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REVIEW

Vitamin D, Osteoclastogenesis and Bone Resorption: from Mechanistic Insight to the Development of New Analogs

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Prologue: 1α,25(OH)$_2$D$_3$ as a bone-resorbing hormone

THE vitamin D hormone 1α,25-dihydroxyvitamin D$_3$ (1α,25(OH)$_2$D$_3$) is a pleiotropic hormone which exerts its effects through the nuclear vitamin D receptor (VDR) [1]. Among these effects, the physiological importance of VDR in maintaining the integrity of mineral and skeletal systems is underscored by the severe hypocalcemia and rickets/osteomalacia observed in VDR gene knockout mice as well as patients with vitamin D deficiency [2]. The connection between vitamin D and bone resorption was first made in 1972, when it was reported that in a classic experiment using bone organ cultures, the so-called "Raisz assay", 1α,25(OH)$_2$D$_3$ stimulates bone resorption, as determined by the release of ^{45}Ca from pre-labeled fetal long bones [3]. This paper formed the basis of the currently prevailing belief that 1α,25(OH)$_2$D$_3$ is a bone-resorbing hormone [4]. In the 1980's, the now well-known co-culture system of hematopoietic cells with osteoblast/stromal cells for generating osteoclasts was developed [5], and together with the demonstration of 1α,25(OH)$_2$D$_3$ as a differentiation-inducing agent, the dogma that 1α,25(OH)$_2$D$_3$ is essential for osteoclast differentiation became firmly established [4].

1997 was a landmark year in the research history of bone biology, especially in terms of the osteoclast. A novel cytokine, named osteoclastogenesis inhibitory factor (OCIF) or osteoprotegerin (OPG), was discovered to protect bone by negatively regulating osteoclast formation. This work was reported by Yukijirushi [6] and Amgen [7]. In the following year, its ligand, OPGL, or currently under the prevailing name of "receptor activator of nuclear factor-κB ligand" (RANKL), was identified by the 2 companies as the long-sought osteoclast differentiation factor (ODF) that is presented on osteoblastic/stromal cells, thereby promoting the terminal differentiation into osteoclasts [8, 9]. Yasuda *et al.* elegantly showed that 1α,25(OH)$_2$D$_3$ at relatively high doses of 10^{-8}–10^{-7} M, induces the expression of RANKL in the ST2 stromal cell line [8], providing the final molecular proof that 1α,25(OH)$_2$D$_3$ is a pro-osteoclastogenic hormone [4]. It then became widely recognized that in addition to 1α,25(OH)$_2$D$_3$, many other bone-resorbing hormones and cytokines, such as PTH, prostaglandins, TNF and IL-1, stimulate osteoclastogenesis indirectly by inducing RANKL in stromal/osteoblastic cells.

Contrary to this prevailing view, however, we in fact observed in various models of accelerated bone resorption that pharmacological doses of active vitamin D drugs unexpectedly inhibit bone resorption *in vivo* [10–12]. I herein summarize what has been learned from these pharmacological experiments, and discuss the role of VDR-based drugs in the treatment of bone disease, especially osteoporosis.

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Osteoclasts in osteoporosis

Osteoclast-mediated bone resorption plays a central pathogenetic role in the development and progression

of osteoporosis [13]. Bisphosphonates, which potently inhibit the bone-resorbing activity of mature osteoclasts, are currently the most widely used treatment for osteoporosis [14, 15]. Multinucleated osteoclasts are derived from hematopoietic precursor cells through the concerted action of 2 cytokines, macrophage-colony-stimulating factor (M-CSF) and RANKL [16, 17]. These cytokines are produced by osteoclastogenesis-supporting marrow stromal cells, and act on osteoclast precursor cells through their cognate receptors, c-fms and RANK, respectively. These cell-surface receptors transmit osteoclastogenic signals through intracellular kinase cascades, which eventually lead to the activation of the transcription factors c-Fos/AP-1 and NF-κB in the nucleus. Accordingly, mice deficient in c-Fos, NF-κB, RANK, RANKL or M-CSF fail to generate osteoclasts and exhibit osteopetrosis [16, 17].

Menopause and aging are thought to cause aberrantly elevated generation of osteoclasts, which elevation involves diverse mechanisms, including the production of bone-resorbing cytokines in response to estrogen deficiency [14, 18]. In addition to estrogen deficiency, a recent report suggests that the pituitary hormone FSH also contributes to the high bone turnover which characteristically follows menopause [19].

