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Increased biological potency of hexafluorinated analogs of 1,25-dihydroxyvitamin D_3 on bovine parathyroid cells

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Abstract

1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) is known to be involved in regulating the proliferation of parathyroid cells and PTH synthesis through reactions involving its nuclear receptor. We evaluated the effects of 1,25-$(OH)_2D_3$ and its hexafluorinated analog, 26,26,26,27,27,27-hexafluoro-1,25-dihydroxyvitamin D_3 (26,27-F_6-1,25-$(OH)_2D_3$), on parathyroid cells. The 1,25-$(OH)_2D_3$ and 26,27-F_6-1,25-$(OH)_2D_3$ each inhibited [^{3}H]thymidine incorporation and ornithine decarboxylase (ODC) activity, which is important in cell proliferation, in primary cultured bovine parathyroid cells. The inhibitory effect of 26,27-F_6-1,25-$(OH)_2D_3$ on PTH secretion from parathyroid cells was significantly more potent than that of 1,25-$(OH)_2D_3$ between 10^{-11} M and 10^{-8} M. Study of 26,27-F_6-1,25-$(OH)_2D_3$ metabolism in parathyroid cells in vitro elucidated its slower degradation than that of 1,25-$(OH)_2D_3$. After 48 h of incubation with [1β-^{3}H]26,27-F_6-1,25-$(OH)_2D_3$, two HPLC peaks, one for [1β-^{3}H]26,27-F_6-1,25-$(OH)_2D_3$, and a second larger peak for [1β-^{3}H]26,27-F_6-1,23(S),25-$(OH)_3D_3$, were detected. No metabolites were detected after the same period of incubation with 1,25-$(OH)_2$[26,27-^{3}H]D_3. We observed that 26,27-F_6-1,23(S),25-$(OH)_3D_3$ was as potent as 1,25-$(OH)_2D_3$ in inhibiting the proliferation of parathyroid cells.

Data suggest that the greater biological activity of 26,27-F_6-1,25-$(OH)_2D_3$ is explained by its slower metabolisms and by the retention of the biological potency of 26,27-F_6-1,25-$(OH)_2D_3$ even after 23(S)-hydroxylation.

1. Introduction

The active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), is a potent regulator of the synthesis and release of parathyroid hormone (PTH) in vivo and in vitro [1–5]. This agent also regulates the proliferation of parathyroid cells. We previously reported that ornithine decarboxylase (ODC) [EC 4.1.1.17], the rate-limiting enzyme in polyamine metabolism, is involved in 1,25-$(OH)_2D_3$-induced inhibition of the parathyroid cell proliferation [6]. It was also reported that ODC activity and its gene expression were induced in uremia and suppressed by a bolus injection of 1,25-$(OH)_2D_3$ [7].

The hexafluorinated vitamin D_3 analog, 26,26,26,27,27,27-hexafluoro-1,25-dihydroxyvitamin D_3 (26,27-F_6-1,25-$(OH)_2D_3$), has been shown to be several times more potent than 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) in curing rickets [8] and in elevating and maintaining plasma calcium levels in patients with hypoparathyroidism [9]. 26,27-F_6-1,25-$(OH)_2D_3$ was reported to be about 10 times as potent as 1,25-$(OH)_2D_3$ in suppressing the proliferation of HL-60 cells and in inducing cell differentiation [10]. The reason for such enhanced biological activities was initially attributed to several mechanisms [11] including a decreased metabolic inactivation by way of 26- and 27-hydroxylation owing to the substituted fluorine groups

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at positions C-26 and C-27 [8]. The substitution of fluorine for hydrogen at C-26 and C-27 alters the chemical reactivity and/or the conformation of carbons C-23, C-24, and C-25 in the 1,25-$(OH)_2D_3$ molecule [12].

We have examined the effects of 26,27-F_6-1,25-$(OH)_2D_3$ on PTH secretion and parathyroid cell proliferation in vitro. We also have investigated its metabolism in parathyroid cells.

