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Evidence for Tissue- and Cell-Type Selective Activation of the Vitamin D Receptor by Ro-26-9228, A Noncalcemic Analog of Vitamin D_3

Sara Peleg,[1]* Ayesha Ismail,[1] Milan R. Uskokovic,[2] and Zafrira Avnur[2]

[1]The University of Texas, M. D. Anderson Cancer Center, Houston, Texas
[2]Roche Bioscience, Palo Alto, California

Abstract Our recent studies have shown that the vitamin D analog Ro-26-9228 restores bone mineral density without inducing hypercalcemia in osteopenic rats. Our ex vivo experiments demonstrated that the analog upregulated gene expression in trabecular bone but not in the duodenum of female rats. We examined the mechanism for the tissue selectivity of Ro-26-9228 in Caco-2, a human cell line of intestinal origin, and hFOB, and a human fetal osteoblast cell line. We found that the abilities of Ro-26-9228 and the natural hormone, 1,25-dihydroxyvitamin D_3 ($1,25D_3$) to induce VDRE-reporter gene expression in transiently transfected human osteoblasts are similar. In contrast, in Caco-2 cells, Ro-26-9228 induces 40-fold less reporter gene expression than $1,25D_3$ does. We also examined the abilities of the vitamin D receptor (VDR)-ligand complexes from these two cell lines to interact with partners of transcription (glucocorticoid receptor-interacting protein, VDR-interacting protein, and retinoid X receptor), in pull-down assays. These assays revealed that $1,25D_3$ induces similar levels of interaction of these co-factors with VDR from both osteoblasts and intestinal cells. In contrast, Ro-26-9228 induces significant interaction of VDR from osteoblast cells with these co-factors, but less of VDR from Caco-2 cells. These results suggest that the cellular environment of intestinal cells, unlike that of osteoblasts, represses the ability of VDR-Ro-26-9228 complexes to interact with transcription partners.
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Key words: vitamin D receptor; vitamin D analogs; Ro-26-9228; osteoporosis; transcription coactivators

Analogs of vitamin D have been used in the past 20 years as molecular probes to determine the structure-function relationship of vitamin D metabolites and their target molecules: the vitamin D receptor (VDR), vitamin D binding protein, and catabolic enzymes of the vitamin D endocrine system [Procsal et al., 1976; Ray et al., 1986; Bouillon et al., 1995; Norman et al., 2001]. In addition, accumulating information on the wide spectrum of the pharmacological actions of vitamin D metabolites has led to experimentation and limited use of vitamin D analogs for treatment of various clinical conditions, including secondary hyperparathyroidism [Funahashi et al., 1998; Tsukamoto et al., 2000], psoriasis [Thaci et al., 2001], autoimmune disease, and cancer [Gulliford et al., 1998; Kubota et al., 1998; Evans et al., 2002]. Most importantly, an increase in world awareness about the health problems and costs of treatment of osteoporosis in aging populations has facilitated the search for drugs that are useful and safe for either preventing or treating it. Vitamin D metabolites [Tanizawa et al., 1999] and analogs [Francis et al., 1996] have been considered side by side with drugs such as bisphosphonates and selective estrogen receptor modulators (SERMs) [Lufkin et al., 1998; Oleksik et al., 2001]. One of the principal limitations of the use of the active metabolite of vitamin D_3 ($1,25D_3$) to treat osteoporosis is that its therapeutic window is very narrow, i.e., concentrations of the hormone that restore bone loss also induce hypercalcemia. To identify compounds that have the desirable pharmacological

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*Correspondence to: Sara Peleg, PhD, Department of Endocrine Neoplasia and Hormonal Disorders, Box 435, The University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030.
E-mail: speleg@mail.mdanderson.org

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properties for treatment of osteoporosis, many analogs were screened by using ovariectomized rats as an animal model for postmenopausal osteopenia [Tsurukami et al., 1994; Ono et al., 1998]. An analog developed by Roche Bioscience, 1α-fluoro-25-hydroxy-16,23E-diene 26,27-bishomo-20-epi-vitamin D_3 (or Ro-26-9228) has these properties. In rats, up to 27 μg/kg it does not increase serum calcium, 9–14 μg/kg only moderately increases urine calcium, but 0.2 μg/kg inhibits bone resorption, and 3 μg/kg induces a significant increase in bone mineral density [Peleg et al., 2002]. In contrast, in the same animal model, $1,25D_3$ induces hypercalcemia and hypercalciurea at concentrations that inhibit bone resorption and increase bone mineral density (0.05–0.5 μg/kg) (Fig. 1). Because the calcium-regulating activities of vitamin D involve the enhancement of duodenal calcium absorption and an increase in bone turnover, we hypothesized that there may be a difference in the sensitivities of duodenum and bone to the analog, whereas both tissues may be equally sensitive to the natural hormone. To examine this hypothesis and to determine if the tissue-selective action is mediated through the VDR, we performed ex vivo experiments with tissues isolated from analog- and $1,25D_3$-treated rats and with human cell lines of osteoblastic and intestinal origin. These tissues and cells were examined for their transcriptional responses to the two compounds, and the results