Native versus active vitamin D

The importance of simple vitamin D as a nutrient for the prevention of osteoporosis is widely recognized, especially in the elderly population, in which simple vitamin D deficiency is often prevalent [20, 21]. Vitamin D, which is both synthesized in the skin and taken up from food, is activated into the hormonal form, $1\alpha,25(OH)_2D_3$, through sequential hydroxylation in the liver and then in the kidney. Although it is generally believed that vitamin D deficiency is a risk factor for osteoporosis and fragile skeleton fractures, precisely how native vitamin D and vitamin D hormone differ in skeletal action, and whether or not vitamin D hormone has any therapeutic advantage over plain vitamin D, especially in patients without vitamin D deficiency or supplemented with native vitamin D, have been and continue to be controversial [22, 23]. In a recent meta analysis, it is concluded that native and/or active vitamin D are effective in reducing vertebral fracture, while the evidence for an effect on non-vertebral fracture is currently lacking [23].

We compared the pharmacological effects of various doses of native vitamin D versus $1\alpha(OH)D_3$ as a prodrug converted to the active form, in ovariectomized rats. The animals were supplemented with calcium and native vitamin D [24]. The results indicate that active vitamin D increases BMD and bone strength more potently than native vitamin D with comparable effects on calcium metabolism, pointing to an advantage of the hormonal over the native form.

Anti-osteoclastogenic action of VDR

The first hint that active vitamin D may actually be an inhibitor of bone resorption was obtained unexpectedly with experiments examining the effects of a vitamin D analog, OCT, on cancer-induced hypercalcemia. To carry out that investigation we had established parathyroidectomized rats rendered hypercalcemic with constant PTHrP infusion so the endogenous PTH levels would be constant. Under these "PTH clamp" conditions, we observed that not only OCT but, surprisingly, $1\alpha,25(OH)_2D_3$, ameliorated hypercalcemia with concomitant inhibition of bone resorption [25]. This prompted us to hypothesize that $1\alpha,25(OH)_2D_3$ has the potential to inhibit bone resorption independently of its effect on PTH. We further studied the pharmacological action of active vitamin D in ovariectomized (OVX) rats, which comprise an established model of high bone turnover osteoporosis due to estrogen deficiency. Using deoxypyridinoline (DPD) as a biochemical marker of bone resorption combined with histomorphometric techniques, we demonstrated that the administration of alfacalcidol, a prodrug metabolized to the natural vitamin D hormone $1\alpha,25(OH)_2D_3$, suppresses the bone resorption elevated by OVX [10]. In the bones of treated rats, the number of osteoclasts was substantially reduced, suggesting that VDR inhibits the differentiation/recruitment of osteoclasts [10]. The anti-osteoclastogenic action of active vitamin D was confirmed by another analog, ED-71 (Fig. 1) [11].

ED-71 has recently been demonstrated in a clinical trial in Japan to reduce a bone resorption marker and increase BMD in osteoporotic patients provided native vitamin D_3 supplementation [26].

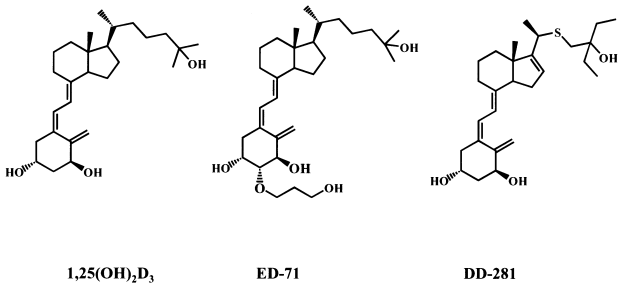

Fig. 1. Chemical structure of $1\alpha,25(OH)_2D_3$ and its analogs.
ED-71: 2β-(3-hydroxypropoxy)-$1\alpha,25(OH)_2D_3$
DD281: (1R,3S,5Z)-5-[(2E)-[(3aS,7aS)-1-[(1R)-1-[(2-ethyl-2-hydroxybutyl)thio] ethyl]-3,3a,5,6,7,7a-hexahydro-7a-methyl-4H-inden-4-ylidene]ethylidene]-4-methylene-1,3-cyclohexanediol
Pharmacological properties of ED-71 and DD281 have been described in ref. [11] and [12], respectively. A clinical trial of ED-71 is now in phase III in Japan [26].

