancer agents.

During the last 30 years, a large number of vitamin D analogs have been synthesized by introducing different types of chemical modifications to calcitriol, such as maxacalcitol, calcipotriol, seocalcitol and lexicalcitol, which were used for the treatment of cancer, osteoporosis and skin disorders.[20, 21] However, most of these classic VDR modulators with a secosteroidal structure can lead to hypercalcemia or hypercalciuria which impedes their application for long-term cancer therapy and the translation of basic and clinical research to therapeutic agents. It was reported that calcitriol was given at a high dose (45 μg) once a week in combination with docetaxel for prostate cancer treatment was terminated in the phase III trial. Part of the reason was the side effects of hypercalcaemia along with myocardial injury, nervous system damage and renal stone resulting in lower survival in the treatment group compared with the placebo group.[22] Moreover, another clinical trial revealed that calcitriol in daily oral doses of more than 2-2.5 μg led to hypercalcaemia in men with recurrent prostate cancer, which limited the ability to raise the administered dose.[23] Hence, there is an urgent clinical need of designing a new generation of VDR modulators without the risk of increasing serum calcium for cancer chemotherapy.

As early as 1999, Boehm et al. reported the first generation of non-steroidal vitamin D modulators which exhibited no hypercalcemic potential *in vivo*. Among them, LG190119 (**Figure 1A**) effectively inhibited LNCaP xenograft tumor growth without increased serum calcium levels or any other apparent side effects.[24] Based on this, various groups had reported their non-steroidal derivatives with different structures.[25, 26] Non-steroidal vitamin D mimics

based on a hydrophobic core of p-carborane have been developed by Fujii, S. et al.[27] Furthermore, other non-steroidal derivatives such as bis and tris-aromatic compounds,[28] carboxylic acid and hydroxamic acid derivatives,[29] hydroxamic acid derivatives[30] have been reported afterwards.

Recently, a series of non-steroidal VDR derivatives with phenyl-pyrrolyl pentane skeleton were designed and synthesized by our group. Among them, sw22 **(Figure 1B)** significantly inhibited the proliferation of human breast adenocarcinoma cells (MCF7 cells, IC_{50} = 320 nM).[31] Based on the study, the pyrrolyl side chains were modified to improve the anti-proliferative activity, and the results showed that designed compounds exhibited promising anti-proliferative activity *in vitro*.[32]

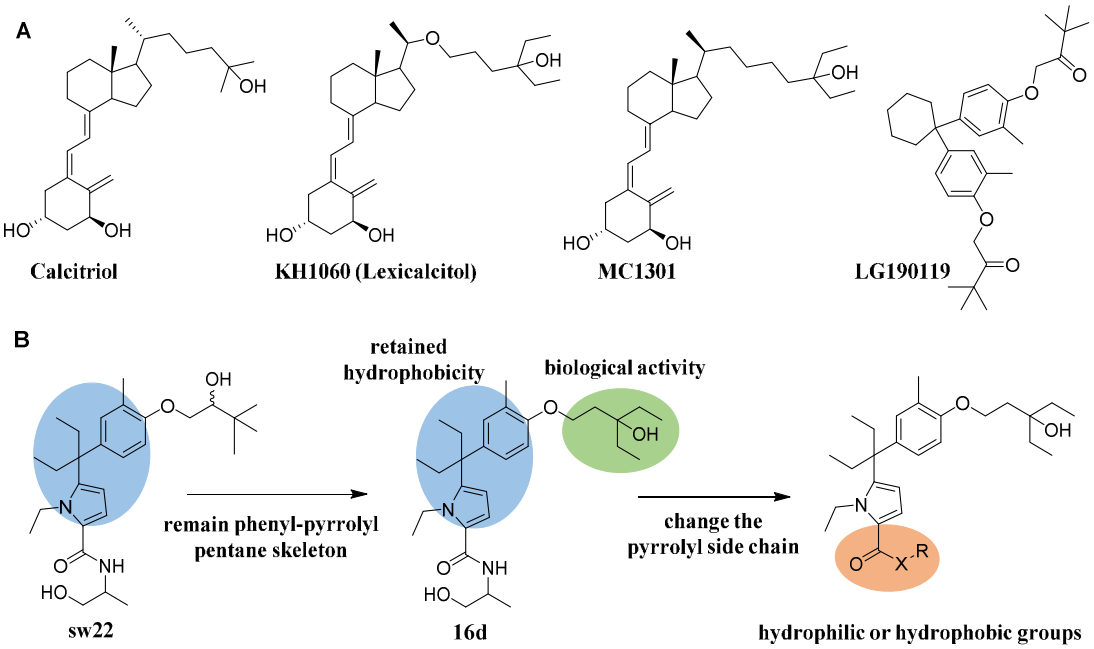


Figure 1. The designed VDR modulators. (A) The structure of calcitriol, lexicalcitol, MC1301 and LG190119. (B) The design of non-steroidal VDR modulators with phenyl-pyrrolyl pentane skeleton.

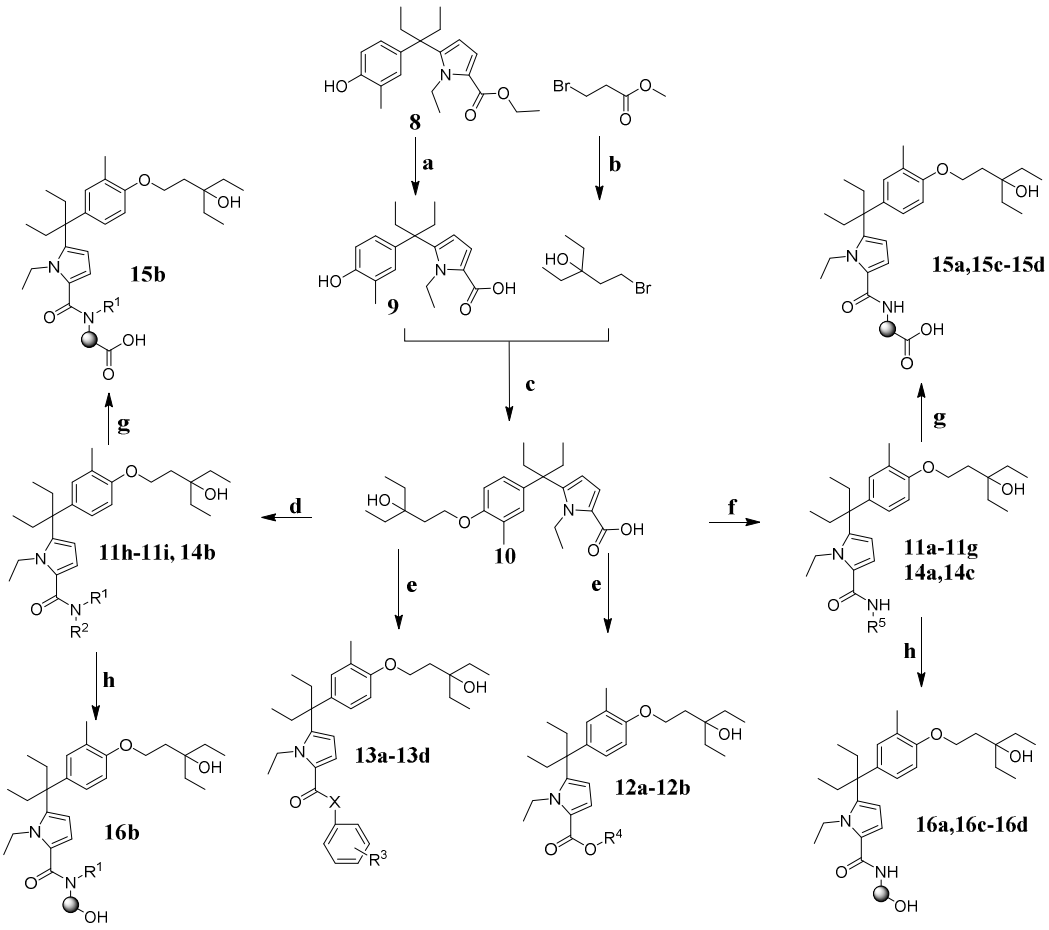
In this study, to further improve the anti-proliferative effect against cancer cells and

pharmacokinetic properties *in vivo*, twenty-six novel vitamin D analogs with phenyl-pyrrolyl pentane skeleton have been designed, synthesized and evaluated their bioactivity in vitro and in vivo. First, we retained the phenyl-pyrrolyl pentane skeleton, which could form strong hydrophobic interactions with the hydrophobic pocket of VDR.[32] Next, we introduced the diethylcarbinol as the terminal hydrophobic group in the phenyl side chain to improve biological activities, as similar modifications have been reported in both secosteroidal and non-steroidal vitamin D analogs.[27, 33, 34] Previous report showed that lexicalcitol (**Figure 1A**) possessing the diethylcarbinol moiety in the side chain is at least 100-fold more effective in the biological activity than calcitriol.[35] Moreover, the compound MC1301 with diethylcarbinol moiety also presented improved differentiation-inducing activity (**Figure 1A**) compared to calcitriol.[35] Therefore, we tried to improve the biological activities by introducing the diethylcarbinol into the benzene ring as the side chain. Then, hydrophilic groups or hydrophobic groups were added in the pyrrolyl side chain to explore the effects of different substitutions on the pyrrole ring. Finally, we investigated whether both the biological activities and the metabolism were enhanced on the basis of the above design and considerations (**Figure 1B**). Here, we describe the sythesis and biological activities of novel non-steroidal VDR modulaters with phenyl-pyrrolyl pentane skeleton and demonstrate their promising therapeutic potential for the treatment of breast cancer and other cancers without hypercalcemia intervenes.

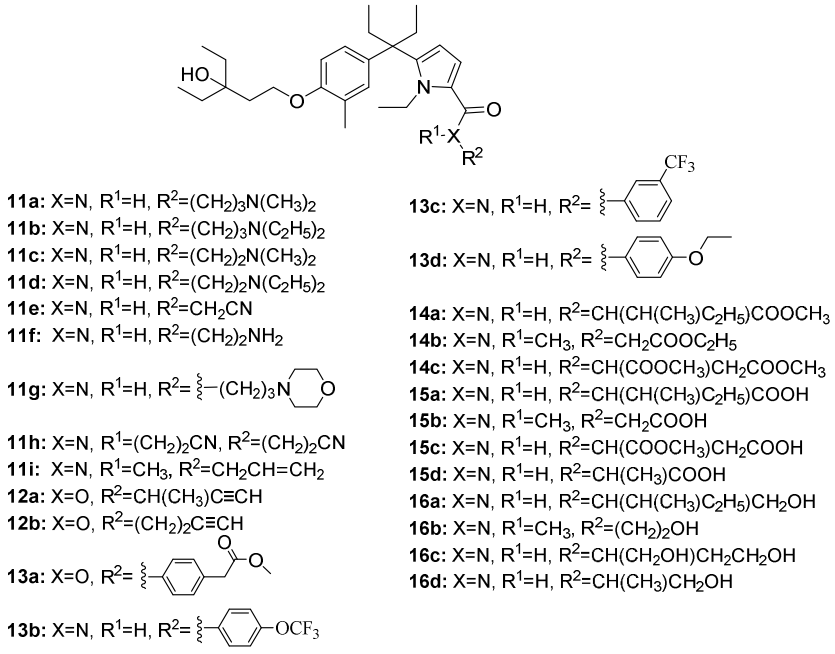
RESULTS

Synthetic Procedures of Target Compounds. The synthetic route of non-steroidal

vitamin D analogs was summarized in **Scheme 1**. Among these compounds, the key intermediate **8** was readily prepared as previously reported by our group.[31, 32] The intermediate **8** underwent potassium hydroxide (KOH) hydrolysis to give intermediate **9** containing the carboxyl group. The hydroxyl of **9** was directly reacted with 1-bromo-3-ethyl-pentan-3-ol to obtain key intermediate **10**. The target compounds **11a-11i**, **12a-12b**, **13a-13d** and **14a-14c** were prepared in a single step by being coupled with intermediate **10** through different amines, alcohols, amino acids methyl ester hydrochloride, aryl amines or esters. Compounds **15a-15d** were synthesized through hydrolysis reaction. The amino acid or ester compounds were reduced to obtain compounds **16a-16d** by sodium borohydride in methanol.



Scheme 1. Synthesis of phenyl-pyrrolyl pentane series of non-steroidal VDR derivatives. Reagents and reaction conditions: (a) KOH, C_2H_5OH, 70 °C, overnight; (b) EtMgBr, Et_2O, 30 °C, 2 hours; (c) NaH, DMF, r.t, 2.5 hours; (d) Amines/Amino acids methyl ester hydrochloride, 4-nitrobenzenesulfonyl chloride, DMAP, CH_3CN, 70 °C, overnight; (e) Aryl amines or esters/Alcohols, EDCI, DMAP, $CHCl_3$, 70 °C, overnight; (f) Amino acid methyl ester hydrochloride/Amines, EDCI, HOBT, Et_3N, $CHCl_3$, 25 °C, overnight; (g) $LiOH·H_2O$, THF, H_2O, 25 °C, overnight; (h) $NaBH_4$, CH_3OH, 25 °C, 6 hours.



Scheme 2. The structure of phenyl-pyrrolyl pentane series of non-steroidal VDR derivatives.

Anti-proliferative Effects on Cancer Cell Lines *in Vitro*. The non-steroidal vitamin D analogs with the phenyl-pyrrolyl pentane skeleton were synthesized and screened for anti-proliferative activity against a panel of four different cancer cell lines [human breast cancer cell line (MCF7 cells)[36], human prostate cancer cell line (PC3 cells)[37], human colorectal adenocarcinoma cell line (Caco2 cells)[38] and human liver cancer cell line (HepG2 cells)[39]] which expressed VDR as previously reported. We confirmed the VDR protein expression in different cell types by western blot and observed the relatively high expression of VDR protein in MCF7 cells (**Figure S1**). As shown in **Table 1**, **11b** exhibited the best anti-proliferative activity with IC_{50} value of 57 nM, 40 nM, 17 nM and 150 nM for MCF7, PC3, Caco2 and HepG2 cells, respectively, being more potent than both the prototype compound sw22 and the positive compound calcitriol. In addition, **11a**, **11c**, **11d**, **11f**, **11g**, **15b** and **16b** also showed better anti-proliferative activity against the four cancer cell lines

than sw22 and calcitriol. It was obvious that R^2 substitutions at the end of pyrrole ring side chain of the compounds (**Scheme 2**) may be crucial for their anticancer activity. In common, compounds with a hydrophilic group such as tertiary amine, morpholine ring, and carboxyl at the end of pyrrole ring side chain (**11a**, **11b**, **11d, 11g** and **15b**) showed more potent anti-proliferative activity than those bearing hydrophobic groups such as trifluoromethyl, benzene, alkynyl and ester group (**12a-12b**, **13a-13d** and **14a-14b**).

Table 1. Cellular anti-proliferative activities of the phenyl-pyrrolyl pentane derivatives

Compound	$IC_{50}(\mu M)^a$				
	MCF7	PC3	Caco2	HepG2	L02
11a	3.35± 1.26*	2.15± 0.93*	0.49± 0.04*	0.11± 0.03*	2.42± 1.35
11b	0.057± 0.018*	0.040± 0.006*	0.017± 0.001*	0.15± 0.05*	0.37± 0.17*
11c	0.43± 0.27*	0.87± 0.26*	1.14± 0.25*	0.24± 0.03*	0.99± 0.21
11d	0.55± 0.16*	3.35± 0.95*	1.63± 0.59*	0.23± 0.12*	2.29± 1.28
11e	>50	17.81± 4.18	>50	>50	>50
11f	0.67± 0.34*	2.42± 0.83*	1.74± 0.43*	1.59± 0.21*	2.94± 0.93
11g	0.26± 0.08*	1.57± 0.61*	2.72± 0.71*	1.76± 0.46*	2.86± 1.12
11h	>50	>50	>50	46.28± 14.28	37.80± 10.28
11i	>50	12.77± 3.86*	>50	>50	1.19± 0.36
12a	>50	14.81± 4.18*	>50	>50	>50
12b	>50	>50	>50	>50	>50
13a	>50	>50	>50	>50	>50
13b	>50	>50	>50	>50	>50
13c	>50	23.77± 7.29	>50	>50	>50
13d	>50	>50	>50	>50	>50
14a	>50	>50	>50	>50	>50
14b	3.21± 1.57*	7.14± 1.63*	30.26± 10.37	25.31± 9.36*	1.99± 0.26
14c	0.39± 0.14*	5.84± 1.42*	3.02± 0.82*	>50	4.28± 1.84
15a	11.27± 2.49	13.79± 2.91*	19.97± 5.15	2.62± 0.91*	9.99± 2.55
15b	0.079± 0.028*	2.25± 0.82*	2.74± 1.16*	5.71± 2.47*	2.15± 0.81
15c	10.21± 3.21	11.17± 1.28*	25.19± 9.36	10.71± 1.64*	7.59± 1.62
15d	19.69± 3.37	11.69± 3.19*	46.47± 12.57	1.74± 0.22*	4.04± 0.74
16a	29.14± 5.76	28.39± 4.25	>50	>50	>50
16b	2.87± 1.04*	3.09± 0.43*	0.074± 0.005*	0.51± 0.06*	2.05± 0.86

16c	31.41± 7.92	8.76± 1.72*	40.24± 11.68	24.81± 10.83*	3.04± 1.51
16d	12.62± 4.63	7.87± 1.45*	>50	43.07± 13.59*	4.84± 1.22
sw22	2.803± 0.52	17.36± 2.65	>50	47.55± 3.27*	17.38± 1.75
calcitriol	5.59± 1.72	17.25± 3.83	4.46± 0.82	>50	0.67± 0.05

[a] Data were presented mean ± SD, n = 3, *P < 0.05, compared with calcitriol.