2. Methods and materials

2.1. Materials

[6-^{3}H]Thymidine (1.15 TBq/mmol) was obtained from Dupont/New England Nuclear (Boston, MA, USA). L-[1-^{14}C]Ornithine (2.04 GBq/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO, USA). 1,25-$(OH)_2D_3$, 26,27-F_6-1,25-$(OH)_2D_3$ and 26,27-F_6-1,23(S),25-$(OH)_3D_3$ were obtained from Sumitomo Pharmaceuticals Co. (Osaka, Japan). Radioactive 1,25-$(OH)_2$[26, 27-^{3}H]D_3 (696 GBq/mmol) and [1β-^{3}H]26,27-F_6-1,25-$(OH)_2D_3$ (6.43 TBq/mmol) were prepared as described previously [11]. Dulbecco's modified eagle's medium, Ham's F-12 medium, bovine serum albumin (BSA), insulin, and transferrin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Streptomycin and penicillin were purchased from ICN Biochemicals, Inc. (Costa Mesa, CA, USA).

2.2. Preparation of parathyroid cell culture

Primary monolayer cell cultures of bovine parathyroid cells were prepared as previously described [6,13]. Briefly, dispersed parathyroid cells were cultured overnight in a culture medium of 1:1 Dulbecco's modified eagle's medium/Ham's F-12 containing 4% heat-inactivated fetal bovine serum (FBS), 15 mM HEPES (pH 7.4), 100 μg/ml streptomycin, 100 U/ml penicillin, 5 μg/ml insulin, 2 mM L-glutamine and 1% nonessential amino acids to allow the cells to adhere to the plates. The medium was replaced by one containing 1 mg/ml BSA and 5 μg/ml transferrin instead of FBS, in the presence or absence of the indicated concentrations of vitamin D_3 analogs for 48 h before determining the PTH secretion, or the addition of 10% FBS to determine the ODC activity and [^{3}H]thymidine incorporation into DNA.

2.3. PTH secretion studies

Primary cultured parathyroid cells were treated with each vitamin D_3 analog for 48 h and then washed twice with ice-cold PBS to remove the previously secreted PTH. Cells treated without vitamin D_3 analog for 48 h were used as control. After another 3 h of incubation in the culture media with the fresh identical medium containing no vitamin D analogs, the media were collected, centrifuged, and stored at −20°C until assayed for PTH [13]. PTH was assayed by using the "Yamasa" PTH kit (Yamasa Shoyu Co., Ltd., Chosi, Japan) [14]. Cellular protein content was measured by the method of Lowry et al. by using BSA as a standard [15].

2.4. Assay of [^{3}H]thymidine incorporation

After treating primary cultured parathyroid cells with vitamin D_3 analogs for 48 h, the cells were incubated in the medium containing 10% FBS, 1 mg/ml BSA, 5 μg/ml transferrin with the same concentration of vitamin D analogs for another 24 h after this 48 h-incubation. Cells treated under same condition without vitamin D_3 analog were used as control. Cells were washed twice with PBS, collected, and suspended in 0.2 ml of ice-cold 5% trichloroacetic acid (TCA). [^{3}H]Thymidine incorporation into DNA was allowed to proceed for 4 h and was assayed as the incorporation of [6-^{3}H]thymidine into the 5% TCA-insoluble fraction as previously reported [6,16].

2.5. Measurement of ODC activity

After treating the primary cultured parathyroid cells with vitamin D_3 analogs for 48 h, the cells were incubated in the medium containing 10% FBS, 1 mg/ml BSA, 5 μg/ml transferrin with the same concentration of vitamin D analogs for another 8 h after this 48 h-incubation. Cells treated under same condition without vitamin D_3 analog were used as control. Cells were washed twice with PBS, collected, then suspended in 0.2 ml of ice-cold buffer containing 10 mM Tris–HCl (pH 7.5), 200 μM pyridoxal phosphate, 0.1 mM EDTA and 2.5 mM dithiothreitol. ODC activity was measured as the release of $^{14}CO_2$ formed from L-[1-^{14}C] ornithine as previously described [17] with minor modifications [6].