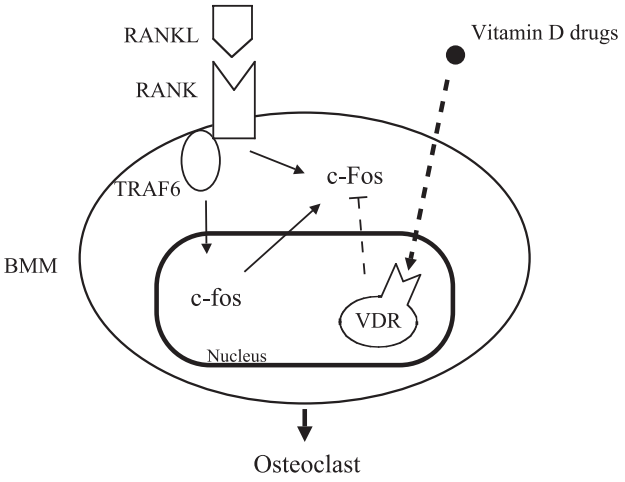

Fig. 2. VDR-based drugs inhibit the differentiation into mature osteoclasts by suppressing c-Fos protein in bone marrow macrophages.
BMM: bone marrow macrophage

Bone marrow macrophages as a target of VDR

The findings that vitamin D hormone, acting through VDR, has the potential to suppress osteoclast development prompted us to explore both the "seeds" and "soil" of osteoclastogenesis in the bone microenvironment of vitamin D-treated animals. With respect to "soil", the expression of RANKL in bone did not increase following $1\alpha,25(OH)_2D_3$ administration *in vivo*, even at the high doses that induce hypercalcemia [27]. In contrast, when the "seeds" for osteoclasts were evaluated by a limiting dilution technique, we found that the number of osteoclast progenitors present in the bone marrow had been increased by the estrogen deficiency conditions, which effect was reduced in the bone marrow of OVX mice treated *in vivo* with active vitamin D [27]. These results suggested that vitamin D hormone acts not on the "soil" but on the "seeds" in bone marrow, that is, on hematopoietic cells.

In order to pinpoint the target cells of $1\alpha,25(OH)_2D_3$, we isolated bone marrow macrophages (BMM) as osteoclast progenitor cells. These cells evidently expressed VDR, and treatment with $1\alpha,25(OH)_2D_3$ potently reduced the differentiation into osteoclasts [12]. Osteoclast development usually proceeds through 2 phases, a stage of M-CSF-dependent growth of osteoclast progenitors, followed by a terminal differentiation stage induced by RANKL in the presence of M-CSF. $1\alpha,25(OH)_2D_3$ had no effect on M-CSF-dependent cell proliferation. That $1\alpha,25(OH)_2D_3$ mainly acts at the latter differentiation stage was confirmed by the result that treatment of the cultures with $1\alpha,25(OH)_2D_3$ was sufficient only during the latter differentiation stage to inhibit osteoclast formation, whereas such treatment in the former period of M-CSF-dependent growth had no effect. Importantly, this action was shown to be mediated through VDR, since in VDR-deficient osteoclast precursor cells isolated from KO mice, the suppressive effect of $1\alpha,25(OH)_2D_3$ on osteoclastogenesis was not observed at all. These data indicate that $1\alpha,25(OH)_2D_3$ acts directly on osteoclast precursors and inhibits their differentiation into mature osteoclasts through the VDR (Fig. 2).

In order to further define the molecular pathway(s) that VDR acts upon, we examined the effects of $1\alpha,25(OH)_2D_3$ in a genetic model of osteoporosis, OPG knockout mice. OPG is a decoy receptor of RANKL, and mice lacking OPG exhibit excessive bone resorption as a result of the unopposed activation of RANKL/RANK signaling [28]. Administration of $1\alpha,25(OH)_2D_3$ to OPG knockout mice caused a reduction in the osteoclast number in bone, concomitantly with a reduction in the urinary level of a biochemical marker of bone resorption, DPD, and resulted in amelioration of bone loss. These results suggest that $1\alpha,25(OH)_2D_3$ suppresses bone resorption by interfering with signaling through RANK receptors on osteoclast precursor cells, BMM (Fig. 2).

VDR suppresses c-Fos protein and function

We then investigated the effects of $1\alpha,25(OH)_2D_3$ on molecules that are known to transmit signals from the RANK receptor in BMM. Among the known signaling molecules involved in osteoclast development downstream of the RANK receptor, the c-Fos protein turned out to be the key target molecule of VDR (Fig. 2). While $1\alpha,25(OH)_2D_3$ did not affect the levels of RANK, TRAF 6, NF-κB and c-Jun, or the phosphorylation of IKK, p38, and JNK, it did inhibit the induction of c-Fos protein by RANKL, which effect was not observed in VDR KO BMM.