Additionally, to evaluate the selective cytotoxicity of designed compounds against normal cells as a surrogate experiment determining their systemic toxicity *in vivo*, the human normal hepatic cell line (L02 cells) was selected. The results revealed that **11a**, **11b**, **11d, 11g** and **15b** were less toxic (IC_{50} = 0.67 μM) against L02 cells, compared with their lead compound sw22 and with calcitriol. Together, these results suggested that **11a**, **11b**, **11d, 11g** and **15b** may possess selective anti-proliferative potential against cancer cells.

Synthesized compounds exhibited VDR-modulating activities.

In Vitro HL-60 Differentiation-inducing Activity. Calcitriol was known to induce HL-60 cells to differentiate into macrophages or monocytes by activating VDR signaling.[40] Recent studies showed that the measurement of the differentiation-inducing activity of HL-60 cells treated with different vitamin D analogs was used for determining their VDR agonistic activity.[41, 42] First, we investigated whether our synthesized compounds were efficient inducers of HL-60 cell differentiation (**Table 2**).

Table 2. The HL-60 differentiation-inducing activity of synthesized compounds

Compound	EC_{50}(nM) [a]	Compound	EC_{50}(nM) [a]
11a	5.000 ± 1.3*	**13d**	1630 ± 486.2
11b	11.36 ± 2.7	**14a**	>10000
11c	82.00 ± 7.8	**14b**	520.0 ± 28.4

11d	2.300 ± 1.1*	**14c**	21.00 ± 8.2
11e	1080 ± 114.9	**15a**	860.0 ± 124.7
11f	156.0 ± 13.7	**15b**	89.00 ± 15.3
11g	238.0 ± 6.2	**15c**	26.00 ± 5.9
11h	>10000	**15d**	66.00 ± 17.9
11i	>10000	**16a**	450.0 ± 26.3
12a	2653± 83.5	**16b**	52.74± 11.9
12b	6033± 491.3	**16c**	2494± 563.2
13a	>10000	**16d**	96.00 ± 7.3
13b	>10000	**sw22**	8.500 ± 1.2
13c	5900 ± 1629.2	**Calcitriol**	9.000 ± 1.2

[a] VDR agonistic activity was measured as HL-60 differentiation inducing affinity. Data were presented as mean ± SD, n = 3, *p < 0.05, compared with calcitriol.

Consistent with their potent effects on cancer cell viability, the compounds (**11a-11d**, **11f**, **11g**, **15b** and **16b)** showed excellent differentiation-inducing activity with EC_{50} values at the nanomolar range (EC_{50} = 2.3-238 nM) when the pyrrolyl side chain contained hydrophilic group (tertiary amine, primary amine, morpholine ring, carboxyl and hydroxyl), suggesting that they may possess VDR agonistic activity. In contrast, compounds **11e**, **11h**, **11i** with no hydrophilic group in the side chain induced HL-60 differentiation with EC_{50} value at the micromole range, indicating that the hydrophilic group in the side chain of pyrrole ring may be important for VDR agonistic activity.

Transactivation and Transcription Activity. VDR transactivation activity of the three compounds (**11b**, **11g** and **15b**) was evaluated by the luciferase reporter assay in human kidney cell lines (HEK293 cells) to further prove their VDR modulating activity. The three compounds showed concentration-dependent transactivation activity and significant partial agonistic activity, compared with the DMSO control. Among them, **15b** appeared to be the

most potent compound while compounds **11b** and **11g** had the similar activity with the natural hormone calcitriol **(Figure 2)**. Furthermore, **11b** and **11g** activated the expression of the endogenous *CYP24a1* gene strongly in MCF7 cells **(Figure S2)**, suggesting that **11b** and **11g** may be novel VDR modulators for further investigation.

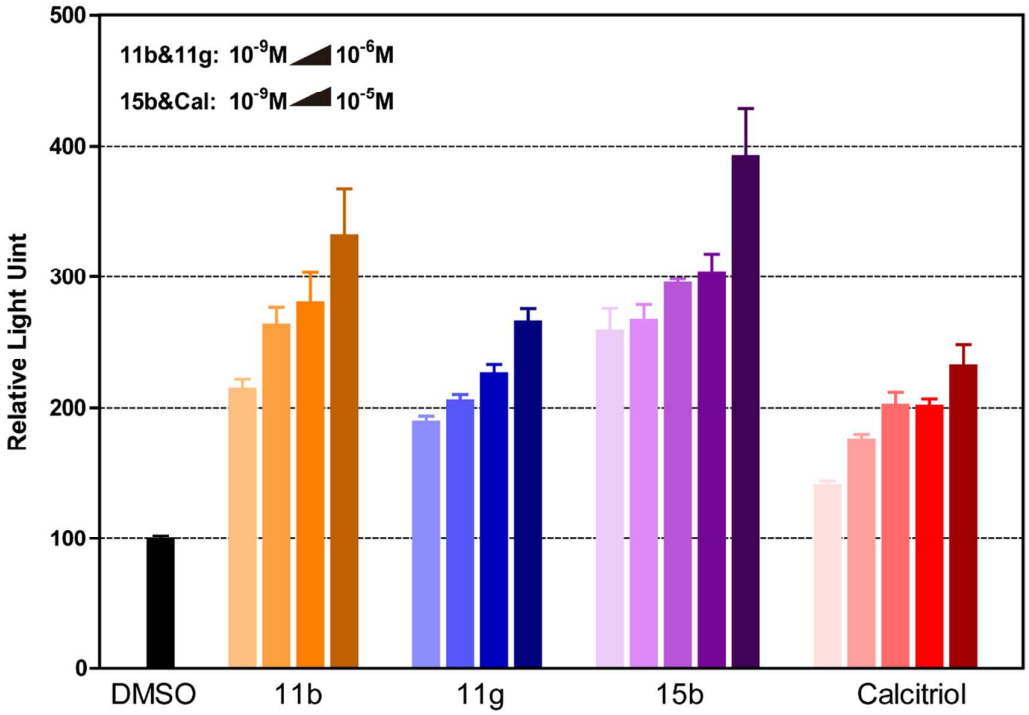


Figure 2. Transcriptional activity of compounds (11b, 11g and 15b) compared with calcitriol in HEK293 cells. HEK293 cells were cotransfected with pGL4.27-SPP×3-Luci reporter plasmid, pCMV-VDR and pCMV-hRXRα. The cells were treated with several concentrations of **11b**, **11g**, **15b** and calcitriol. After 24 hours, the cells were harvested to test the luciferase activity.

VDR-binding Affinity. Due to the potency of these vitamin D analogs with phenyl-pyrrolyl pentane skeleton (**11b**, **11d, 11g** and **15b)** on cancer cell viability (**Table 1**), HL-60 cells differentiation (**Table 2**) and VDR signaling activation (**Figure 2**), we further examined whether these analogs could bind directly to VDR protein in vitro by the ligand-binding assay.

Compounds **11e** and **13d** with little differentiation-inducing activity and anti-proliferative activity for cancer cells showed lower potency (IC_{50} >500 nM) than sw22 and calcitriol under equilibrium conditions in vitro, whereas the more potent analogs (**11b**, **11d** and **11g**) in the cell-based assay (HL-60 differentiation in **Table 2** and VDR transactivation in **Figure 2**) did demonstrate binding to VDR protein; with **11b** and **11d** being the most potent (**Figure 3**). We hypothesized that the lower VDR binding affinity of phenyl-pyrrolyl pentane derivatives might result from less hydrogen bonds, the most important bond for ligands and VDR protein interaction, formed with the ligand binding domain of VDR (VDR-LBD) than the natural hormone calcitriol.

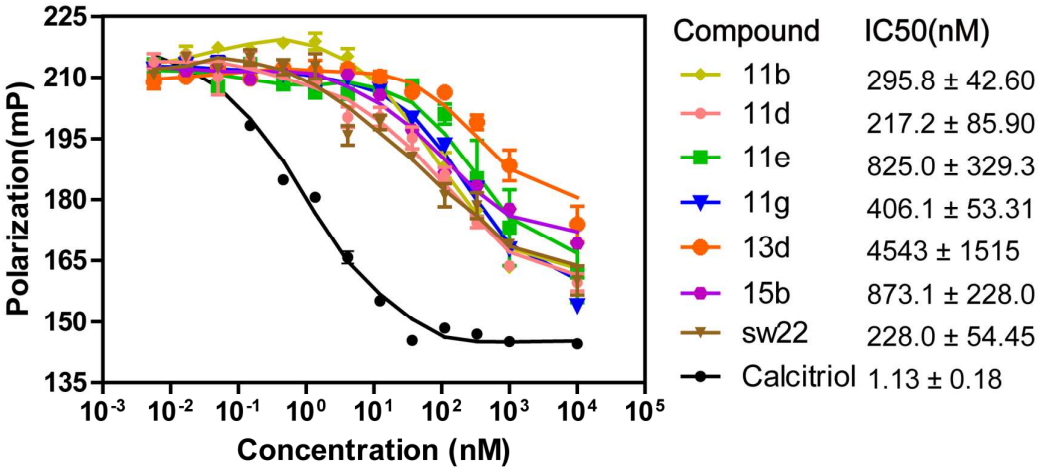


Figure 3. The VDR ligand binding ability for compounds measured by fluorescence polarization assay. Fluorescence polarization competition assay was performed under equilibrium conditions *in vitro* and the competence of the Fluormone VDR Red ligand from the VDR-LBD by different compounds are shown (n = 3).

Molecular Docking Study. Docking study was performed to investigate VDR-binding

characteristics of phenyl-pyrrolyl pentane derivatives. As the vitamin D analogs (**11b**, **11d** and **11g**) possessed a flexible side chain in the benzene ring, we first investigated the feasibility of our docking model for predicting the structure complex between such flexible analogs and VDR-LBD. LG190178 is a representative non-steroidal vitamin D analog possessing a similar flexible 2-hydroxy-3,3-dimethylbutoxy phenyl side chain (**Figure S3A and S3B**), which embedded in the ligand-binding pocket in the same position as calcitriol in the VDR-LBD-calcitriol complex.[26, 43, 44] Therefore, we determined whether the in silico docking structure of the VDR-LBD-LG190178 could reproduce the X-ray crystal structure of the VDR-LBD-LG190178 complex (PDB ID: 2ZFX).[43] The docking results showed that in silico docking structure of the VDR-LBD-LG190178 complex superimposed on the X-ray crystal structure of the VDR-LBD-LG190178 (**Figure S3C**), with the root-mean-squared deviation (r.m.s.d.) equals 0.5031, suggesting that the docking model was repeatable and reliable for predicting the structure complex between the VDR-LBD and flexible non-steroidal vitamin D analogs.

Then the binding models of **11g** and **15b** with VDR-LBD (PDB ID: 2ZFX) were predicted and the most suitable conformations of the ligands were selected based on the calculated docking scores by means of the bonding strength (**Figure 4**). The results showed that **11g** could form hydrogen bonds with VDR-LBD, including the hydroxyl group at the terminal of phenyl side chain with His301 and His393, and the O at the morpholine ring of pyrrolyl side chain with Asp144 and Tyr232. Besides, the hydroxyl group at the terminal of phenyl side chain of **15b** formed the same hydrogen bond with His393 and His301, and the terminal carboxyl group of pyrrolyl side chain formed a weak hydrogen bond with Arg270. Moreover,

11g and **15b** also had similar intense van der Waals interactions between VDR-LBD and the phenyl-pyrrolyl pentane skeleton to those of calcitriol.[45]

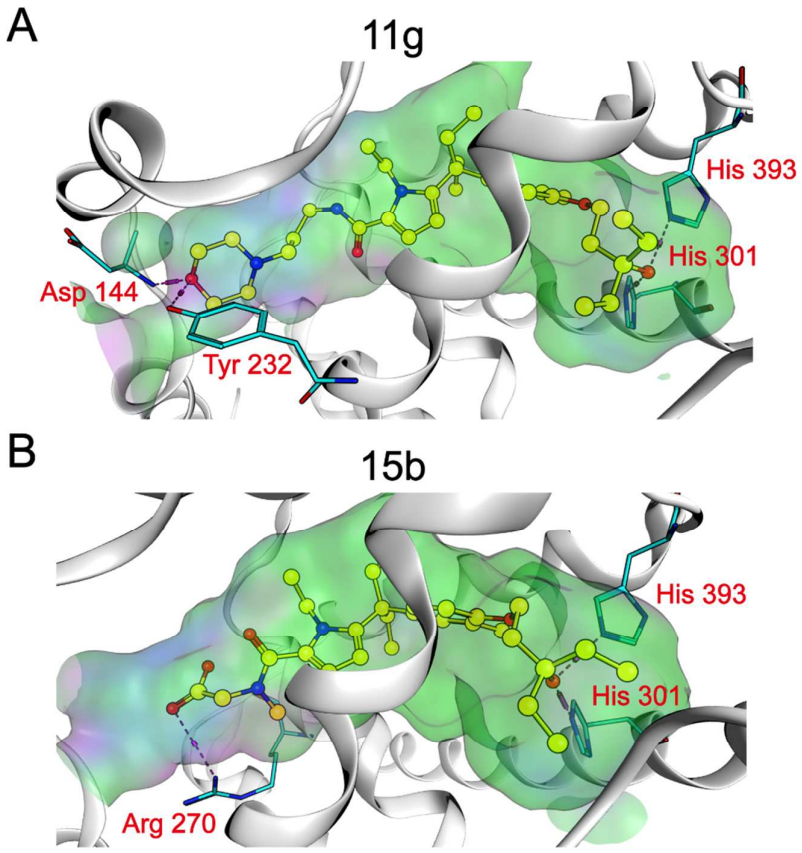


Figure 4. The predicted binding model of 11g (A) and 15b (B). VDR-LBD of PDB reference 2ZFX is applied for molecular docking using Glide 5.5 in Schrödinger 2009. The most suitable conformations of the ligands were selected based on the calculated docking scores by means of the bonding strength. **(A)** Hydroxyl group at the end of phenyl ring side chain of compound **11g** was able to form hydrogen bond with the His393 and His301 of VDR-LBD. On the other side of the structure, the O of morpholine ring bound with Asp144 and Try232. **(B)** Hydroxyl group beside phenyl of compound **15b** was able to form hydrogen bond with the His393 and His301, and the terminal carboxyl group formed hydrogen bond with Arg270.

It was reported that calcitriol could form hydrogen bonds with VDR-LBD, including the 25-OH group with His301 and His393, the 1-OH group with Ser233 and Arg270, and the 3-OH group with Tyr139 and Ser274.[44] In comparison, the hydrogen bonds formed by the hydroxyl group at the terminal of phenyl side chain of **11g** and **15b** interacting with His393 and His301 were the same as those of calcitriol. It is interesting that the pyrrolyl side chain of **11g** formed hydrogen bonds with Asp144 and Tyr232 but not Ser233 or Arg270 on the other side, resulting from the structure of spatial configuration reversal of the morpholine ring. However, the 3-OH group of calcitriol also bound with Tyr139 and Ser274 to form additional hydrogen bonds compared to **11g** and **15b**, suggesting lower binding affinity of **11g** and **15b** than calcitriol with VDR-LBD.[44]

11b, 11d and 11g Inhibited MCF7 Cell Growth by Inducing Cell Cycle Arrest and Apoptosis. As compounds **11b**, **11d**, **11g** and **15b** showed the most potent VDR binding, transactivation and anti-proliferative activities for cancer cells, they were selected for further evaluation of biological activities.