2.6. HPLC analyses of the metabolites of 1,25-$(OH)_2$[26,27-^{3}H]D_3 and [1β-^{3}H]26,27-F_6-1,25-$(OH)_2D_3$ in parathyroid cells

Parathyroid cells were treated with 10^{-8} M 1,25-$(OH)_2$[26, 27-^{3}H]D_3 (384,096 dpm) or 10^{-8} M [1β-^{3}H]26,27-F_6-1,25-$(OH)_2D_3$ (407,370 dpm) for 48 h. After the cells were washed twice with PBS, 0.6 ml of methanol/chloroform (2/1 v/v) was added to the cell pellets, and stored at −20°C until the time of extraction. Lipid extraction was performed by the method of Bligh and Dyer [12,18,19]. The chloroform layer containing the lipid-soluble metabolites of 1,25-$(OH)_2D_3$

or 26,27-F_6-1,25-$(OH)_2D_3$ was dried under a stream of nitrogen at 37°C. Samples were stored in absolute ethanol at −20°C until analyzed on HPLC. At this point, putative metabolites were pure enough for HPLC analysis. Automatic analytical HPLC was performed using a modular instrument comprising a Model 600 pump, a Model 440 fixed wavelength ultraviolet detector (254 nm; all from Waters Associates, Inc., MA, USA). Effluent was collected in 30-s fractions using programmable fraction collector (Bio-Rad Laboratories, CA, USA). Separations were performed using Zorbax-Sil 4.6 mm × 25 cm (DuPont Instruments, DE, USA). The solvent system of choice was dependent on the substrate used. Purification of 26,27-F_6-1,25-$(OH)_2D_3$ and analysis of metabolites generated from this were chromatographed on Zorbax-Sil using *n*-hexane-dichloromethane-methanol (49:48:3, v/v/v) at a flow rate of 1.5 ml/min. Purification of 1,25-$(OH)_2D_3$ was chromatographed on Zorbax-Sil using *n*-hexane-2-propanol (92/8) at a flow rate of 2.0 ml/min. Fractions obtained by HPLC separation were evaporated at room temperature in a fume hood and were used to quantitate the radioactivity by scintillation spectroscopy. Scintillation counting was performed using an organically based scintillant, ACS II (Amersham, IL, USA) in an automatic counter Minaxi Tri-Carb B4450 (Packard, IL, USA).

2.7. Statistical analysis

Statistical analyses were performed by using a Student's *t*-test.

3. Results

3.1. Effects of 1,25-$(OH)_2D_3$ and 26,27-F_6-1,25-$(OH)_2D_3$ on the inhibition of PTH secretion from bovine parathyroid cells

After treatment with the vitamin D_3 analogs, we examined the primary cultured parathyroid cells for their ability to secrete PTH. As shown in Fig. 1, 1,25-$(OH)_2D_3$ and its fluorinated analog each inhibited the secretion of PTH from the cells in a concentration-dependent manner. The agent, 26,27-F_6-1,25-$(OH)_2D_3$, was significantly more potent than 1,25-$(OH)_2D_3$ in inhibiting the secretion of PTH between 10^{-11} M and 10^{-8} M.

3.2. Effects of 1,25-$(OH)_2D_3$, 26,27-F_6-1,25-$(OH)_2D_3$, and 26,27-F_6-1,23(S),25-$(OH)_3D_3$ on inhibition of bovine parathyroid cell proliferation

Fig. 2 shows the inhibitory effects of vitamin D_3 analogs on parathyroid cell proliferation. The inhibitory effects of 1,25-$(OH)_2D_3$ and 26,27-F_6-1,25-$(OH)_2D_3$ on the serum-induced rise in [^{3}H]thymidine incorporation were concentration-dependent between 10^{-11} M and 10^{-8} M. The agent, 26,27-F_6-1,25-$(OH)_2D_3$, was significantly more potent than 1,25-$(OH)_2D_3$ between 10^{-11} M and 10^{-8} M. Interestingly, the potency of 26,27-F_6-1,23(S),25-$(OH)_3D_3$, the major metabolite of 26,27-F_6-1,25-$(OH)_2D_3$, was equivalent to that of 1,25-$(OH)_2D_3$, indicating the retention of the biological activity even after 23(S)-hydroxylation.