$1\alpha,25(OH)_2D_3$-mediated suppression of the c-Fos protein is thought to dampen the transcription function of c-Fos/AP-1 in the nucleus. In fact, stimulation with RANKL induced 2 notable c-Fos target genes, NFATc1 and IFN-β, which regulate osteoclast differentiation positively and negatively, respectively, and simultaneous treatment with $1\alpha,25(OH)_2D_3$ inhibited the induction of these c-Fos target molecules. Importantly, the suppression of c-Fos protein through VDR was proven to be critical for the pharmacological action of vitamin D, since the forced expression of c-Fos blocked the suppressive effect of vitamin D on osteoclast formation [12].

Synthesis of new vitamin D analogs

The VDR-mediated c-Fos suppression was employed as a readout to screen a library of vitamin D compounds, and 2 analogs, DD280 and DD281 (Fig. 1) were identified as having more potent activity than $1\alpha,25(OH)_2D_3$. These analogs suppressed osteoclast development more potently than the natural hormone *in vitro*, and we set out to test the efficacy of DD281 *in vivo*.

DD281 has a binding affinity for the VDR similar to $1\alpha,25(OH)_2D_3$, whereas it has a very short half life in the circulation compared with the natural hormone, presumably due to its very low affinity for vitamin D-binding protein (DBP). The results of preclinical studies indicate that for the same degree of effect on calcium metabolism, DD281 prevents bone loss more potently than $1\alpha,25(OH)_2D_3$, with a reduction in the number of osteoclasts. Thus, DD281 is a bone-selective analog that may be useful for the treatment of bone diseases with excessive osteoclastic activity [12].

Conclusion

The major action of vitamin D is the stimulation of intestinal calcium absorption, and the therapeutic effect of vitamin D on bone is believed to be indirect, this increase in intestinal calcium absorption correcting negative calcium balance, and hence normalizing the abnormal, sustained PTH secretion frequently seen in elderly patients [29]. We have demonstrated in a series of pharmacological experiments that hematopoietic cells of the monocyte/macrophage lineage express VDR and are important target cells of vitamin D activity *in vivo*.

Vitamin D hormone is similar to estrogens and bisphosphonates in that they all inhibit bone resorption. Unlike the bisphosphonates, however, which suppress the coupling of resorption to formation, active vitamin D has the peculiar property of stimulating or at least maintaining osteoblastic bone formation [30]. Future research should aim at clarifying the cellular and molecular mechanisms underlying this interesting potential of VDR-based drugs on bone formation per se, as well as on the coupling of formation to resorption [31].

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References

1. Reichel H, Koeffler HP, Norman AW (1989) The role of the vitamin D endocrine system in health and disease. *N Engl J Med* 320: 980–991.
2. Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto

T, Kato S (1997) Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nat Genet* 16: 391–396.