Previous study showed that vitamin D analogs such as calcitriol could prevent tumor progression by inhibiting cell proliferation and inducing apoptosis.[1, 4, 39, 46] To explore the underlying mechanism for the reduced cell viability of MCF7 cells, we investigated the effect of **11b**, **11d**, **11g** and **15b** on the cell cycle by flow cytometry. Treatment with **11b**, **11d** and **11g** on MCF7 cells effectively arrested cells in the G0/G1 phase of the cell cycle. The population of cells in G0/G1 phase dramatically increased as compared to that of calcitriol

(**Figure 5A and 5B**). The effect of cell cycle arrest was also observed in HepG2 cells after treatment with compounds **11b**, **11g** and **15b** (**Table S1**). Then we investigated whether **11b**, **11d**, **11g** and **15b** might induce apoptosis of MCF7 cells. MCF7 cells were treated with each compound at the concerntration of 1 μM for 24 h. The percentage of apoptotic cells in MCF7 was measured by staining Annexin V-FITC and propidium iodide (PI). After that, we analyzed the DNA content of the cells by flow cytometry as described in the section of "Experimental Section". We discovered that **11b**, **11d**, **11g** and **15b** could promote the apoptosis of MCF7 cells compared with the DMSO control group. Moreover, the proportion of early apoptotic cells (Annexin V-FITC positive and propidium iodide negative) signifcantly increased in the groups after **11d**, **11g** and **15b** treatment compared with the calcitriol treatment group (**Figure 5C and 5D**).

Previous studies reported that MART-10, a new analog of calcitriol, could up-regulate the protein level of p21 and p27, arrest cancer cells at the G_0/G_1 phase and promote cell apoptosis. Moreover, other studies also determined that VDR modulators could cause cell apoptosis and the accumulation of cells in G_0/G_1 phase which was associated with the up-regulation of p21 and p27 in the protein level in cells.[39, 46-48] Hence, we speculated that our derivatives could arrest the tumor cells in G_0/G_1 phase through the up-regulation of p21 and p27. After MCF7 cells were treated with **11b**, **11g** at the concentration of 1 μM for 24 hours, the expression levels of p21 and p27 were examined. Western blot assay showed that treatment with **11b** and **11g** increased p21 and p27 expression levels in MCF7 cells (**Figure S5**). The real-time quantitative PCR assay revealed that **11b** and **11g** also increased the mRNA levels of p21 and p27 (**Figure S4**). Furthermore, relative expression levels of Bax and Bak mRNA were

elevated 24 hours after treatment with different compounds in MCF7 cells. The results revealed that treatment with **11b** and **11g** dramatically increased the levels of pro-apoptotic Bax and Bak expression in MCF7 cells (**Figure S4**). Taken together, the results demonstrated that **11b**, **11d** and **11g** inhibited the growth of MCF7 cells by inducing cell cycle arrest and cell apoptosis.

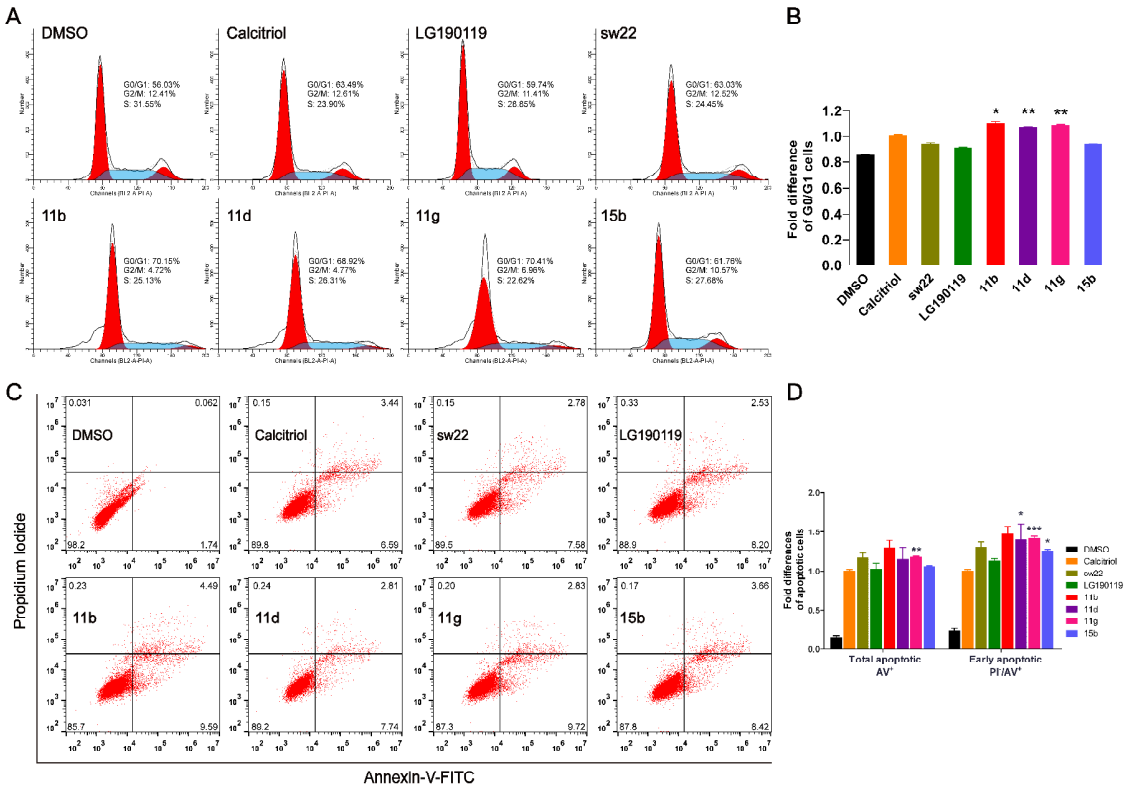


Figure 5. Cell cycle arrest and apoptosis after treatment with vitamin D modulators in MCF7 cells. (A) Cell cycle phase distribution was determined by flow-cytometry. The MCF7 cells were treated with each compound at 1 μM for 24 hours. (B) The fold differences of cell population in the G0/G1 phase of the cell cycle after treatment of each compound (n = 4 for each compound). The relative difference was normalized to the group of calcitriol treatment. (C) Representative flow cytometry dot plot of Annexin V-FITC and PI staining for the detection of apoptotic MCF7 cells. The

MCF7 cells were treated with each compound at 1 μM for 24 hours. (D) The fold differences of apoptotic quantification after exposure to each compound for 24 hours (n = 3 for each compound). The relative difference was normalized to the group of calcitriol treatment.

Cellular Uptake and Metabolic Stability of 11b, **11d** and **11g.** To provide more basic information on drug-like properties, cellular uptake and metabolic stability of **11b**, **11d** and **11g** in MCF7 cells were further investigated. MCF7 cells were cultured with 1 μM of **11b**, **11d**, **11g**, sw22, LG190119 and calcitriol in the presence of 10% FBS for 10 and 30 min, and the concentrations of these compounds in both the culture medium and cells were determined by HPLC. Cellular uptake of **11g** was 37.9% and 38.2% at 10 and 30 min, respectively, while that of calcitriol was 6.5% and 10.4% at 10 and 30 min, respectively. Thus, compound **11g** was shown to be 4-5 times more effectively incorporated into the MCF7 cells than calcitriol, and 2-3 times more effectively than sw22 and LG190119. In addition, more effective uptake of **11b** and **11d** than calcitriol incorporated into the MCF7 cells was also observed (**Figure 6A**).

When MCF7 cells were cultured up to 24 hours, 27% of calcitriol was metabolically consumed, while only 6% of **11g** was consumed, indicating that compound **11g** was four times more stable than calcitriol in MCF7 cells (**Figure 6B**). Overall, the results suggested that **11b**, **11d** and **11g** possessed comparable uptake activity and metabolic stability to that of calcitriol.

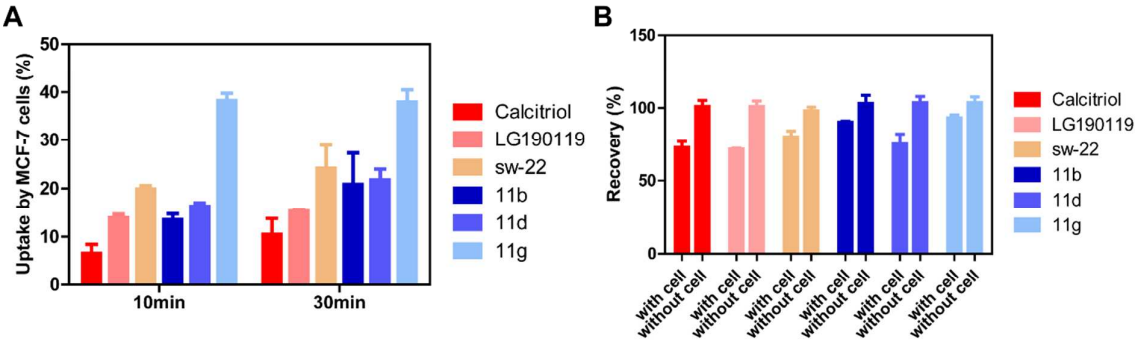


Figure 6. Uptake and metabolism stability of 11b, 11d, 11g, calcitriol, LG190119 and sw22 in MCF7 cells. (A) MCF7 cells were incubated with 1 μM of different compounds for 10 minutes or 30 minutes. The cells and medium were extracted separately by chloroform/methanol (3:1, v/v). The extracts were analyzed by HPLC. (B) MCF7 cells were incubated with 1 μM of different compounds for 24 hours (with cells). Similarly, each of the substrates (**11b**, **11d**, **11g**, calcitriol, LG190119 and sw22) was incubated with medium only for 24 hours (without cells). Each experimental mixture was extracted with chloroform/methanol (3:1, v/v). The extracts were analyzed by HPLC. Data were shown as the mean ± SEM (n = 3).

***In vivo* Calcemic Activity Assay.** *In vivo* calcemic activity assay was performed to evaluate the potential hypercalcemic possibility of designed non-steroidal modulators. The compounds **11b**, **11d**, **11g** and **15b** were chosen for calcemic activity assay *in vivo*, with calcitriol, sw22 and LG190119 as the positive controls and vehicle as the negative control. The results were shown in **Figure 7**.

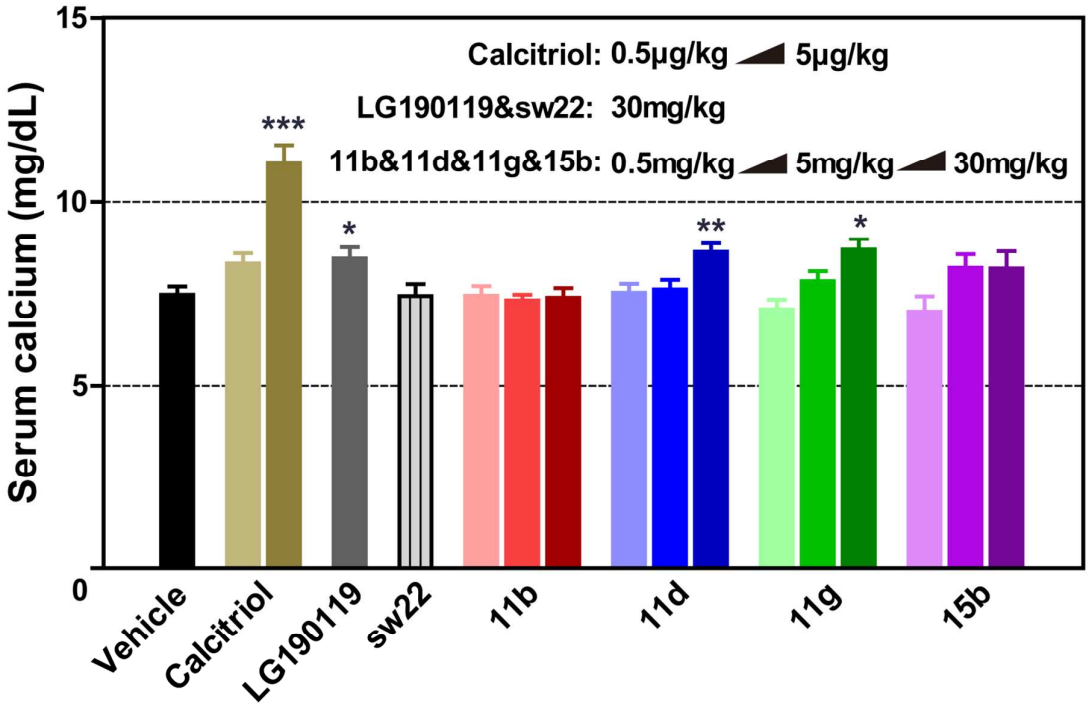


Figure 7. ***In vivo*** **hypercalcemic effects of calcitriol, LG190119, sw22 and compounds 11b, 11d, 11g and 15b.** Mice were treated with **11b**, **11d**, **11g and 15b** in different concentration (0.5 mg/kg, 5 mg/kg and 30 mg/kg. i.p.) over a 7 day period. Calcitriol (0.5 μg/kg or 5 μg/kg. i.p.) and LG190119 (30 mg/kg. i.p.), sw22 (30 mg/kg. i.p.) were employed as reference drugs. Data were shown as the mean ± SEM (n = 6). Statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) for each concentration of serum calcium compared to the vehicle control.

A significant increase in serum calcium was observed after the administration of calcitriol (5 μg/kg) for 7 days (11.04 mg/dL for calcitriol *vs* 7.05 mg/dL for saline; $p < 0.001$), while a dose of 0.5 μg/kg showed normal serum calcium level compared with the vehicle group. Furthermore, **11b**, **11d**, **11g** and **15b** at the dosage of 0.5 mg/kg/day and 5 mg/kg/day had no effect on serum calcium. Intraperitoneal administration of **11d** and **11g** even at the dosage of 30 mg/kg/day resulted in a slight elevation of serum calcium level compared with the vehicle

group. Therefore, the designed non-steroidal modulators had no hypercalcemic effect *in vivo*.

***In vivo* antitumor activity assay.** To investigate the *in vivo* antitumor effect of **11b**, **11d** and **11g**, breast tumor mouse model was established by orthotopic transplantation of human MCF7 cells in athymic nude mice.[49] LG190119 and calcitriol as controls were selected as positive controls because of their antitumor effects in animal models or human clinical trials. LG190119, the representative of nonsteroidal compounds,[44] is the only reported compound of the non-steroidal analogs that displayed antitumor effects in animal models of cancer. Specifically, previous report showed that LG190119 effectively inhibited LNCaP prostate cancer xenograft tumor growth without causing hypercalcemia.[24] In addition, calcitriol has been used in a number of animal models of cancer and clinical trials for cancer treatment, such as prostate cancer and breast cancer.[1, 4] The results showed that **11b**, **11d** and **11g** significantly inhibited the tumor growth compared with calcitriol (0.5 μg/kg, in which concentration antitumor effects but no significant increase of the serum calcium were observed[49] as shown in **Figure 7**) and sw22 (**Figure 8B and 8C**). In addition, these three compounds also have better antitumor efficacy compared with LG190119 (10 mg/kg, in which concentration the inhibition of prostate tumor growth was observed[24]). However, there was no significant change of the body weight in the treatment of compounds **11b**, **11d** and **11g** compared with the saline or vehicle group (**Figure 8A**), indicating that the three compounds have no apparent toxicity to mice during the period of cancer treatment.

Importantly, the treatment of **11b**, **11d** and **11g** did not increase mouse serum calcium levels in comparison with the control groups in breast tumor-bearing mice (**Figure 8D**). We

further determined the proliferation and apoptosis rate of MCF7 cells by Ki67 immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling assay (TUNEL). There was a significantly low intensity of Ki67 staining in sections of MCF7 orthotopic tumor after treatment with **11b** and **11g** compared with calcitriol group (**Figure 8E and 8F**), which was consistent with tumor volume results (**Figure 8B and 8C**), suggesting that **11b** and **11g** inhibited MCF7 cells proliferation *in vivo*. In addition, TUNEL staining showed significantly increasing apoptosis in the breast tumor after treatment with **11b**, **11d** and **11g** compared with sw22, LG190119 and calcitriol treatment (**Figure 8G and 8H**). Collectively, these findings suggested that **11b** and **11g** could inhibit the growth of breast cancer through suppressing proliferation and inducing apoptosis of cancer cells.