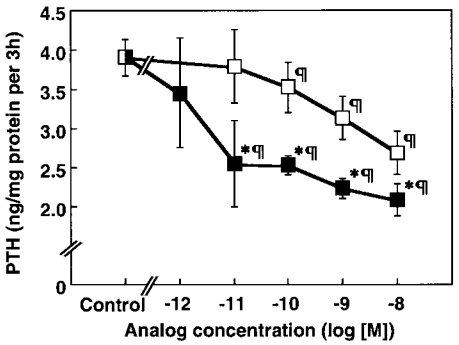

Fig. 1. Dose-response effects of 1,25-$(OH)_2D_3$ and 26,27-F_6-1,25-$(OH)_2D_3$ on the inhibition of PTH secretion. Parathyroid cells were treated with 10^{-12} to 10^{-8} M 1,25-$(OH)_2D_3$ (— □ —) or 10^{-11} to 10^{-8} M 26,27-F_6-1,25-$(OH)_2D_3$ (— ■ —) for 48 h. Culture media were collected after a 3 h incubation and assayed for PTH by using a RIA. Results represent the mean ± SD of six dishes. $^*p < 0.001$ vs 1,25-$(OH)_2D_3$; $^¶p < 0.005$ vs control.

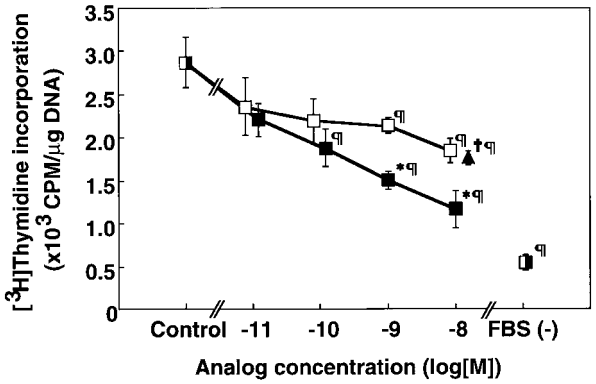

Fig. 2. Dose-response effects of 1,25-$(OH)_2D_3$ and 26,27-F_6-1,25-$(OH)_2D_3$ on the inhibition of [^{3}H] thymidine incorporation stimulated by 10% FBS. Parathyroid cells were treated with 10^{-11} to 10^{-8} M 1,25-$(OH)_2D_3$ (— □ —), or 26,27-F_6-1,25-$(OH)_2D_3$ (— ■ —), or 10^{-8} M 26,27-F_6-1,23(S),25-$(OH)_3D_3$ (▲) for 48 h. The medium was then replaced by fresh medium containing 10% FBS. [^{3}H] Thymidine incorporation for 4 h was assayed 24 h after FBS was added. Results represent the mean ±SD of three dishes. $^*p < 0.01$ vs 1,25-$(OH)_2D_3$; †$p < 0.01$ vs 26,27-F6-1,25-$(OH)_2D_3$; ¶$p < 0.05$ vs control.

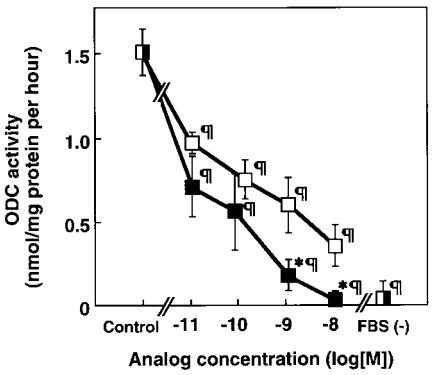

Fig. 3. Dose-response effects of 1,25-$(OH)_2D_3$ and 26,27-F_6-1,25-$(OH)_2D_3$ on the inhibition of ODC activity stimulated by 10% FBS. Parathyroid cells were treated with 10^{-11} to 10^{-8} M 1,25-$(OH)_2D_3$ (— □ —) or 26,27-F_6-1,25$(OH)_2D_3$ (— ■ —) for 48 h. ODC activity was determined 8 h after FBS was added. Results represent the mean ±SD of three dishes. $^*p < 0.01$ vs 1,25-$(OH)_2D_3$; $^¶p < 0.05$ vs control.