3. Raisz LG, Trummel CL, Holick MF, DeLuca HF (1972) 1,25-dihydroxycholecalciferol: a potent stimulator of bone resorption in tissue culture. *Science* 175: 768–769.
4. Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ (1999) Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr Rev* 20: 345–357.
5. Takahashi N, Yamana H, Yoshiki S, Roodman GD, Mundy GR, Jones SJ, Boyde A, Suda T (1988) Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. *Endocrinology* 122: 1373–1382.
6. Tsuda E, Goto M, Mochizuki S, Yano K, Kobayashi F, Morinaga T, Higashio K (1997) Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochem Biophys Res Commun* 234: 137–142.
7. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarpley J, Derby P, Lee R, Boyle WJ (1997) Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 89: 309–319.
8. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T (1998) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* 95: 3597–3602.
9. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93: 165–176.
10. Shiraishi A, Takeda S, Masaki T, Higuchi Y, Uchiyama Y, Kubodera N, Sato K, Ikeda K, Nakamura T, Matsumoto T, Ogata E (2000) Alfacalcidol inhibits bone resorption and stimulates formation in an ovariectomized rat model of osteoporosis: distinct actions from estrogen. *J Bone Miner Res* 15: 770–779.
11. Uchiyama Y, Higuch IY, Takeda S, Masaki T, Shira-Ishi A, Sato K, Kubodera N, Ikeda K, Ogata E (2002) ED-71, a vitamin D analog, is a more potent inhibitor of bone resorption than alfacalcidol in an estrogen-deficient rat model of osteoporosis. *Bone* 30: 582–588.
12. Takasu H, Sugita A, Uchiyama Y, Katagiri N, Okazaki M, Ogata E, Ikeda K (2006) c-Fos protein as a target of anti-osteoclastogenic action of vitamin D, and synthesis of new analogs. *J Clin Invest* 116: 528–535.
13. Cummings SR, Melton LJ (2002) Epidemiology and outcomes of osteoporotic fractures. *Lancet* 359: 1761–1767.
14. Parfitt AM, Mundy GR, Roodman GD, Hughes DE, Boyce BF (1996) A new model for the regulation of bone resorption, with particular reference to the effects of bisphosphonates. *J Bone Miner Res* 11: 150–159.
15. Rodan GA, Martin TJ (2000) Therapeutic approaches to bone diseases. *Science* 289: 1508–1514.
16. Teitelbaum SL (2000) Bone resorption by osteoclasts. *Science* 289: 1504–1508.
17. Karsenty G, Wagner EF (2002) Reaching a genetic and molecular understanding of skeletal development. *Dev Cell* 2: 389–406.
18. Raisz LG (2005) Clinical practice. Screening for osteoporosis. *N Engl J Med* 353: 164–171.
19. Sun L, Peng Y, Sharrow AC, Iqbal J, Zhang Z, Papachristou DJ, Zaidi S, Zhu LL, Yaroslavskiy BB, Zhou H, Zallone A, Sairam MR, Kumar TR, Bo W, Braun J, Cardoso-Landa L, Schaffler MB, Moonga BS, Blair HC, Zaidi M (2006) FSH directly regulates bone mass. *Cell* 125: 247–260.
20. Thomas MK, Lloyd-Jones DM, Thadhani RI, Shaw AC, Deraska DJ, Kitch BT, Vamvakas EC, Dick IM, Prince RL, Finkelstein JS (1998) Hypovitaminosis D in medical inpatients. *N Engl J Med* 338: 777–783.
21. Trivedi DP, Doll R, Khaw KT (2003) Effect of four monthly oral vitamin D3 (cholecalciferol) supplementation on fractures and mortality in men and women living in the community: randomised double blind controlled trial. *BMJ* 326: 469.
22. Hauselmann HJ, Rizzoli R (2003) A comprehensive review of treatments for postmenopausal osteoporosis. *Osteoporos Int* 14: 2–12.
23. Papadimitropoulos E, Wells G, Shea B, Gillespie W, Weaver B, Zytaruk N, Cranney A, Adachi J, Tugwell P, Josse R, Greenwood C, Guyatt G (2002) Meta-analyses of therapies for postmenopausal osteoporosis. VIII: Meta-analysis of the efficacy of vitamin D treatment in preventing osteoporosis in postmenopausal women. *Endocr Rev* 23: 560–569.
24. Shiraishi A, Higashi S, Ohkawa H, Kubodera N, Hirasawa T, Ezawa I, Ikeda K, Ogata E (1999) The advantage of alfacalcidol over vitamin D in the treatment of osteoporosis. *Calcif Tissue Int* 65: 311–316.
25. Endo K, Katsumata K, Hirata M, Masaki T, Kubodera N, Nakamura T, Ikeda K, Ogata E (2000) 1,25-dihydroxyvitamin D3 as well as its analogue OCT

lower blood calcium through inhibition of bone resorption in hypercalcemic rats with continuous parathyroid hormone-related peptide infusion. *J Bone Miner Res* 15: 175–181.

26. Matsumoto T, Miki T, Hagino H, Sugimoto T, Okamoto S, Hirota T, Tanigawara Y, Hayashi Y, Fukunaga M, Shiraki M, Nakamura T (2005) A new active vitamin D, ED-71, increases bone mass in osteoporotic patients under vitamin D supplementation: a randomized, double-blind, placebo-controlled clinical trial. *J Clin Endocrinol Metab* 90: 5031–5036.
27. Shibata T, Shira-Ishi A, Sato T, Masaki T, Masuda A, Hishiya A, Ishikura N, Higashi S, Uchida Y, Saito MO, Ito M, Ogata E, Watanabe K, Ikeda K (2002) Vitamin D hormone inhibits osteoclastogenesis in vivo by decreasing the pool of osteoclast precursors in bone marrow. *J Bone Miner Res* 17: 622–629.
28. Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL, Boyle WJ, Simonet WS (1998) Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 12: 1260–1268.
29. Lips P (2001) Vitamin D deficiency and secondary hyperparathyroidism in the elderly: consequences for bone loss and fractures and therapeutic implications. *Endocr Rev* 22: 477–501.
30. Ikeda K, Ogata E (2000) Modulation of bone remodeling by active vitamin D: its role in the treatment of osteoporosis. *Mech Ageing Dev* 116: 103–111.
31. Martin TJ, Sims NA (2005) Osteoclast-derived activity in the coupling of bone formation to resorption. *Trends Mol Med* 11: 76–81.