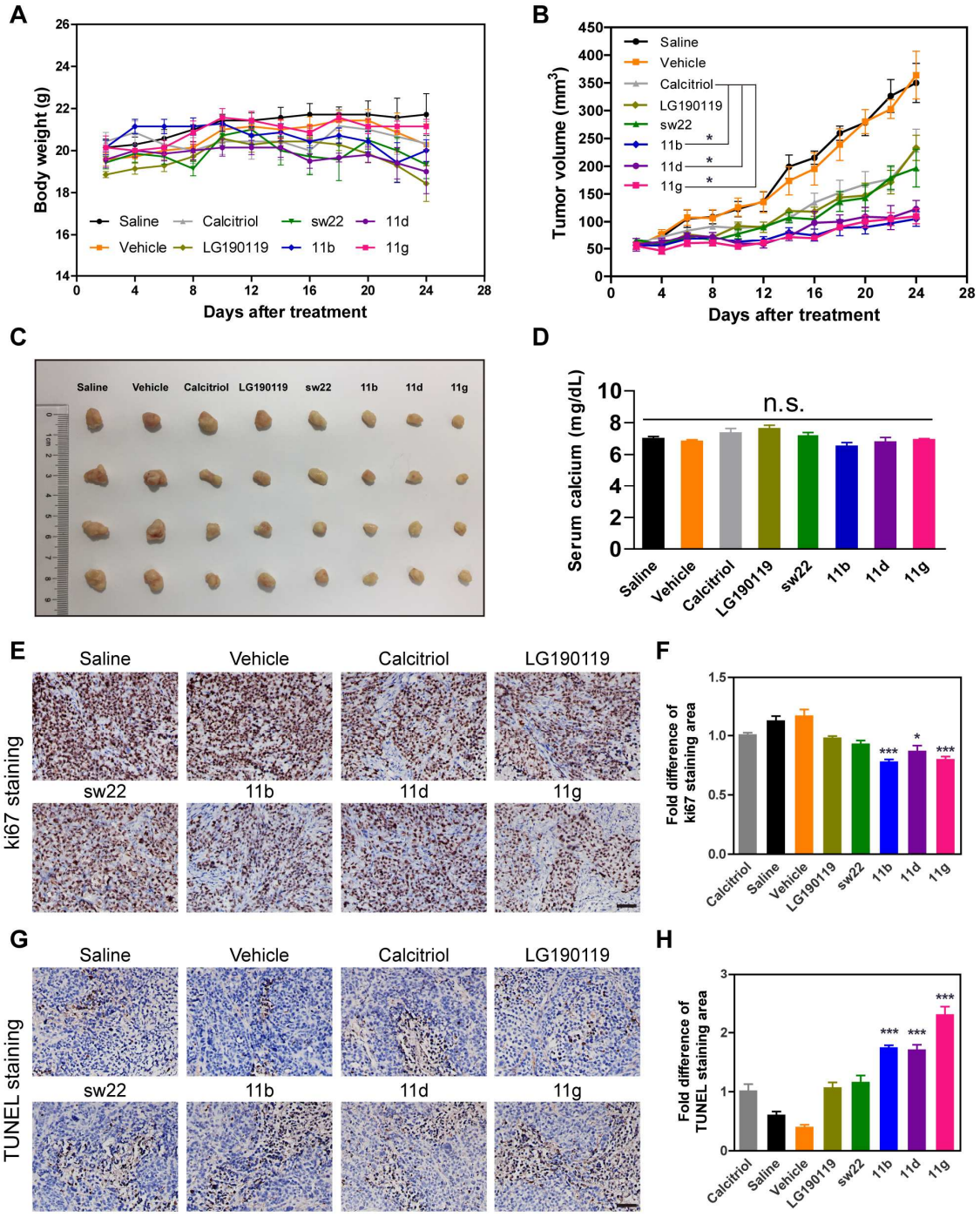


Figure 8. Antitumor effects of compounds 11b, 11d and 11g *in vivo*. (A) The change in the body weight, (B) tumor volume of MCF7-bearing mice after treatment with vehicle (polyoxyethylated castor oil (EL)/ethanol/saline=1:1:18), saline, sw22 (10 mg/kg, i.p.), LG190119 (10 mg/kg, i.p.), **11b**

(10 mg/kg, i.p.), **11d** (10 mg/kg, i.p.), **11g** (10 mg/kg, i.p.) and calcitriol (0.5 μg/kg, i.p.). (C) The images of excised tumors in each group. (D) The hypercalcemic effects of calcitriol, LG190119, sw22 and **11b**, **11d** and **11g** in the MCF7 tumor-bearing mice. (E) Immunohistochemistry of Ki-67 in tumor sections of MCF7-bearing mice. Scale bar, 50 μm. (F) Quantification of percentages of positive Ki-67 staining area in total nuclei area in each field (n=10, 400 fold magnification). The relative difference was normalized to the group of calcitriol treatment. (G) Immunohistochemistry of TUNEL in tumor sections of MCF7-bearing mice. Scale bar, 50 μm. (H) Quantification of percentages of positive TUNEL staining area in total nuclei area in each field (n=10, 400 fold magnification). The relative difference was normalized to the group of calcitriol treatment. Statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) for **11b**, **11d** and **11g** compared to calcitriol.

Pharmacokinetics Study *In Vivo*. Given their promising *in vivo* anti-tumor activities, preliminary *in vivo* pharmacokinetic (PK) studies of compounds **11g** and **11b** were determined in rats and sw22 was used as the control. The results were summarized in **Table 3**. **11b** and **11g** displayed better pharmacokinetic properties than sw22, with an oral bioavailability of 36.8% and a $T_{1/2}$ value of 11.31 hours after oral administration. Moreover, the $T_{1/2}$ value of **11g** ($T_{1/2}$ = 4.75 h) was similar to calcitriol ($T_{1/2}$ = 4.27 h) after intravenous administration.[50] The results suggested that **11b** and **11g** could possess therapeutic potential for cancer treatment.

Table 3. The pharmacokinetic profile of compounds 11b, 11g, sw22 in rats[a]

Parameter	11b			11g			sw22		
	i.v.	i.p.	p.o.	i.v.	i.p.	p.o.	i.v.	i.p.	p.o.
	5 mg/kg	20 mg/kg	20 mg/kg	5 mg/kg	20 mg/kg	20 mg/kg	5 mg/kg	20 mg/kg	20 mg/kg
AUC(mg/L*h)	25.21±3.40	30.47±5.09	21.47±1.26	10.37±1.91	25.91±2.57	15.27±1.68	13.40±1.07	24.76±2.26	13.49±1.75
$T_{1/2}$(h)	2.97±0.16	6.21±1.62	6.82±1.18	4.75±0.85	11.73±1.6	11.31±2.75	2.44±0.43	3.96±0.53	6.89±2.52
MRT(h)	3.85±0.49	9.30±2.32	10.97±1.53	7.55±0.78	17.59±2.42	15.90±3.18	2.82±0.04	8.15±0.97	10.76±3.18
CL(L/h)	0.21±0.032	0.68±0.15	0.93±0.065	0.49±0.089	0.78±0.082	1.31±0.14	0.41±0.036	0.81±0.085	1.50±0.21
F(%)		30.22%	21.29%		62.46%	36.80%		46.19%	25.17%

[a] SD rats (male, 4 animals per group) weighted 180−220 g were used for the study (n = 4). Data were presented as Mean ± SD.

DISCUSSION AND CONCLUSIONS

In this study, a series of novel vitaimin D analogs with phenyl-pyrrolyl pentane skeleton were synthesized and a comprehensive series of experiments were performed to validate their VDR modulating activities and in vivo anticancer activities. This study was the first to show that **11b** and **11g** with the phenyl-pyrrolyl pentane skeleton markedly inhibited MCF7 breast tumor growth in athymic nude mice without causing increased serum calcium level or animal weight loss, suggesting that they may be suitable for long-term cancer chemotherapy.

sw22, previously reported by our group,[31] was selected as the lead compound. First, we retained the phenyl-pyrrolyl pentane skeleton to keep the VDR binding affinity. Then, we introduced the diethylcarbinol as the terminal hydrophobic group into the phenyl side chain to give **16d**. The *in vitro* anti-proliferation results revealed **16d** had similar IC_{50} range against different cancer cells compared to those of sw22 (**Table 1**). Based on this, we further

explored the effects of substitutions groups on the pyrrole ring, **11a-11i**, **12a-12b**, **13a-13d, 14a-14c, 15a-15d** and **16a-16c** were designed and synthesized.

Based on the results of the *in vitro* anti-proliferative assay and HL-60 differentiation-inducing assay, we summarized the following structure-activity relationships (SARs) of phenyl-pyrrolyl pentane derivatives. (1) Introducing the 3-ethyl-3-hydroxypentoxy into the phenyl side chain could retain the anti-proliferative activity against cancer cells. (2) Introducing the hydrophilic moieties at the R^2 group is important to improve the anti-proliferative and HL-60 differentiation-inducing activities, such as introducing the hydroxy, amines and carboxyl groups to give compounds **11a-11d, 15a-15d** and **16a-16d**, while the introduction of hydrophobic segments may lead to a remarkable decrease or even loss of anti-proliferative and HL-60 differentiation-inducing activities, such as **11e** and **11h**. (3) The introduction of large groups such as benzene ring into R^2 group cannot be tolerated, nearly leading to a loss of the anti-proliferative and HL-60 differentiation-inducing activities, such as **13a-13d**. (4) Introducing alkenyl and alkynyl to the pyrroyl side chain could result in a complete disappearance of anti-proliferative and HL-60 differentiation-inducing activities, such as **11i**, **12a-12b**.

11b and **11g** showed significant VDR agonistic activity determined by both dual luciferase reporter assay (**Figure 2)** and human leukemia cell line (HL-60 cells) differentiation assay (**Table 2**), though the new analogs (**11b**, **11d** and **11g**) showed lower competence with Fluormone VDR Red ligand (PolarScreen™ Vitamin D Receptor Competitor Assay, Invitrogen) than calcitriol in the VDR binding assay performed under equilibrium conditions in vitro. Therefore, the VDR-dependent cell-based assay may be more sensitive than the in

vitro VDR equilibrium-binding assay in detection of VDR activators. We speculated that detection of VDR binding of the less potent non-steroidal vitamin D analogs under equilibrium conditions in the competition binding assay with the Fluormone VDR Red ligand, may be precluded by the high on-rate and low off-rate of the high-affinity Fluormone VDR Red ligand. Similar results have been reported in the VDR-binding characteristics of non-steroidal vitamin D analogs including LG190119, LG190155 and LG190178.[26, 29] For the transcriptional activity of VDR downstream target gene *Cyp24a1*, *Cyp24a1* could be strongly induced by calcitriol and often serves as a marker of VDR activation in the cell. In the present study, the activation of *Cyp24a1* transcription after treatment of **11b** and **11g** is lower than that of calcitriol treatment group (**Figure S2**). The differences of the activation level of *Cyp24a1* gene between calcitriol and the non-steroidal VDR modulators could be due to their distinctions in metabolism and coactivator recruitment.[51-54]

Both preclinical and clinical studies support the development of vitamin D analogs as preventative and therapeutic anticancer agents,[1] however, clinically approved anticancer vitamin D drugs remain elusive. Part of the reason for the clinical failure is that drug concentrations, which are thought to be necessary for antitumor activity on the basis of preclinical results, are not achievable in the blood/tumor of patients as well as hypercalcemia intervenes.[4, 55, 56] One possibility for an effective cancer therapy is attempting to overcome poor bioavailability and designing novel VDR modulators without the risk of increasing serum calcium. Another would be the combination of a low-calcemic vitamin D analog with other anticancer agents to achieve a synergistic effect on cancer therapy.[1, 55] In this study, we found that **11b** and **11g** possessing good bioavailability could inhibit breast tumor growth

without hypercalcemia problems, which makes them promising leads for further anticancer-drug development. In addition, cancer treatment in combination of **11b** and **11g** with other antitumor agents (such as taxols and cisplatins) is worth a further evaluation.

In summary, a series of novel phenyl-pyrrolyl pentane derivatives as the VDR modulators were synthesized and analyzed for biological activities. Among them, **11b** and **11g** are more tumor selective in action and displayed superior therapeutic indices *in vivo* in the orthotopic breast tumor model without hypercalcemia intervenes, suggesting that **11b** and **11g** may be suitable for long-term treatment and/or chemoprevention. Overall, the structurally novel non-steroidal tumor-selective VDR modulators with phenyl-pyrrolyl pentane skeleton provides us promising lead compounds for further anticancer drug discovery.

EXPERIMENTAL SECTION

General Methods for Chemistry. All commercially available chemicals and solvents were used without further purification. All ^{1}H NMR and ^{13}C NMR were recorded on Bruker AV-300 or AV-500 instruments with $CDCl_3$ or DMSO-d_6. Chemical shifts were reported in ppm (d) with reference to the internal standard tetramethylsilane (TMS). The signals were designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet. High-resolution mass spectra (HRMS) were recorded on QSTAR XL Hybrid MS/MS mass spectrometer. The reactions were monitored by thin layer chromatography (TLC). The homogeneity of the compounds was separated by column chromatography on silica gel (200-300 mesh), visualized by ultraviolet light (UV) or iodine chamber. Purity of all tested compounds was greater than 95%, as estimated by high-performance liquid chromatography

(HPLC) analysis. The major peak of the compounds analyzed by HPLC accounted for ≥ 95% of the combined total peak area when monitored by a UV detector at 254 nm.

***N*-(3-(dimethylamino)propyl)-1-ethyl-5-(3-(4-((3-ethyl-3-hydroxypentyl)oxy)-3-methylphenyl)pentan-3-yl)-1*H*-pyrrole-2-carboxamide (11a).** To a solution of compound **10** (1 g, 2.32 mmol) in DMF (20 mL) was added EDCI (0.54 g, 2.82 mmol) and HOBt (0.38 g, 2.82 mmol), the mixture was activated with stirring at r.t for 0.5 hours. Then the mixture was added Et_3N (1.14 g, 10.21 mmol, 1.58 mL), followed by N^1, N^1-dimethylpropane-1,3-diamine (0.26 g, 2.56 mmol) and reacted at r.t for 4 hours. Water and ethyl acetate were added and the two phases were separated. The aqueous phase was extracted with ethyl acetate and the combined organic phase was washed with water, dried over Na_2SO_4, and evaporated to get yellow oil which was further purified to afford compound **11a** as yellow oil (0.63 g, 55%). HRMS, ESI^+, m/z: calcd for $C_{31}H_{51}N_3O_3$ $(M+H)^+$ 514.4003, found 514.3998. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.04 (1 H, d, *J* = 8.7 Hz), 7.01 (1 H, s), 6.74 (1 H, d, *J* = 8.7 Hz), 6.52 (1 H, d, *J* = 1.8 Hz), 6.34 (1 H, d, *J* = 1.8 Hz), 4.30 (2 H, q, *J* = 7.2 Hz), 4.13 (2 H, t, *J* = 6.0 Hz), 3.45 (2 H, t, *J* = 6.0 Hz), 3.00 (2 H, t, *J* = 7.2 Hz), 2.70 (6 H, s), 2.17 (3 H, s), 2.07 (2 H, m), 1.56 (4 H, m), 1.34 (3 H, t, *J* = 6.9 Hz), 0.90 (6 H, t, *J* = 7.5 Hz), 0.65 (3 H, t, *J* = 7.2 Hz). ^{13}C NMR(75 MHz, $CDCl_3$) δ: 162.56, 154.32, 140.17, 131.41, 130.21, 125.90, 125.05, 112.32, 109.65, 64.69, 45.82, 44.83, 43.69, 36.90, 36.33, 30.92, 30.24, 25.10, 17.26, 8.57.

***N*-(3-(diethylamino)propyl)-1-ethyl-5-(3-(4-((3-ethyl-3-hydroxypentyl)oxy)-3-methylphenyl)pentan-3-yl)-1*H*-pyrrole-2-carboxamide (11b).** The same method as **11a** and the starting materials were **10** and N^1, N^1-diethylpropane-1,3-diamine. Yellow oil, 0.52 g, 42% yield. HRMS, ESI^+, m/z: calcd for $C_{33}H_{55}N_3O_3$ $(M+H)^+$ 542.4316, found 542.4316. ^1H NMR

(300 MHz, $CDCl_3$) δ: 7.04 (1 H, d, *J* = 8.4 Hz), 7.01 (1 H, s), 6.74 (1 H, d, *J* = 8.4 Hz), 6.51 (1 H, d, *J* = 1.5 Hz), 6.45 (1 H, d, *J* = 1.5 Hz), 4.30 (2 H, q, *J* = 7.2 Hz), 4.13 (3 H, t, *J* = 6.0 Hz), 3.46 (2 H, m), 3.02 (6 H, m), 2.21 (3 H, s), 2.08 (2 H, m), 1.07 (6 H, m), 1.55 (4 H, m), 1.34 (9 H, m), 0.90 (6 H, t, *J* = 7.5 Hz), 0.65 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 162.57, 154.40, 140.19, 131.41, 130.22, 125.88, 125.02, 123.54, 112.42, 109.62, 64.70, 49.84, 46.38, 44.83, 43.66, 36.89, 30.92, 30.22, 24.29, 17.25, 8.75, 8.56.