3.3. Effects of 1,25-$(OH)_2D_3$ and 26,27-F_6-1,25-$(OH)_2D_3$ on inhibition of ODC activity in bovine parathyroid cells

The effects of the two vitamin D_3 analogs on serum-induced ODC activity were examined. It has been reported that, at 8 h after a medium change, ODC activity rapidly increased to its maximum level and declined to the basal levels by 24 h [6]. We therefore determined the biological potencies of the vitamin D_3 analogs 8 h after the media were changed. As shown in Fig. 3, the inhibitory effects of vitamin D_3 analogs were concentration-dependent. The agent 26,27-F_6-1,25-$(OH)_2D_3$ was significantly more potent than 1,25-$(OH)_2D_3$ between 10^{-11} M and 10^{-8} M. Therefore, the enhanced inhibitory potency of 26,27-F_6-1,25-$(OH)_2D_3$ on ODC activity was closely associated with its effects on the proliferation of parathyroid cells.

3.4. Metabolism of 1,25-$(OH)_2$[26,27-^{3}H]D_3 and [1β-^{3}H]26,27-F_6-1,25-$(OH)_2D_3$ in bovine parathyroid cells

We evaluated the metabolism of 26,27-F_6-1,25-$(OH)_2D_3$ and of 1,25-$(OH)_2D_3$ in parathyroid cells. The radioactive profile of the lipid extract derived from incubating the cells with 1,25-$(OH)_2$[26,27-^{3}H]D_3 for 48 h is shown in Fig. 4A. The radioactivity of the metabolites of 1,25-$(OH)_2$[26,27-^{3}H]D_3 was too low for identification. In contrast, a large amount of [1β-^{3}H]26,27-F_6-1,25-$(OH)_2D_3$ remained (Fig. 4B). The major metabolite was identified as authentic 26,27-F_6-1,23(S),25-$(OH)_3D_3$, which corroborates the findings previously reported in other cell systems [12].

4. Discussion

The present study clearly indicates an enhancement of the biological potency of 1,25-$(OH)_2D_3$, by fluorination at positions C-26 and C-27, on parathyroid glands in vitro. An active form of vitamin D_3, 1,25-$(OH)_2D_3$, is a potent regulator of parathyroid cell proliferation as well as the synthesis and release of PTH [1–5]. In this study, 26,27-F_6-1,25-$(OH)_2D_3$ was significantly more potent than 1,25-$(OH)_2D_3$ in suppressing the proliferation of parathyroid cells and the secretion of PTH (Figs. 1 and 2). The inhibitory effect of 26,27-F_6-1,25-$(OH)_2D_3$ on ODC activity was closely associated with its inhibitory effect on parathyroid cell proliferation (Figs. 2 and 3). Together with our previous data

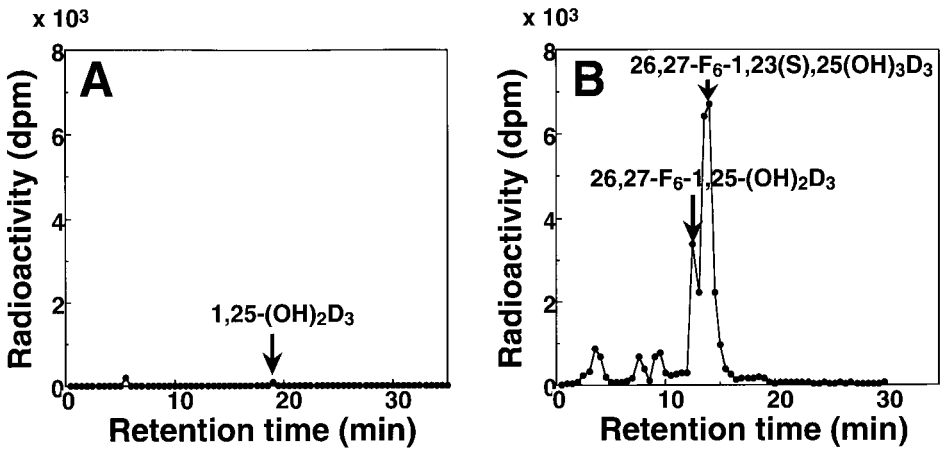

Fig. 4. Metabolites of vitimin D_3 produced by in vitro incubation of parathyroid cells with 1,25-$(OH)_2$[26,27-^{3}H]D_3 or [1β-^{3}H]26,27-F_6-1,25-$(OH)_2D_3$. Parathyroid cells were treated with 10^{-8} M 1,25-$(OH)_2D_3$ (128,032 dpm) (A) and 10^{-8} M 26,27-F_6-1,25-$(OH)_2D_3$ (135,790 dpm) (B) for 48 h. After the cells were washed twice with PBS, methanol: chloroform (2:1, v/v) was added to extract the sterols. Sterols were then subjected to straight-phase HPLC on a Zorbax-Sil column. The elution position of each victim D_3 compound was assessed with radio-inert standards by measuring absorbance at 254 nm.