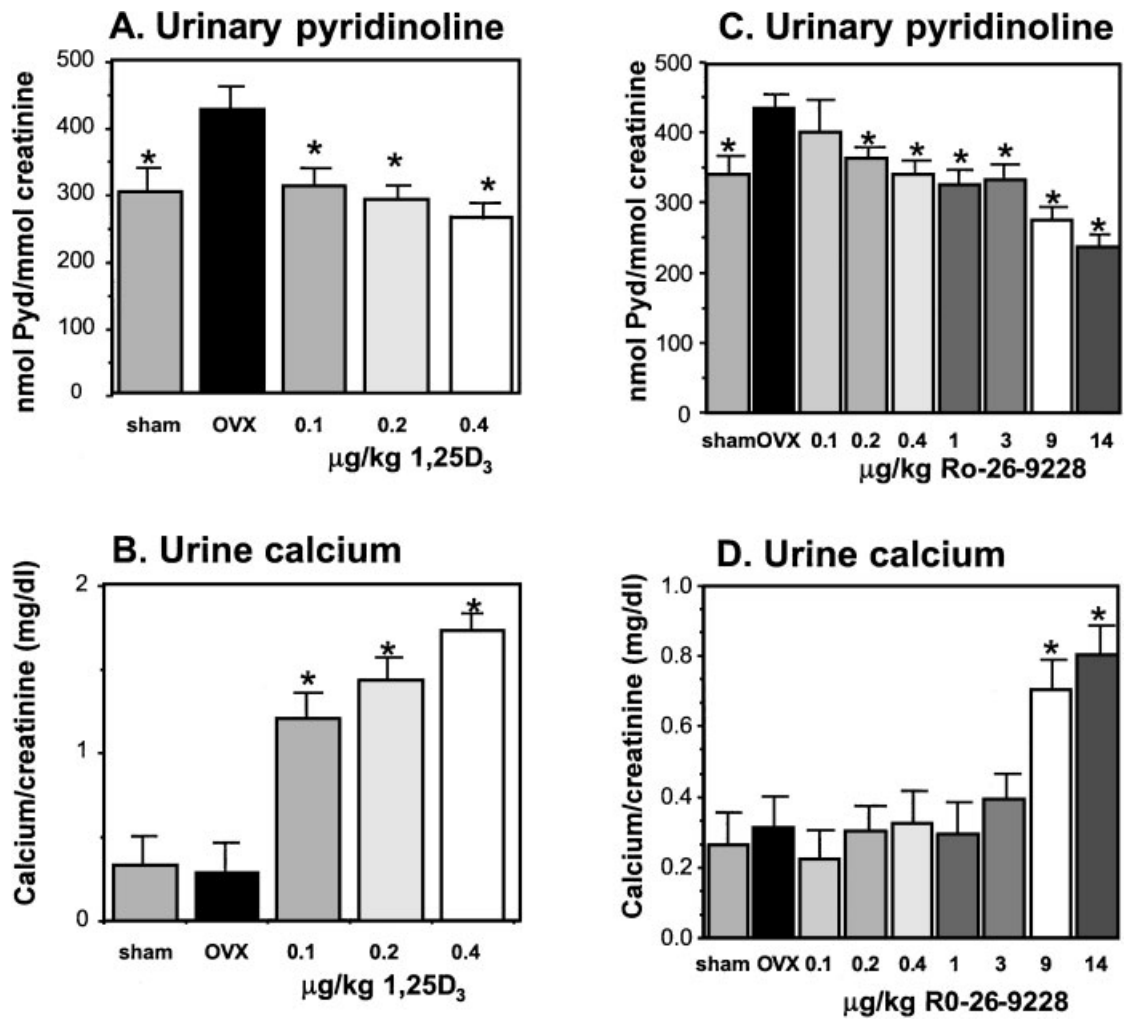

Fig. 1. Regulation of bone resorption and calcium excretion by $1,25D_3$ and by Ro-26-9228. Urinary pyridinoline (**A** and **C**) and urinary calcium (**B** and **D**) levels in sham-operated or ovariectomized (OVX) female rats treated daily either with $1,25D_3$ or Ro-26-9228. The urinary pyridinoline and urinary calcium values were normalized against creatinine levels. [n = 10], $*P < 0.05$ compared to ovariectomized rats. Adapted with permission from Peleg et al. [2002]. Copyright owner: The Endocrine Society.

(described below) suggested that Ro-26-9228 might be a tissue- and cell-type selective VDR modulator.