***N*-(2-(dimethylamino)ethyl)-1-ethyl-5-(3-(4-((3-ethyl-3-hydroxypentyl)oxy)3-methylphenyl)pentan-3-yl)-1*H*-pyrrole-2-carboxamide (11c).** The same method as **11a** and the starting materials were **10** and N^1, N^1-dimethylethane-1,2-diamine. Yellow oil, 0.59 g, 51% yield. HRMS, ESI^+, m/z: calcd for $C_{30}H_{49}N_3O_3$ $(M+H)^+$ 500.3847, found 500.3841. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.04 (1 H, d, *J* = 8.4 Hz), 7.02 (1 H, s), 6.73 (1 H, d, *J* = 8.4 Hz), 6.62 (1 H, d, *J* = 1.5 Hz), 6.51 (1 H, d, *J* = 1.5 Hz), 4.29 (2 H, q, *J* = 7.2 Hz), 4.13 (2 H, t, *J* = 6.0 Hz), 3.68 (2 H, m), 3.02 (2 H, m), 2.67 (6 H, s), 2.17 (3 H, s), 1.95 (6 H, m), 1.56 (4 H, m), 1.32 (3 H, t, *J* = 7.2 Hz), 0.90 (6 H, t, *J* = 7.5 Hz), 0.65 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 162.42, 154.37, 140.15, 131.40, 130.22, 125.92, 125.09, 123.20, 112.99, 109.65, 64.67, 58.23, 44.14, 36.09, 35.06, 30.92, 17.24, 8.59.

***N*-(3-(diethylamino)ethyl)-1-ethyl-5-(3-(4-((3-ethyl-3-hydroxypentyl)oxy)-3-methylphenyl)pentan-3-yl)-1*H*-pyrrole-2-carboxamide (11d).** The same method as **11a** and the starting materials were **10** and N^1, N^1-dimethylethane-1,2-diamine. Yellow oil, 0.68 g, 55% yield. HRMS, ESI^+, m/z: calcd for $C_{32}H_{53}N_3O_3$ $(M+H)^+$ 528.4160, found 528.4152. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.05 (1 H, d, *J* = 8.1 Hz), 7.02 (1 H, s), 6.73 (2 H, m), 6.47 (1 H, d, *J* = 1.5 Hz), 4.29 (2 H, q, *J* = 7.2 Hz), 4.13 (2 H, t, *J* = 6.0 Hz), 3.73 (2 H, m), 3.09 (6 H, m), 2.17

(3 H, s), 1.95 (6 H, m), 1.57 (4 H, m), 1.34 (9 H, m), 0.90 (6 H, t, *J* = 7.5 Hz), 0.66 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 162.49, 154.37, 140.10, 131.51, 130.23, 125.87, 125.18, 123.01, 113.45, 109.64, 74.33, 64.68, 52.59, 48.05, 45.84, 44.84, 43.67, 36.90, 30.91, 17.22, 16.80, 8.59, 8.00.

1-Ethyl-5-^{20}-1*H*-pyrrole-2-carboxylic acid cyanomethyl-amide (11e). The same method as **11a** and the starting materials were **10** and amino-acetonitrile. Yellow oil, 0.96 g, 89% yield. HRMS, ESI$^+$, m/z: calcd for $C_{28}H_{41}N_3O_3$ $(M+Na)^+$ 490.3040, found 490.3031. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.01 (1H, d, *J* = 8.4 Hz), 6.98 (1 H, s), 6.73 (1 H, d, *J* = 8.4 Hz), 6.64 (1 H, d, *J* = 1.5 Hz), 6.24 (1 H, d, *J* = 1.5 Hz), 4.32 (2 H, q, *J* = 7.2 Hz), 4.21 (2 H, s), 4.13 (2 H, d, *J* = 6.0 Hz), 2.17 (3 H, s), 1.95 (6 H, m), 1.57 (4 H, m), 1.37 (3 H, t, *J* = 7.2 Hz), 0.90 (6 H, t, *J* = 7.2 Hz), 0.65 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR(75 MHz, $CDCl_3$) δ: 161.11, 154.54, 139.91, 131.88, 130.23, 125.88, 112.85, 109.62, 74.41, 64.68, 44.92, 43.96, 36.88, 30.25, 27.20, 17.14, 8.51, 8.01.

1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carboxylic acid (2-amino-ethyl)-amide (11f)

Synthesis of (2-Amino-ethyl)-carbamic acid tert-butyl ester (*N*-Boc). Et_3N (15.17 g, 0.15 mol, 20.96 mL) was dropped into the solution of ethylenediamine in CH_3OH (150 mL), then the reaction solution was added a solution of di-tert-butyl dicarbonate (10.88 g, 49.94 mmol) in CH_3OH at r.t, which was stirred for 5 hours at r.t. Then aqueous solution of 1M NaH_2PO_4 (60 mL) and ether (60 mL) were added and the two phases were separated. Then the aqueous phase was adjusted to approximately pH 9 with 1M NaOH and extracted with ethyl acetate. The combined organic phase was washed with brine, dried over Na_2SO_4 and

evaporated to get white solid (4.37 g, 55%).

{2-[(1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carbonyl)-amino]-ethyl}-carbamic acid tert-butyl ester (10-*N*-Boc). The same method as **11a** and the starting materials were **10** and **N-Boc**, white oil, 0.13 mg, 50% yield.

1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carboxylic acid (2-amino-ethyl)-amide (11f). To a solution of compound **10-*N*-Boc** (0.13 mg, 0.23 mmol) in saturated ethyl acetate solution of hydrochloric acid (3 mL) at 0 °C for overnight. Then the reaction solution was adjusted to approximately pH 7 with 1M $NaHCO_3$ and extracted with ethyl acetate. The combined organic phase was washed with brine, dried over Na_2SO_4 and the white oil was obtained which was further purified by column chromatography with dichloromethane/methanol (15:1, v/v) to afford compound **11f** as a white solid (0.094 mg, 87%). HRMS, ESI^+, m/z: calcd for $C_{28}H_{45}N_3O_3$ $(M+H)^+$ 472.3534, found 472.3527. ^1H NMR (300 MHz, $CDCl_3$) δ: 6.99 (2 H, m), 6.72 (1 H, d, *J* = 7.8 Hz), 6.52 (1 H, d, *J* = 1.5 Hz), 6.25 (1 H, d, *J* = 1.5 Hz), 4.29 (2 H, m), 4.11 (2 H, m), 3.44 (2 H, m), 2.96 (2 H, m), 2.15 (3 H, s), 1.95 (6 H, m), 1.56 (4 H, m), 1.31 (3 H, m), 0.89 (6 H, t, *J* = 7.2 Hz), 0.63 (6 H, m). ^{13}C NMR(75 MHz, $CDCl_3$) δ: 160.76, 154.47, 140.12, 131.38, 130.90, 125.93, 125.08, 125.48, 112.45, 109.67, 74.39, 68.15, 64.61, 44.80, 43.73, 39.10, 35.94, 30.93, 30.14, 29.70, 23.73, 17.22, 16.81, 8.54, 8.02.

1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carboxylic acid (3-morpholin-4-yl-propyl)-amide (11g). The same method as **11a** and the starting materials were **10** and 3-morpholin-4-yl-propylamine, Yellow oil, 0.14 g, 52%

yield. HRMS, ESI$^+$, m/z: calcd for $C_{33}H_{53}N_3O_4$ $(M+H)^+$ 556.4109, found 556.4104. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.02 (1 H, d, *J* = 8.4 Hz), 7.00 (1 H, s), 6.72 (1 H, d, *J* = 8.4 Hz), 6.57 (1 H, d, *J* = 1.8 Hz), 6.20 (1 H, d, *J* = 1.8 Hz), 4.32 (2 H, q, *J* = 6.9 Hz), 4.12 (2 H, t, *J* = 6.0 Hz), 3.59 (4 H, t, *J* = 4.2 Hz), 3.39 (2 H, m), 2.58 (6 H, m), 2.16 (3 H, s), 1.94 (6 H, m), 1.78 (2 H, m), 1.57 (4 H, m), 1.36 (3 H, t, *J* = 7.2 Hz), 0.90 (6 H, t, *J* = 7.5 Hz), 0.62 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 162.18, 154.26, 140.24, 131.32, 130.18, 125.81, 125.07, 124.30, 111.36, 109.58, 74.32, 66.04, 64.61, 58.09, 53.44, 44.83, 43.65, 38.89, 36.90, 30.94, 24.43, 17.27, 8.49, 8.01.

1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carboxylic acid bis-(2-cyano-ethyl)-amide (11h). To a solution of compound **10** (0.5 g, 1.16 mmol) in CH_3CN (10 mL) was added DMAP (0.59 g, 4.8 mmol) at 60 °C for 30 minutes. The mixture was added 4-nitrobenzenesulfonyl chloride (0.28 g, 1.3 mmol) and was heated up to 70 °C for 3 hours. Then 3-(2-cyano-ethylamino)-propionitrile (0.16 g, 1.3 mmol) was dropped into the mixture and was refluxed for 12 hours. Water was added and the two phases were separated. The aqueous phase was extracted with ethyl acetate and the combined organic phase was washed with water, dried over Na_2SO_4, and evaporated to get yellow oil which was further purified by column chromatography with ethyl acetate / hexane (1:5, v/v) as the eluent to get a white oil (0.43 g, 68%). HRMS, ESI$^+$, m/z: calcd for $C_{32}H_{46}N_4O_3$ $(M+Na)^+$ 557.3462, found 557.3465. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.02 (1 H, d, *J* = 8.4 Hz), 7.00 (1 H, s), 6.75 (1 H, d, *J* = 8.4 Hz), 6.60 (1 H, d, *J* = 1.5 Hz), 6.04 (1 H, d, *J* = 1.5 Hz), 4.12 (4 H, m), 3.83 (4 H, t, *J* = 6.6 Hz), 2.67 (4 H, t, *J* = 6.6 Hz), 2.18 (3 H, s), 1.95 (6 H, m), 1.56 (4 H, m), 1.37 (3 H, t, *J* = 7.2 Hz), 0.91 (6 H, t, *J* = 7.5 Hz), 0.67 (6 H, t, *J* = 7.2 Hz). ^{13}C

NMR (75 MHz, $CDCl_3$) δ: 165.07, 154.55, 139.72, 131.51, 130.16, 125.95, 123.80, 122.25, 117.50, 112.40, 109.67, 74.34, 64.68, 45.07, 43.11, 36.91, 30.95, 17.12, 8.64, 8.01.

1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carboxylic acid allyl-methyl-amide (11i). The same method as **11h**, **11i** was prepared from **10** and allyl-methyl-amine. white oil, 0.42 g, 76% yield. HRMS, ESI^+, m/z: calcd for $C_{30}H_{46}N_2O_3$ $(M+H)^+$ 483.3581, found 483.3574. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.03 (1 H, d, *J* = 8.1 Hz), 7.01 (1 H, s), 6.72 (1 H, d, *J* = 8.1 Hz), 6.50 (1 H, d, *J* = 1.5 Hz), 6.08 (1 H, d, *J* = 1.5 Hz), 5.80 (1 H, m), 5.19 (2 H, m), 4.11 (6 H, m), 3.02 (3 H, s), 2.17 (3 H, s), 1.94 (6 H, m), 1.58 (4 H, m), 1.34 (3 H, t, *J* = 7.2 Hz), 0.91 (6 H, t, *J* = 7.5 Hz), 0.65 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 162.87, 154.38, 140.18, 133.34, 130.40, 125.97, 124.94, 122.69, 117.13, 112.68, 109.47, 74.34, 64.69, 45.06, 42.96, 36.89, 30.94, 17.25, 16.69, 8.67, 8.01.

1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carboxylic acid 1-methyl-prop-2-ynyl ester (12a). To a solution of compound **10** (0.2 g, 0.47 mmol) in $CHCl_3$ (10 mL) was added DMAP (11.37 mg, 0.093 mmol), the mixture was activated with stirring at r.t for 1 hours. Then EDCI (99.11 mg, 0.52 mmol) was added at 0 °C for 1 hours, the mixture was reacted for 2 hours at r.t. After that 3-Butyn-2-ol was dropped into the mixture and was refluxed for 12 hours and cooled, then the mixture was evaporated solvent to afford crude product which was further purified to afford compound **12a** as yellow oil (87 mg, 35%). HRMS, ESI^+, m/z: calcd for $C_{30}H_{43}NO_4$ $(M+Na)^+$ 504.3084, found 504.3083. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.02 (1 H, d, *J* = 7.8 Hz), 7.00 (1 H, s), 6.75 (1 H, s), 6.71 (1 H, d, *J* = 7.8 Hz), 6.57 (1 H, s), 4.28 (2 H, q, *J* = 7.2 Hz), 4.14 (2 H, t, *J* = 6.0

Hz), 3.76 (1 H, s), 2.18 (3 H, s), 1.95 (6 H, m), 1.56 (7 H, m), 1.35 (3 H, t, *J* = 6.9 Hz), 0.91 (6 H, t, *J* = 7.5 Hz), 0.65 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 160.07, 154.47, 139.99, 131.94, 130.22, 126.53, 128.88, 117.30, 109.60, 72.56, 64.69, 59.59, 44.87, 44.03, 30.94, 21.42, 17.05, 8.55, 8.00.

1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carboxylic acid but-3-ynyl ester (12b). The same method as **12a** and the starting materials were **10** and 3-butyn-1-ol. Yellow oil, 98 mg, 43% yield. HRMS, ESI$^+$, m/z: calcd for $C_{30}H_{43}NO_4$ $(M+Na)^+$ 504.3084, found 504.3078. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.02 (1 H, d, *J* = 6.6 Hz), 7.00 (1 H, s), 6.73 (1 H, d, *J* = 6.6 Hz), 6.69 (1 H, d, *J* = 2.1 Hz), 6.58 (1 H, d, *J* = 2.1 Hz), 4.28 (4 H, m), 4.14 (2 H, t, *J* = 6.0 Hz), 2.60 (2 H, m), 2.18 (3 H, s), 1.93 (7 H, m), 1.54 (4 H, m), 0.90 (6 H, t, *J* = 7.5 Hz), 0.65 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 160.15, 154.47, 139.95, 131.89, 130.23, 126.87, 125.87, 125.14, 1117.87, 109.59, 80.29, 74.34, 69.84, 64.68, 61.44, 44.87, 44.01, 36.90, 30.95, 19.18, 17.07, 8.55, 8.00.

1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-4*H*-pyrrole-2-carboxylic acid 4-ethoxycarbonylmethyl-phenyl ester (13a). The same method as **12a**, **13a** was prepared from **10** and (4-Hydroxy-phenyl)-acetic acid methyl ester. Yellow oil, 103 mg, 37% yield. HRMS, ESI$^+$, m/z: calcd for $C_{35}H_{47}NO_6$ $(M+NH_4)^+$ 595.3742, found 595.3741. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.28 (2 H, m), 7.11 (4 H, m), 6.93 (1 H, d, *J* = 2.4 Hz), 6.76 (1 H, d, *J* = 8.4 Hz), 6.68 (1 H, d, *J* = 2.4 Hz), 4.29 (2 H, q, *J* = 7.2 Hz), 4.15 (2 H, t, *J* = 6.0 Hz), 3.68 (3 H, s), 3.62 (2 H, s), 2.21 (3 H, s), 1.97 (6 H, m), 1.56 (4 H, m), 1.37 (3 H, t, *J* = 7.2 Hz), 0.91 (6 H, t, *J* = 7.5 Hz), 0.69 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 172.43, 154.50, 149.81, 139.89, 132.40, 131.04, 130.19, 125.87, 125.89, 122.12, 118.82,

115.49, 109.69, 74.64, 64,73, 52.11, 44.92, 44.15, 40.64, 36.84, 30.90, 17.00, 8.57, 8.01.