[6] that indicated the involvement of ODC in parathyroid cell proliferation, vitamin D_3 seemed to inhibit parathyroid cell proliferation by suppressing ODC activity. We previously showed that polyamine is intimately involved in the proliferation of parathyroid cells; (i) the suppression of ODC activity by 1,25-$(OH)_2D_3$ was preceded by the suppression of [^{3}H]thymidine incorporation in parathyroid cells and (ii) α-difluoromethylornithine (DFMO), a specific inhibitor of ODC, inhibited the serum-stimulated [^{3}H]thymidine incorporation. Furthermore, simultaneous addition of DFMO with putrescine, the product of ODC, reversed the inhibitory effect of DFMO, clearly showing an important role of ODC in the proliferation of parathyroid cells [6].

We previously reported that the enhanced biological activities of the fluorinated vitamin D_3 analog were accounted for by several mechanisms [11,20], including a decrease in metabolic inactivation by way of 26- and 27-hydroxylation owing to the substituted fluoro groups at these carbons [8]. The major metabolite of 26,27-F_6-1,25-$(OH)_2D_3$, whether in vivo [21] or in vitro [12], is reportedly 26,27-F_6-1,23(S),25-$(OH)_3D_3$. This substance differs from 1,25-$(OH)_2D_3$ in that the major metabolic pathway of 1,25-$(OH)_2D_3$ involves hydroxylation at C-24. We examined the metabolites of 26,27-F_6-1,25-$(OH)_2D_3$ in parathyroid cells in parallel with 1,25-$(OH)_2D_3$. As reported in other cell types [10–12], the degradation rate of 26,27-F_6-1,25-$(OH)_2D_3$ was significantly slower than 1,25-$(OH)_2D_3$, with the major metabolite being 26,27-F_6-1,23(S),25-$(OH)_3D_3$ (Fig. 4). Furthermore, 23(S)-hydroxylation of 26,27-F_6-1,25-$(OH)_2D_3$ did not produce a total loss of vitamin D_3 activity in parathyroid cells in contrast to that of 1,25-$(OH)_2D_3$ [22], as evidenced by Fig. 2. Therefore, the higher potency of 26,27-F_6-1,25-$(OH)_2D_3$ was explained not only by its slower metabolism but also by the retention of the biological activity of its metabolite. We postulate that the reason for the alteration in metabolism might be explained by the inhibition of hydroxylation at C-24 and C-25 and by the conformational change at C-23 in 1,25-$(OH)_2D_3$ caused by fluorine substitution at these positions [12]. It has recently been reported that the biological activity of 26,27-F_6-1,25-$(OH)_2D_3$ is potentiated by an enhanced transcriptional activity of 26,27-F_6-1,25-$(OH)_2D_3$-vitamin D receptor complex as compared with 1,25-$(OH)_2D_3$-vitamin D receptor complex, probably resulting from the tighter binding of the former complex to DNA [23]. In parathyroid cells, 26,27-F_6-1,25-$(OH)_2D_3$ may affect at transcriptional step.

In summary, vitamin D_3 analogs inhibited the parathyroid cell proliferation that was stimulated by serum through an inhibition of ODC activity. The biological effects of 26,27-F_6-1,25-$(OH)_2D_3$ on parathyroid cells exceeded that of 1,25-$(OH)_2D_3$. This enhanced activity could be explained in part by the alterations in metabolic inactivation.

In clinical study, 26,27-F_6-1,25-$(OH)_2D_3$ was reported to be several times as potent as 1,25-$(OH)_2D_3$ in maintaining plasma calcium levels in the patients with primary or secondary hypoparathyroidism [9]. Now this compound is under clinical trial for the treatment of secondary hyperparathyroidism often observed in the patients with chronic renal failure [24]. We hope that this compound will be potent enough to inhibit PTH secretion and parathyroid gland enlargement in uremia.

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