1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-4*H*-pyrrole-2-carboxylic acid (4-methoxy-phenyl)-amide (13b). The same method as **11a**, **13b** was prepared from **10** and 4-methoxy-phenylamine. Yellow oil, 0.57 g, 46% yield. HRMS, ESI^+, m/z: calcd for $C_{33}H_{43}F_3N_2O_4$ $(M+Na)^+$ 611.3067, found 611.3072. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.56 (2 H, m), 7.17 (2 H, m), 7.04 (1 H, d, *J* = 7.8 Hz), 7.02 (1 H, s), 6.77 (1 H, d, *J* = 7.8 Hz), 6.68 (1 H, d, *J* = 1.8 Hz), 6.35 (1 H, d, *J* = 1.8 Hz), 4.37 (2 H, q, *J* = 6.9 Hz), 4.15 (2 H, t, *J* = 6.0 Hz), 2.18 (3 H, s), 1.97 (6 H, m), 1.56 (4 H, m), 1.42 (3 H, t, *J* = 7.2 Hz), 0.91 (6 H, t, *J* = 7.5 Hz), 0.68 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 160.35, 154.59, 139.99, 136.90, 131.72, 130.28, 126.45, 125.28, 123.75, 121.22, 117.32, 112.22, 109.61, 74.35, 64.68, 44.98, 44.03, 36.90, 30.97, 30.40, 17.23, 8.53, 8.00.

1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carboxylic acid (4-trifluoromethyl-phenyl)-amide (13c). The same method as **11a** and the starting materials were **10** and 4-trifluoromethyl-phenylamine, yellow oil, 0.68 g, 51% yield. HRMS, ESI^+, m/z: calcd for $C_{33}H_{43}F_3N_2O_3$ $(M+H)^+$ 573.32, found 573.3526. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.23 (2 H, m), 7.03 (1 H, d, *J* = 8.4 Hz), 7.02 (1 H, s), 6.93 (1 H, s), 6.86 (1 H, m), 6.74 (1 H, d, *J* = 8.4 Hz), 6.69 (1 H, d, *J* = 2.1 Hz), 6.58 (1 H, d, *J* = 2.1 Hz), 4.26 (2 H, q, *J* = 8.1 Hz), 4.14 (2 H, d, *J* = 2.1 Hz), 2.18 (3 H, s), 1.96 (6 H, m), 1.57 (4 H, m), 1.35 (3 H, t, *J* = 8.1 Hz), 0.91 (6 H, t, *J* = 7.5 Hz), 0.66 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 154.78, 140.09, 130.24, 126.45, 125.89, 117.30, 109.58, 74.36, 64.69, 59.60, 44.87, 43.94, 36.89, 30.40, 17.08, 14.45, 8.55, 8.00.

1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-p

yrrole-2-carboxylic acid (4-ethoxy-phenyl)-amide (13d). The same method as **11a**, **13d** was prepared from **10** and 4-ethoxy-phenylamine. Brown solid, 1.08 g, 85% yield. HRMS, ESI^+, m/z: calcd for $C_{34}H_{48}N_2O_4$ $(M+H)^+$ 549.3687, found 549.3679. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.40 (2 H, m), 7.05 (1 H, d, *J* = 8.4 Hz), 7.03 (1 H, s), 6.85 (2 H, m), 6.76 (1 H, d, *J* = 8,4 Hz), 6.64 (1 H, d, *J* = 1.8 Hz), 6.30 (1 H, d, *J* = 1.8 Hz), 4.36 (2 H, q, *J* = 6.9 Hz), 4.15 (2 H, t, *J* = 6.0 Hz), 4.00 (2 H, q, *J* = 6.9 Hz), 2.19 (3 H, s), 1.96 (6 H, m), 1.56 (4 H, m), 1.37 (3 H, t, *J* = 6.9 Hz), 0.88 (9 H, m), 0.67 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 159.81, 155.47, 154.54, 140.13, 131.42, 125.95, 124.27, 121.81, 114.78, 111..65, 109.58, 74.35, 64.68, 63.68, 44.97, 43.89, 37.11, 30.28, 27.09, 17.29, 16.87, 8.56, 8.01.

2-[(1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carbonyl)-amino]-3-methyl-pentanoic acid methyl ester (14a). The same method as **11a** and the starting materials were **10** and L-isoleucine methyl ester hydrochloride, white oil, 0.95 g, 74% yield. HRMS, ESI^+, m/z: calcd for $C_{33}H_{52}N_2O_5$ $(M+H)^+$ 557.3949, found 557.3939. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.03 (1 H, d, *J* = 8.4 Hz), 7.02 (1 H, s), 6.75 (1 H, d, *J* = 8.4 Hz), 6.56 (1 H, d, *J* = 1.8 Hz), 6.29 (1 H, d, *J* = 1.8 Hz), 4.67 (1 H, m), 4.29 (2 H, q, *J* = 7.8 Hz), 4.15 (3 H, t, *J* = 6.0 Hz), 3.74 (3 H, s), 2.19 (3 H, s), 1.96 (8 H, m), 1.56 (5 H, m), 1.34 (3 H, t, *J* = 6.9 Hz), 0.92 (12 H, m), 0.66 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR(75 MHz, $CDCl_3$) δ: 172.96, 161.58, 154.48, 140.12, 131.18, 130.28, 125.94, 125.04, 111.54, 109.58, 74.35, 64.68, 56.14, 52.03, 44.92, 43.74, 38.22, 25.41, 17.21, 16.84, 11.58, 8.56, 8.01.

[(1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carbonyl)-methyl-amino]-acetic acid ethyl ester (14b). The same method as

11h and the starting materials were **10** and sarcosine ethyl ester hydrochloride. White oil, 0.06 g, 29% yield. HRMS, ESI^+, m/z: calcd for $C_{31}H_{48}N_2O_5$ $(M+H)^+$ 529.3636, found 529.3627. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.04 (1 H, d, *J* = 8.4 Hz), 7.01 (1 H, s), 6.72 (1 H, d, *J* = 8.4 Hz), 6.52 (1 H, d, *J* = 1.5 Hz), 6.07 (1 H, d, *J* = 1.5 Hz), 4.15 (8 H, m), 3.15 (3 H, s), 2.17 (3 H, s), 1.94 (6 H, m), 1.62 (4 H, m), 1.34 (3 H, t, *J* = 7.2 Hz), 1.25 (3 H, t, *J* = 6.9 Hz), 0.91 (6 H, t, *J* = 7.5 Hz), 0.65 (6 H, t, *J* = 7.5 Hz). ^{13}C NMR(75 MHz, $CDCl_3$) δ: 169.40, 154.39, 140.11, 130.37, 126.00, 124.96, 123.04, 109.50, 74.34, 64.69, 61.17, 45.07, 43.07, 36.89, 30.94, 17.14, 14.14, 8.66, 8.00.

2-[(1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carbonyl)-amino]-succinic acid dimethyl ester (14c). The same method as **11a**, **14c** was prepared from **10** and L-aspartic acid dimethyl ester hydro-chloride. Yellow oil, 0.62 g, 78% yield. HRMS, ESI^+, m/z: calcd for $C_{32}H_{48}N_2O_7$ $(M+H)^+$ 573.3534, found 573.3526. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.03 (1 H, d, *J* = 8.4 Hz), 7.02 (1 H, s), 6.68 (1 H, d, *J* = 8.4 Hz), 6.56 (1 H, d, *J* = 1.8 Hz), 6.32 (1 H, d, *J* = 1.8 Hz), 4.95 (1 H, m), 4.29 (2 H, m), 4.15 (2 H, t, *J* = 6.0 Hz), 3.77 (3 H, s), 3.68 (3 H, s), 2.95 (2 H, m), 2.18 (3 H, s), 1.96 (6 H, m), 1.64 (4 H, m), 1.38 (3 H, t, *J* = 7.2 Hz), 0.91 (6 H, t, *J* = 7.5 Hz), 0.66 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ6: 171.66, 161.39, 154.47, 140.05, 131.43, 130.23, 125.87, 125.31, 125.13, 112.13, 109.58, 74.35, 64.68, 52.80, 52.03, 48.29, 44.86, 43.84, 36.88, 36.45, 30.92, 30.28, 17.19, 8.54, 8.02.

2-[(1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carbonyl)-amino]-3-methyl-pentanoic acid (15a). To a solution of compound **14a** (0.2 g, 0.36 mmol) in THF (10 mL) and water (2 mL) was added $LiOH{\cdot}2H_2O$ (150 mg,

3.55 mmol) at r.t. Then the mixture was stirred for overnight. The reacted solution was added water (20 mL) which was adjusted to approximately pH 4 with 1M HCl and extracted with ethyl acetate. The combined organic phase was washed with brine and dried over Na_2SO_4. Then the organic phase was evaporated and the crude product was obtained which was further purified by column chromatography using dichloromethane/methanol (40:1, v/v) to afford compound **15a** as a yellow oil (0.16 g, 83%). HRMS, ESI^+, m/z: calcd for $C_{32}H_{50}N_2O_5$ $(M+H)^+$ 543.3792, found 543.3792. ^1H NMR (300 MHz, $CDCl_3$) δ: 6.99 (1 H, s), 6.71 (1 H, d, *J* = 9.0 Hz), 6.54 (1 H, d, *J* = 9.0 Hz), 6.42 (1 H, s), 6.34 (1 H, s), 4.12 (5 H, m), 2.17 (3 H, s), 1.92 (6 H, m), 1.56 (6 H, m), 1.23 (3 H, t, *J* = 6.9 Hz), 1.05 (1 H, m), 0.85 (12 H, m), 0.62 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR(75 MHz, $CDCl_3$) δ: 173.15, 162.38, 154.46, 140.02, 131.16, 130.16, 126.01, 125.05, 123.79, 112.22, 109.66, 74.34, 64.63, 59.43, 44.87, 36.94, 30.91, 25.29, 17.14, 11.34, 8.57, 8.00.

[(1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carbonyl)-methyl-amino]-acetic acid (15b). The same method as **15a** and the starting materials were **14b**. Pink oil, 0.1 g, 77% yield. HRMS, ESI^+, m/z: calcd for $C_{29}H_{44}N_2O_5$ $(M+H)^+$ 501.3323, found 501.3313. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.01 (1 H, d, *J* = 8.4 Hz), 6.99 (1 H, s), 6.72 (1 H, d, *J* = 8.4 Hz), 6.52 (1 H, d, *J* = 1.5 Hz), 6.09 (1 H, d, *J* = 1.5 Hz), 4.15 (6 H, m), 3.15 (3 H, s), 2.17 (3 H, s), 1.95 (6 H, m), 1.58 (4 H, m), 1.38 (3 H, m), 0.90 (6 H, t, *J* = 7.5 Hz), 0.65 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR(75 MHz, $CDCl_3$) δ: 171.35, 165.62, 154.36, 140.02, 130.77, 125.98, 125.04, 122.32, 109.61, 74.35, 64.65, 45.04, 43.12, 36.78, 30.84, 29.70, 17.01, 8.64, 7.99.

2-[(1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1

***H*-pyrrole-2-carbonyl)-amino]-succinic acid 1-methyl ester (15c).** The same method as **15a**, **15c** was prepared from **14c**. White oil, 0.13 g, 62% yield. HRMS, ESI^+, m/z: calcd for $C_{31}H_{46}N_2O_7$ $(M+H)^+$ 559.3378, found 559.3366. ^1H NMR (300 MHz, $CDCl_3$) δ: 6.99 (1 H, d, *J* = 8.4 Hz), 6.85 (1 H, s), 6.75 (1 H, d, *J* = 8.4 Hz), 6.59 (1 H, d, *J* = 1.8 Hz), 6.33 (1 H, d, *J* = 1.8 Hz), 5.30 (1 H, m), 4.27 (2 H, m), 4.16 (2 H, t, *J* = 6.0 Hz), 3.71 (3 H, s), 3.05 (2 H, m), 2.17 (3 H, s), 1.96 (6 H, m), 1.64 (4 H, m), 1.38 (3 H, t, *J* = 7.2 Hz), 0.91 (6 H, t, *J* = 7.5 Hz), 0.66 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ6: 171.66, 161.39, 154.47, 140.05, 131.43, 130.23, 125.87, 125.31, 125.13, 112.13, 109.58, 74.35, 64.68, 52.80, 52.03, 48.29, 44.86, 43.84, 36.88, 36.45, 30.92, 30.28, 17.19, 8.54, 8.02.

2-[(1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carbonyl)-amino]-propionic acid (15d). The same method as **11a** and the starting materials were **10** and L-alanine methyl ester hydrochloride. Intermediate **77** was obtained through the reaction. Then the same method as **15a**, **15d** was prepared from **77**. Yellow oil, 0.1 g, 71% yield. HRMS, ESI^+, m/z: calcd for $C_{29}H_{44}N_2O_5$ $(M+H)^+$ 501.3323, found 501.3317. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.02 (1 H, d, *J* = 8.1 Hz), 7.00 (1 H, s), 6.74 (1 H, d, *J* = 8.1 Hz), 6.55 (1 H, d, *J* = 2.1 Hz), 6.28 (1 H, d, *J* = 2.1 Hz), 4.61 (1 H, m), 4.33 (2 H, q, *J* = 6.6 Hz), 4.16 (2 H, t, *J* = 6.0 Hz), 2.18 (3 H, s), 1.93 (6 H, m), 1.58 (4 H, m), 1.49 (3 H, d, *J* = 7.2 Hz), 1.35 (3 H, t, *J* = 6.6 Hz), 0.91 (6 H, m), 0.65 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 175.38, 162.20, 154.45, 140.09, 131.57, 130.27, 125.53, 123.00, 112.48, 109.62, 74.78, 64.67, 48.32, 44.91, 43.88, 36.79, 30.25, 17.91, 17.18, 8.53, 7.99.

1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carboxylic acid (1-hydroxymethyl-2-methyl-butyl)-amide (16a). $NaBH_4$ (0.14 g,

3.8 mmol) was added to a solution of compound **14a** (0.21 g, 0.38 mmol) in CH_3OH (10 mL) at r.t and the mixture was stirred for 0.5 hours. Water was added and the two phases were separated. Then the aqueous phase was extracted with ethyl acetate and the combined organic phase which was dried over Na_2SO_4, and evaporated to get white oil which was further purified by column chromatography using ethyl acetate / hexane (1:1, v/v) as eluent to get a white solid (0.18 g, 89%). HRMS, ESI^+, m/z: calcd for $C_{32}H_{52}N_2O_4$ $(M+H)^+$ 529.4000, found 529.3998. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.03 (1 H, d, *J* = 8.4 Hz), 7.02 (1 H, s), 6.75 (1 H, d, *J* = 8.4 Hz), 6.56 (1 H, d, *J* = 1.8 Hz), 6.29 (1 H, d, *J* = 1.8 Hz), 4.67 (1 H, m), 4.29 (2 H, q, *J* = 7.8 Hz), 4.15 (3 H, t, *J* = 6.0 Hz), 3.73 (2 H, m), 2.19 (3 H, s), 1.96 (8 H, m), 1.56 (5 H, m), 1.34 (3 H, t, *J* = 6.9 Hz), 0.92 (12 H, m), 0.66 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR(75 MHz, $CDCl_3$) δ: 172.96, 161.58, 154.48, 140.12, 131.18, 130.28, 125.94, 125.04, 111.54, 109.58, 74.35, 64.68, 56.14, 52.03, 44.92, 43.74, 38.22, 36.89, 30.93, 25.41, 17.21, 16.84, 11.58, 8.56, 8.01.

1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carboxylic acid (2-hydroxy-ethyl)-methyl-amide (16b). The same method as **16a**, **16b** was prepared from **14b**. Yellow oil, 0.09 g, 60% yield. HRMS, ESI^+, m/z: calcd for $C_{29}H_{46}N_2O_4$ $(M+H)^+$ 487.3530, found 487.3521. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.02 (1 H, d, *J* = 8.4 Hz), 7.01 (1 H, s), 6.72 (1 H, d, *J* = 8.4 Hz), 6.55 (1 H, d, *J* = 1.5 Hz), 6.11 (1 H, d, *J* = 1.5 Hz), 4.13 (4 H, m), 3.81 (2 H, t, *J* = 4.8 Hz), 3.62 (2 H, t, *J* = 4.8 Hz), 3.16 (3 H, s), 2.17 (3 H, s), 1.95 (6 H, m), 1.58 (4 H, m), 1.34 (3 H, t, *J* = 7.2 Hz), 0.91 (6 H, t, *J* = 7.5 Hz), 0.66 (6 H, t, *J* = 7.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 166.41, 154.42, 140.08, 130.54, 128.84, 125.99, 124.99, 123.34, 113.89, 109.49, 74.36, 64.66, 61.17, 51.69, 45.07, 43.11,

36.88, 30.78, 17.17, 16.81, 8.65, 8.01.

1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carboxylic acid (3-hydroxy-1-hydroxymethyl-propyl)-amide (16c). The same method as **16a** and the starting materials were **14c**. White oil, 0.07 g, 54% yield. HRMS, ESI^+, m/z: calcd for $C_{30}H_{48}N_2O_5$ $(M+H)^+$ 517.3636, found 517.3624. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.02 (1 H, d, *J* = 8.1 Hz), 7.00 (1 H, s), 6.73 (1 H, d, *J* = 8.1 Hz), 6.59 (1 H, d, *J* = 1.5 Hz), 6.26 (1 H, d, *J* = 1.5 Hz), 4.32 (2 H, m), 4.20 (1 H, m), 4.13 (2 H, t, *J* = 6.0 Hz), 3.73 (4 H, m), 2.17 (3 H, s), 1.95 (6 H, m), 1.58 (4 H, m), 1.35 (3 H, t, *J* = 7.2 Hz), 0.90 (6 H, t, *J* = 7.5 Hz), 0.64 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 163.13, 154.44, 140.15, 131.42, 130.24, 125.94, 125.21, 125.42, 111.95, 109.61, 74.49, 65.39, 64.66, 58.74, 48.17, 44.88, 43.88, 36.85, 34.56, 30.91, 30.19, 17.24, 8.53, 8.01.

1-Ethyl-5-{1-ethyl-1-[4-(3-ethyl-3-hydroxy-pentyloxy)-3-methyl-phenyl]-propyl}-1*H*-pyrrole-2-carboxylic acid (2-hydroxy-1-methyl-ethyl)-amide (**16d**). The same method as **16a**, **16d** was prepared from **77**. Yellow oil, 0.4 g, 67% yield. HRMS, ESI^+, m/z: calcd for $C_{29}H_{46}N_2O_4$ $(M+H)^+$ 487.3530, found 487.3525. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.02 (1 H, d, *J* = 8.4 Hz), 7.00 (1 H, s), 6.74 (1 H, d, *J* = 8.4 Hz), 6.59 (1 H, d, *J* = 1.5 Hz), 6.19 (1 H, d, *J* = 1.5 Hz), 4.31 (2 H, q, *J* = 6.9 Hz), 4.14 (2 H, t, *J* = 6.0 Hz), 3.74 (1 H, m), 3.63 (2 H, m), 2.18 (3 H, s), 1.95 (6 H, m), 1.57 (4 H, m), 1.39 (3 H, t, *J* = 6.9 Hz), 1.21 (3 H, d, *J* = 6.9 Hz), 0.91 (6 H, t, *J* = 7.5 Hz), 0.67 (6 H, t, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 162.83, 154.48, 140.18, 5.93, 131.28, 130.27, 123.78, 111.58, 109.54, 74.38, 67.82, 64.66, 47.84, 44.90, 43.80, 36.88, 30.93, 17.23, 8.54, 8.01.

Cell Lines and Cell Cultures. The cell lines (HL-60, HepG2, MCF7, PC3, Caco2 and L02)

were purchased from ATCC. HL-60 cells, HepG2 cells and L02 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. MCF7 cells and PC3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Caco2 cells were cultured in DMEM containing 10% FBS, 1% nonessential amino acid (NEAA), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2.

Inhibition on Cell Proliferation by MTT Assay. 200 μL 1×10^5 cells/mL of cells suspension was added to each well of 96-well culture plates and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 24 hours. Then the culture medium was discarded and the cells were treated with different concentrations of each compound dissolved in 200 μL serum-free medium and incubated at 37 °C for 48 hours. After that, 20 μL of methylthiazolyldiphenyl-tetrazolium bromide (MTT) was added to each well and incubated for additional 4 hours. The medium was replaced by 150 mL DMSO to solubilize the purple formazan crystals and the absorbance was measured on a microplate reader at 570 nm. The inhibition of cell growth was evaluated as the ratio of the absorbance of the sample to that of the control. Finally, the IC_{50} value of each compound was calculated using GraphPad Prism 5.0.

Measurement of VDR activation by Leukemic Cell Differentiation. HL-60 cells were plated 1×10^4 cells/mL in 96-well plates and were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 24 hours. Then, ligands were dissolved in DMSO, diluted to different concentrations with the RPMI-1640 medium (the final concentration of DMSO was

0.1%), and were incubated with HL-60 cells for 96 hours. After incubation, cells were harvested for determining the differentiation level.

The nitroblue tetrazolium (NBT) and 12-O-tetradecanoylphorbol-13-acetate (TPA) was added to the cells, with the final concentrations of 0.1% NBT and 100 ng/mL TPA. After the mixture was incubated at 37 °C for another 3 hours, NBT was bio-reduced and turned to blue-black by the differentiated cells. The differentiated cells were counted and percentages of differentiated cells was determined. Finally, the EC_{50} value of each ligand was calculated using GraphPad Prism 5.0 (GraphPad Inc., La Jolla, CA, USA).

Transfection and Transactivation Assay. Human kidney HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone™) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin (HyClone™), and 0.1 mg/mL streptomycin (HyClone™) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Transfections of 100 ng of pGL4.27-SPP×3-Luci reporter plasmid containing three copies of the mouse osteopontin VDRE (5'-GGTTCAcgaGGTTCA-3'), 20 ng of pRL-TK (Promega), 30 ng of pENTER-CMV-hVDR, and 30 ng of pENTER-CMV-hRXRα for each well of a 48-well plate were performed using a ExFect Transfection Reagent Kit (Vazyme, Nanjing, China) according to the manufacturer's protocol. After a 12 hours incubation, the cells were treated with either the ligand or DMSO vehicle and cultured for 24 hours. Cells in each well were harvested with a cell lysis buffer, and the luciferase activity was measured with a dual luciferase assay kit (Promega, WI, U.S.). Transactivation measured by the luciferase activity was normalized with the internal Renilla luciferase expression.

VDR Binding Characteristics of Phenyl-Pyrrolyl Pentane Derivatives. Full-length

human VDR, Fluormone™ VDR Red, and VDR Red Screening Buffer were purchased from Invitrogen (PolarScreen™ Vitamin D Receptor Competitor Assay, Invitrogen). Assays were performed in 384-well black polypropylene plates. The test compounds and calcitriol were dissolved in DMSO and diluted with VDR Red Screening Buffer with 1% DMSO to different concentrations. Receptor/tracer complex was added to ligands with various concentrations or DMSO solvent control to final concentrations of 0.7 nM of VDR and 1 nM of Fluormone VDR Red. The mixture was incubated for 4 hours at room temperature. Then fluorescence polarization was measured on an Ultra384 microplate reader (Tecan) using a 535 nm excitation filter (25 nm bandwidth) and 590 nm emission filter (20 nm bandwidth). Finally, the IC_{50} value of each compound was calculated using GraphPad Prism 5.0.

Cell Cycle Assay and Cell Apoptosis. MCF7 and HepG2 cells were plated in 6-well plate and treated with each compound (1 μM) for 24 hours. For cell cycle assay, cells were washed twice with ice-cold PBS, and treated with ice-cold 70% ethanol while vortexing fixed overnight at 4 °C. After centrifuging for 5 minutes at 1000 g, cells were washed with 1 mL ice-cold PBS, and stained with 0.5 mL mixture solution containing 465 μL 1× PBS, 25 μL propidium iodide (PI), and 10 μL RNaseA at 37 °C for 30 min. Data were collected using Attune NxT Acoustic Focusing Cytometer (Life Technologies, Carlsbad, CA, USA) and analyzed using the ModFit LT software (Verity Software House, Inc).

For analysis of cell apoptosis, the cells were collected and suspended in 0.5 mL of 1 × binding buffer, followed by two washes with ice-cold PBS. The Annexin V-FITC Apoptosis Detection Kit (Vazyme, Nanjing, China) according to the manufacturer's protocol was used for apoptosis assay. The stained cells were analyzed by a BD Accuri™ C6 Plus flow

cytometer equipped with BD Accuri C6 Software (Becton Dickinson, San Jose, CA).

Western Blot. Cells were washed twice with PBS and extracted using 1% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Na-deoxycholate, 1 mM phenyl methyl sulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitors (Roche). Protein lysates (25 μg) were separated by SDS-PAGE and transferred onto PVDF membranes (Merck Millipore). After blocking, the membranes were incubated at 4 °C overnight with the following primary antibodies: anti-VDR (1:1000, Santa Cruz Biotechnology), anti-p21 (1:500, Bioworld), anti-p27 (1:500, Santa Cruz Biotechnology) and anti-β-actin (1:5000, Cell Signalling Technology).

Cellular Uptake and Metabolic Stability. For measurement of cellular uptake of compounds, MCF7 cells were plated in 12-well plate at a density 2×10^5 cells/well. After incubated for 24 hours, the cells were incubated with 1 μM calcitriol, sw22, LG190119, **11b**, **11d** and **11g** for 10 or 30 minutes, respectively. After the incubation, compounds were extracted using three volumes of chloroform/methanol (3:1, v/v) from cells and medium separately. The organic phase was recovered and dried under reduced pressure. The residue was dissolved in acetonitrile and applied to HPLC for analyze. For estimation of the stability of compounds in the culture medium, the MCF7 cells plated in 12-well plate at a density 2×10^5 cells/well were incubated with 1 μM calcitriol, sw22, LG190119, **11b**, **11d** and **11g** for 24 hours. After the incubation, compounds were extracted using three volumes of chloroform/methanol (3:1, v/v) from cells and medium together. After evaporating the organic phase, the residue was dissolved in acetonitrile and applied to HPLC for analysis.

***In vivo* Calcemic Activity Assay.** ICR mice (obtained from Shanghai Silaike Laboratory

Animal Ltd, 7 weeks, 18-22 g) were housed under normal lighting and received a vitamin D deficient, calcium replete diet (0.2% calcium, 1% phosphorus, 2000 units vitamin) and water for 7 days prior to treatment with various concentrations of compounds. Compounds dissolved in ethanol/EL/saline (1:1:18) respectively were injected intraperitoneally daily for 7 days. Mice were fasted overnight and venous blood were withdrawn on the 8th day. Then, total calcium ion concentration was colorimetric determined by Methyl Timolol Blue (MTB) method in venous blood using a calcium assay kit (Nanjing Jiancheng Bioengineering institute, China).

In vivo **Antitumor Activity Assay.** The female BALB/c nude mice weighing approximately 20 g obtained from the Beijing Vital River Laboratory Animal Technology Co.Ltd (Beijing, China). To generate the MCF7 tumor-bearing mice model, 1×10^7 cells were inoculated in the left fourth mammary fat pad of nude mice. Upon reaching an average tumor volume of 50 mm^3 (7 days after injecting), animals were randomly divided into eight groups (1) saline; (2) vehicle; (3) calcitriol; (4) sw22; (5) LG190119; (6) **11b**; (7) **11d**; (8) **11g** (n=7). sw22, LG190119, **11b**, **11d**, **11g** (10 mg/kg were prepared in ethanol/EL/saline = 1:1:18), calcitriol (0.5 μg/kg were prepared in ethanol/EL/saline = 1:1:18), vehicle (ethanol/EL/saline = 1:1:18) and saline were given intraperitoneal injection every other day. Body weight was also monitored every other day during the experiments. Tumor volumes were measured periodically throughout the experiments using the digital caliper and the tumor volume was calculated by the following formula: tumor volume (mm^3) = (L*W*W)/2, where L= length and W= width in mm. One day after the last injection, bloods were collected and calcium ion concentration was determined using calcium assay kit. Then, tumors were

collected from the euthanized mice and washed with saline several times. Furthermore, immunohistochemistry of Ki-67 was performed on paraffin sections by rabbit–anti-mouse Ki-67 at 1:200 (Cell Signalling Technology Inc., Beverly, MA), stained with the HRP-DAB SPlink Detection Kit (ZSGB-Bio, Beijing, China). Apoptosis in tumor was evaluated by TUNEL staining by the In Situ Cell Death Detection Kit (Roche Diagnostics, USA) according to the manufacturer's protocol. Ki-67 staining and TUNEL staining were photographed using Microscope BX53 (Olympus) and analyzed by ImageJ 1.51n with ImmunoRatio 1.0 c plugin. The Animal Care and Use Committee of China Pharmaceutical University approved all of the animal studies and animal protocols.

Pharmacokinetics Study. Compounds **11b** and **11g** were dissolved in ethanol/EL/saline (1:1:18) for intraperitoneal and intravenous injection or ethanol/ medium chain triglycerides (MCT) (1:9) for oral administration. Male Sprague-Dawley (SD) rats weighing 180-220 g were injected intravenously or intraperitoneal, administrated orally at doses of 5 mg/kg (i.v.) or 20 mg/kg (p.o., i.p.), respectively (n = 4). After administration, blood samples were collected from posterior orbital venous plexus at the time point of 5.0 min, 15 min, 30 min, 1.0 h, 2.0 h, 4.0 h, 6.0 h, 8.0 h, 12.0 h, 24.0 h and 48.0 h, and then immediately centrifuged (12,000 rpm, 10 min) to obtain plasma samples. The compound in plasma was extracted with acetonitrile and stored at -20 °C until tested by HPLC to measure the concentration. The pharmacokinetic parameters were calculated by Kinetica 4.4 software.

Statistical Analysis. Data were presented as the means ± SEM from at least three independent experiments. Student's unpaired t-test was used for two-group comparison in appropriate condition. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the website of ACS Publications and contains biological data, procedures for intermediates preparation, ^{1}H NMR, ^{13}C NMR and HRMS spectrums of phenyl-pyrrolyl pentane derivatives, and molecular formula strings for studied compounds (CSV).

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. $^{+}$These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

VDR, Vitamin D receptor; EC_{50}, half maximal (50%) effect concentration of a substance; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; IC_{50}, half maximal (50%) inhibitory concentration of a substance; PK, pharmacokinetic; LBD, ligand binding domain; TMS, tetramethylsilane; HRMS, high-resolution mass spectra; TLC, thin layer chromatography; UV, ultraviolet light; DMF, *N,N*- dimethylformamide; FBS, fetal bovine serum; DMEM, Dulbecco's Modified Eagle's Medium; NEAA, nonessential amino acid; NBT, nitroblue tetrazolium; TPA, 12-O-tetradecanoylphorbol-13-acetate; PI, propidium iodide; MCT, medium-chain triglycerides; EL, polyoxyethylenated castor oil

REFERENCES

(1) Deeb, K. K.; Trump, D. L.; Johnson, C. S. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nat Rev Cancer* **2007,** *7*, 684-700.

(2) Plum, L. A.; DeLuca, H. F. Vitamin D, disease and therapeutic opportunities. *Nat Rev Drug Discov* **2010,** *9*, 941-955.

(3) Khanal, R.; Nemere, I. Membrane receptors for vitamin D metabolites. *Crit Rev Eukaryot*

Gene Expr **2007,** *17*, 31-47.

(4) Feldman, D.; Krishnan, A. V.; Swami, S.; Giovannucci, E.; Feldman, B. J. The role of vitamin D in reducing cancer risk and progression. *Nat Rev Cancer* **2014,** *14*, 342-357.

(5) Sherman, M. H.; Yu, R. T.; Engle, D. D.; Ding, N.; Atkins, A. R.; Tiriac, H.; Collisson, E. A.; Connor, F.; Van Dyke, T.; Kozlov, S.; Martin, P.; Tseng, T. W.; Dawson, D. W.; Donahue, T. R.; Masamune, A.; Shimosegawa, T.; Apte, M. V.; Wilson, J. S.; Ng, B.; Lau, S. L.; Gunton, J. E.; Wahl, G. M.; Hunter, T.; Drebin, J. A.; O'Dwyer, P. J.; Liddle, C.; Tuveson, D. A.; Downes, M.; Evans, R. M. Vitamin D receptor-mediated stromal reprogramming suppresses pancreatitis and enhances pancreatic cancer therapy. *Cell* **2014,** *159*, 80-93.

(6) Garland, C. F.; Comstock, G. W.; Garland, F. C.; Helsing, K. J.; Shaw, E. K.; Gorham, E. D. Serum 25-hydroxyvitamin D and colon cancer: eight-year prospective study. *Lancet* **1989,** *2*, 1176-1178.

(7) Carballa, D. M.; Seoane, S.; Zacconi, F.; Perez, X.; Rumbo, A.; Alvarez-Diaz, S.; Larriba, M. J.; Perez-Fernandez, R.; Munoz, A.; Maestro, M.; Mourino, A.; Torneiro, M. Synthesis and biological evaluation of 1 alpha,25-Dihydroxyvitamin D-3 Analogues with a Long Side Chain at C12 and Short C17 Side Chains. *J Med Chem* **2012,** *55*, 8642-8656.

(8) Berger, U.; McClelland, R. A.; Wilson, P.; Greene, G. L.; Haussler, M. R.; Pike, J. W.; Colston, K.; Easton, D.; Coombes, R. C. Immunocytochemical determination of estrogen receptor, progesterone receptor, and 1,25-dihydroxyvitamin D3 receptor in breast cancer and relationship to prognosis. *Cancer Res* **1991,** *51*, 239-244.

(9) Bertone-Johnson, E. R.; Chen, W. Y.; Holick, M. F.; Hollis, B. W.; Colditz, G. A.; Willett, W. C.; Hankinson, S. E. Plasma 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D and risk

of breast cancer. *Cancer Epidemiol Biomarkers Prev* **2005,** *14*, 1991-1997.

(10) Hendrickson, W. K.; Flavin, R.; Kasperzyk, J. L.; Fiorentino, M.; Fang, F.; Lis, R.; Fiore, C.; Penney, K. L.; Ma, J.; Kantoff, P. W.; Stampfer, M. J.; Loda, M.; Mucci, L. A.; Giovannucci, E. Vitamin D receptor protein expression in tumor tissue and prostate cancer progression. *J Clin Oncol* **2011,** *29*, 2378-2385.

(11) Ahonen, M. H.; Tenkanen, L.; Teppo, L.; Hakama, M.; Tuohimaa, P. Prostate cancer risk and prediagnostic serum 25-hydroxyvitamin D levels (Finland). *Cancer Causes Control* **2000,** *11*, 847-852.

(12) Bao, Y.; Ng, K.; Wolpin, B. M.; Michaud, D. S.; Giovannucci, E.; Fuchs, C. S. Predicted vitamin D status and pancreatic cancer risk in two prospective cohort studies. *Br J Cancer* **2010,** *102*, 1422-1427.

(13) Erber, E.; Maskarinec, G.; Lim, U.; Kolonel, L. N. Dietary vitamin D and risk of non-Hodgkin lymphoma: the multiethnic cohort. *Br J Nutr* **2010,** *103*, 581-584.

(14) Zinser, G. M.; Sundberg, J. P.; Welsh, J. Vitamin D(3) receptor ablation sensitizes skin to chemically induced tumorigenesis. *Carcinogenesis* **2002,** *23*, 2103-2109.

(15) Garland, C. F.; Garland, F. C. Do sunlight and vitamin D reduce the likelihood of colon cancer? *Int J Epidemiol* **1980,** *9*, 227-231.

(16) Lappe, J. M.; Travers-Gustafson, D.; Davies, K. M.; Recker, R. R.; Heaney, R. P. Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial. *Am J Clin Nutr* **2007,** *85*, 1586-1591.

(17) Dalhoff, K.; Dancey, J.; Astrup, L.; Skovsgaard, T.; Hamberg, K. J.; Lofts, F. J.; Rosmorduc, O.; Erlinger, S.; Bach Hansen, J.; Steward, W. P.; Skov, T.; Burcharth, F.; Evans,

T. R. A phase II study of the vitamin D analogue seocalcitol in patients with inoperable hepatocellular carcinoma. *Br J Cancer* **2003,** *89*, 252-257.

(18) Srinivas, S.; Feldman, D. A phase II trial of calcitriol and naproxen in recurrent prostate cancer. *Anticancer Res* **2009,** *29*, 3605-3610.

(19) Beer, T. M.; Ryan, C. W.; Venner, P. M.; Petrylak, D. P.; Chatta, G. S.; Ruether, J. D.; Redfern, C. H.; Fehrenbacher, L.; Saleh, M. N.; Waterhouse, D. M.; Carducci, M. A.; Vicario, D.; Dreicer, R.; Higano, C. S.; Ahmann, F. R.; Chi, K. N.; Henner, W. D.; Arroyo, A.; Clow, F. W.; Investigators, A. Double-blinded randomized study of high-dose calcitriol plus docetaxel compared with placebo plus docetaxel in androgen-independent prostate cancer: a report from the ASCENT Investigators. *J Clin Oncol* **2007,** *25*, 669-674.

(20) Leyssens, C.; Verlinden, L.; Verstuyf, A. The future of vitamin D analogs. *Front Physiol* **2014,** *5*, 122.

(21) Christakos, S.; Dhawan, P.; Verstuyf, A.; Verlinden, L.; Carmeliet, G. Vitamin D: metabolism, molecular mechanism of action, and pleiotropic effects. *Physiol Rev* **2016,** *96*, 365-408.

(22) Galsky, M. D.; Vogelzang, N. J. Docetaxel-based combination therapy for castration-resistant prostate cancer. *Ann Oncol* **2010,** *21*, 2135-2144.

(23) Gross, C.; Stamey, T.; Hancock, S.; Feldman, D. Treatment of early recurrent prostate cancer with 1,25-dihydroxyvitamin D3 (calcitriol). *J Urol* **1998,** *159*, 2035-2039; discussion 2039-2040.

(24) Polek, T. C.; Murthy, S.; Blutt, S. E.; Boehm, M. F.; Zou, A.; Weigel, N. L.; Allegretto, E. A. Novel nonsecosteroidal vitamin D receptor modulator inhibits the growth of LNCaP

xenograft tumors in athymic mice without increased serum calcium. *Prostate* **2001,** *49*, 224-233.

(25) Watarai, Y.; Ishizawa, M.; Ikura, T.; Zacconi, F. C. M.; Uno, S.; Ito, N.; Mourino, A.; Tokiwa, H.; Makishima, M.; Yamada, S. Synthesis, biological activities, and X-ray crystal structural analysis of 25-Hydroxy-25(or 26)-adamantyl-17420(22),23-diynyl]-21-norvitamin D compounds. *J Med Chem* **2015,** *58*, 9510-9521.

(26) Boehm, M. F.; Fitzgerald, P.; Zou, A.; Elgort, M. G.; Bischoff, E. D.; Mere, L.; Mais, D. E.; Bissonnette, R. P.; Heyman, R. A.; Nadzan, A. M.; Reichman, M.; Allegretto, E. A. Novel nonsecosteroidal vitamin D mimics exert VDR-modulating activities with less calcium mobilization than 1,25-dihydroxyvitamin D3. *Chem Biol* **1999,** *6*, 265-275.

(27) Fujii, S.; Masuno, H.; Taoda, Y.; Kano, A.; Wongmayura, A.; Nakabayashi, M.; Ito, N.; Shimizu, M.; Kawachi, E.; Hirano, T.; Endo, Y.; Tanatani, A.; Kagechika, H. Boron cluster-based development of potent nonsecosteroidal vitamin D receptor ligands: direct observation of hydrophobic interaction between protein surface and carborane. *J Am Chem Soc* **2011,** *133*, 20933-20941.

(28) Ciesielski, F.; Sato, Y.; Chebaro, Y.; Moras, D.; Dejaegere, A.; Rochel, N. Structural basis for the accommodation of bis- and tris-aromatic derivatives in vitamin D nuclear receptor. *J Med Chem* **2012,** *55*, 8440-8449.

(29) Ma, Y.; Khalifa, B.; Yee, Y. K.; Lu, J.; Memezawa, A.; Savkur, R. S.; Yamamoto, Y.; Chintalacharuvu, S. R.; Yamaoka, K.; Stayrook, K. R.; Bramlett, K. S.; Zeng, Q. Q.; Chandrasekhar, S.; Yu, X. P.; Linebarger, J. H.; Iturria, S. J.; Burris, T. P.; Kato, S.; Chin, W. W.; Nagpal, S. Identification and characterization of noncalcemic, tissue-selective,

nonsecosteroidal vitamin D receptor modulators. *J Clin Invest* **2006,** *116*, 892-904.

(30) Tavera-Mendoza, L. E.; Quach, T. D.; Dabbas, B.; Hudon, J.; Liao, X.; Palijan, A.; Gleason, J. L.; White, J. H. Incorporation of histone deacetylase inhibition into the structure of a nuclear receptor agonist. *Proc Natl Acad Sci U S A* **2008,** *105*, 8250-8255.

(31) Shen, W.; Xue, J.; Zhao, Z.; Zhang, C. Novel nonsecosteroidal VDR agonists with phenyl-pyrrolyl pentane skeleton. *Eur J Med Chem* **2013,** *69*, 768-778.

(32) Ge, Z.; Hao, M.; Xu, M.; Su, Z.; Kang, Z.; Xue, L.; Zhang, C. Novel nonsecosteroidal VDR ligands with phenyl-pyrrolyl pentane skeleton for cancer therapy. *Eur J Med Chem* **2016,** *107*, 48-62.

(33) Gill, H. S.; Londowski, J. M.; Corradino, R. A.; Zinsmeister, A. R.; Kumar, R. The synthesis and biological activity of 25-hydroxy-26,27-dimethylvitamin D3 and 1,25-dihydroxy-26,27-dimethylvitamin D3: highly potent novel analogs of vitamin D3. *J Steroid Biochem* **1988,** *31*, 147-160.

(34) Wang, B.; Hao, M. X.; Zhang, C. Design, synthesis and biological evaluation of nonsecosteroidal vitamin D-3 receptor ligands as anti-tumor agents. *Bioorg Med Chem Lett* **2017,** *27*, 1428-1436.

(35) Binderup, L.; Latini, S.; Binderup, E.; 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.

(36) Chesney, R. W.; Han, X. Differential regulation of TauT by calcitriol and retinoic acid via VDR/RXR in LLC-PK1 and MCF-7 cells. *Adv Exp Med Biol* **2013,** *776*, 291-305.

(37) Yin, Y.; Ni, J.; Chen, M.; Guo, Y. L.; Yeh, S. Y. RRR-alpha-Vitamin E succinate

potentiates the antitumor effect of calcitriol in prostate cancer without overt side effects. *Clinical Cancer Research* **2009,** *15*, 190-200.

(38) Larriba, M. J.; Munoz, A. SNAIL vs vitamin D receptor expression in colon cancer: therapeutics implications. *Br J Cancer* **2005,** *92*, 985-989.

(39) 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-2alpha-(3-hydroxypropyl)-1alpha,25-dihydroxyvitamin D3 (MART-10) is a potent cell growth regulator with enhanced chemotherapeutic potency in liver cancer cells. *Steroids* **2011,** *76*, 1513-1519.

(40) Haussler, M. R.; McCain, T. A. Basic and clinical concepts related to vitamin D metabolism and action (first of two parts). *N Engl J Med* **1977,** *297*, 974-983.

(41) Maruyama, K.; Noguchi-Yachide, T.; Sugita, K.; Hashimoto, Y.; Ishikawa, M. Novel selective anti-androgens with a diphenylpentane skeleton. *Bioorg Med Chem Lett* **2010,** *20*, 6661-6666.

(42) Thomas, E.; Brion, J. D.; Peyrat, J. F. Synthesis and preliminary biological evaluation of new antiproliferative aromatic analogues of 1alpha,25-dihydroxyvitamin D3. *Eur J Med Chem* **2014,** *86*, 381-393.

(43) Kakuda, S.; Okada, K.; Eguchi, H.; Takenouchi, K.; Hakamata, W.; Kurihara, M.; Takimoto-Kamimura, M. Structure of the ligand-binding domain of rat VDR in complex with the nonsecosteroidal vitamin D3 analogue YR301. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **2008,** *64*, 970-973.

(44) Yamada, S.; Makishima, M. Structure-activity relationship of nonsecosteroidal vitamin D receptor modulators. *Trends Pharmacol Sci* **2014,** *35*, 324-337.

(45) Ciesielski, F.; Rochel, N.; Mitschler, A.; Kouzmenko, A.; Moras, D. Structural investigation of the ligand binding domain of the zebrafish VDR in complexes with 1alpha,25(OH)2D3 and Gemini: purification, crystallization and preliminary X-ray diffraction analysis. *J Steroid Biochem Mol Biol* **2004,** *89-90*, 55-59.

(46) Jensen, S. S.; Madsen, M. W.; Lukas, J.; Binderup, L.; Bartek, J. Inhibitory effects of 1alpha,25-dihydroxyvitamin D(3) on the G(1)-S phase-controlling machinery. *Mol Endocrinol* **2001,** *15*, 1370-1380.

(47) Caputo, A.; Pourgholami, M. H.; Akhter, J.; Morris, D. L. 1,25-Dihydroxyvitamin D(3) induced cell cycle arrest in the human primary liver cancer cell line HepG2. *Hepatol Res* **2003,** *26*, 34-39.

(48) Flores, O.; Wang, Z.; Knudsen, K. E.; Burnstein, K. L. Nuclear targeting of cyclin-dependent kinase 2 reveals essential roles of cyclin-dependent kinase 2 localization and cyclin E in vitamin D-mediated growth inhibition. *Endocrinology* **2010,** *151*, 896-908.

(49) Swami, S.; Krishnan, A. V.; Wang, J. Y.; Jensen, K.; Peng, L.; Albertelli, M. A.; Feldman, D. Inhibitory effects of calcitriol on the growth of MCF-7 breast cancer xenografts in nude mice: selective modulation of aromatase expression in vivo. *Horm Cancer* **2011,** *2*, 190-202.

(50) Yoon, I. S.; Son, J. H.; Kim, S. B.; Choi, M. K.; Maeng, H. J. Effects of 1alpha,25-Dihydroxyvitamin D3 on Intestinal Absorption and Disposition of Adefovir Dipivoxil and Its Metabolite, Adefovir, in Rats. *Biol Pharm Bull* **2015,** *38*, 1732-1737.

(51) Choi, M.; Yamada, S.; Makishima, M. Dynamic and ligand-selective interactions of vitamin D receptor with retinoid X receptor and cofactors in living cells. *Mol Pharmacol* **2011,** *80*, 1147-1155.

(52) 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.

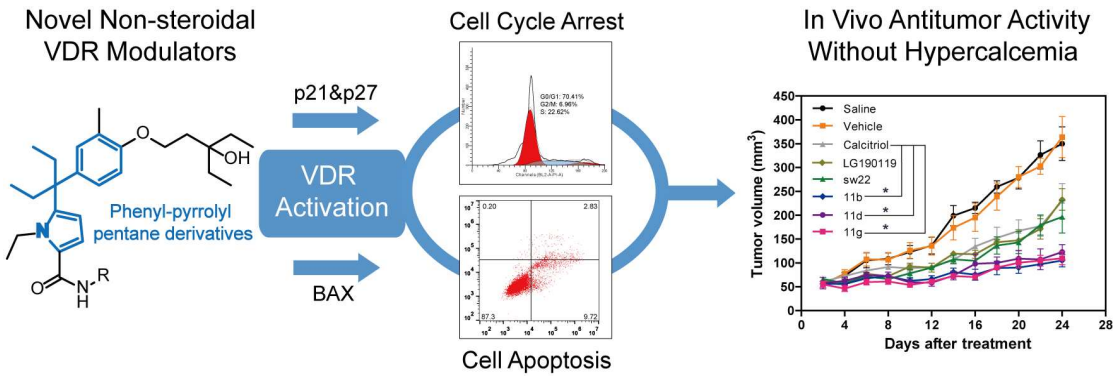
(53) Kim, S.; Shevde, N. K.; Pike, J. W. 1,25-Dihydroxyvitamin D3 stimulates cyclic vitamin D receptor/retinoid X receptor DNA-binding, co-activator recruitment, and histone acetylation in intact osteoblasts. *J Bone Miner Res* **2005,** *20*, 305-317.

(54) Issa, L. L.; Leong, G. M.; Sutherland, R. L.; Eisman, J. A. Vitamin D analogue-specific recruitment of vitamin D receptor coactivators. *J Bone Miner Res* **2002,** *17*, 879-890.

(55) Masuda, S.; Jones, G. Promise of vitamin D analogues in the treatment of hyperproliferative conditions. *Mol Cancer Ther* **2006,** *5*, 797-808.

(56) Scher, H. I.; Jia, X.; Chi, K.; de Wit, R.; Berry, W. R.; Albers, P.; Henick, B.; Waterhouse, D.; Ruether, D. J.; Rosen, P. J.; Meluch, A. A.; Nordquist, L. T.; Venner, P. M.; Heidenreich, A.; Chu, L.; Heller, G. Randomized, open-label phase III trial of docetaxel plus high-dose calcitriol versus docetaxel plus prednisone for patients with castration-resistant prostate cancer. *J Clin Oncol* **2011,** *29*, 2191-2198.

TABLE OF CONTENTS GRAPHIC:


Novel Non-steroidal
VDR Modulators
Phenyl-pyrrolyl
pentane derivatives
p21&p27
VDR
Activation
BAX
Cell Cycle Arrest
Cell Apoptosis
In Vivo Antitumor Activity
Without Hypercalcemia
Saline
Vehicle
Calcitriol
LG190119
sw22
11b
11d
11g
Tumor volume (mm3)
Days after treatment