TISSUE-SELECTIVE GENE REGULATION IN OSTEOPENIC RATS TREATED WITH RO-26-9228

To determine whether the wider safe concentration range of the analog actions in rats is associated with differences in tissue sensitivity to Ro-26-9228 and $1,25D_3$, the abilities of the two compounds to increase gene expression in the duodenum and in the bone were examined by Northern blot analysis of mRNAs expressing VDR target genes. These included transforming growth factor-β (TGF-β), osteocalcin [Mahonen et al., 1990], and osteopontin [Noda et al., 1990] in the bone, and 24-hydroxylase [Chen et al., 1993], calbindin D 9K [Dupret et al., 1987], and plasma membrane calcium pump (PMCA-1) [Zelinski et al., 1991] in the duodenum. Figure 2 shows that a concentration of $1,25D_3$ that saturates 50% of VDR binding sites (0.2 μg/kg) significantly increases mRNA expression of 24-hydroxylase in the duodenum, whereas a concentration of Ro-26-9288 that saturates 50% of the VDR binding sites (5 μg/kg) has no significant effect on the expression of this gene. In the same animals, both $1,25D_3$ and Ro-26-9228 induce significant increases in TGF-β mRNA levels and the analog is more efficacious than $1,25D_3$ in that respect. Similar results were obtained with other gene-regulatory events: in the duodenum of animals treated with Ro-26-9228, there is no increase in calbindin D 9K or PMCA-1, but in the bone, there is a significant increase in osteopontin mRNA and some increase in osteocalcin mRNA. In contrast, $1,25D_3$ increases these mRNAs in both duodenum and bone [Peleg et al., 2002].

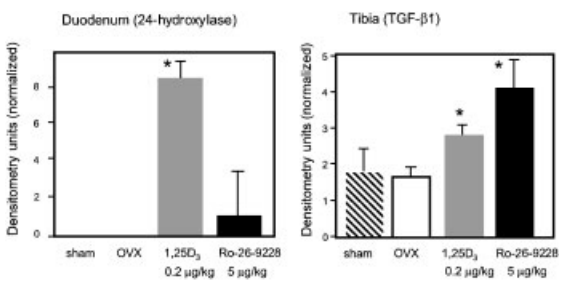

Fig. 2. Expression of vitamin D-responsive genes in the duodenum and bone of $1,25D_3$-treated and Ro-26-9228-treated rats. The mRNA levels in the duodenum were measured by Northern blotting for 24-hydroxylase (**A**) and by semiquantitative reverse transcription-polymerase chain reaction for TGF-β1 (**B**) in the trabecular bone of ovariectomized and sham-operated rats treated with a single dose of $1,25D_3$ (0.2 μg/kg) or Ro-26-9228 (5 μg/kg). The data were normalized against expression of glyceraldehyde-3-phosphate dehydrogenase (G3P) mRNA and are expressed as mean ± SD of six individually tested samples from each group. Adapted with permission from Peleg et al. [2002]. Copyright owner: The Endocrine Society.

SELECTIVE REGULATION OF GENE EXPRESSION BY RO-26-9228 IN OSTEOBLASTIC AND INTESTINE-LIKE CELLS

To further investigate the possibility that Ro-26-9228 regulates VDR-mediated gene expression in a cell-type selective manner, we examined its action in two human cell lines. The colon carcinoma Caco-2, has transcriptionally active VDR, can transport calcium apically (mucosa to serosa) in response to $1,25D_3$, and expresses calbindin D 9K in a vitamin D-regulated fashion [Fleet and Wood, 1994, 1999]. The other cell line, the human fetal osteoblast line, hFOB, can undergo programmed proliferation and differentiation to mature calcified matrix-producing cells. The hFOB cells also have transcriptionally active VDR and express the osteoblast-specific protein osteocalcin, in a vitamin D-regulated fashion [Harris et al., 1995]. The two cell lines were examined for either endogenous gene expression or transgene expression by using the growth hormone reporter attached to a vitamin D-responsive element and the thymidine kinase promoter. We found that the effective doses required to reach 50% of maximal expression (ED_{50}) of both osteocalcin and the growth hormone reporter in hFOB cells are 2–4 nM for $1,25D_3$ and 2–6 nM for the analog. In contrast, in Caco-2 cells, the ED_{50}s for expression of the calbindin D 9K mRNA by $1,25D_3$ and the analog are 5 and 500 nM, respectively. The ED_{50}s for expression of the transgene are 5 nM for $1,25D_3$ and 160 nM for the analog. These results show that the ability of the analog to induce gene expression through the VDR is similar to that of $1,25D_3$ in osteoblastic cells but 30–100 times lower in intestinal cells.

EFFECT OF CELLULAR ENVIRONMENT ON ACTIVATION OF VDR BY RO-26-9228

To further explore the mechanism for the differences in the sensitivities of intestinal and osteoblastic cells to Ro-26-9228, we examined the possibility that the VDR-analog complexes

in Caco-2 cells have a limited ability to interact with dimerization partners and transcription coactivators, whereas the cellular environment in osteoblasts is more permissive for these interactions. To test this possibility we incubated Caco-2 and hFOB cells with receptor-saturating concentrations of either $1,25D_3$ or Ro-26-9228 and then prepared whole-cell extracts. The extracts were incubated with glutathione-sepharose beads and fusion proteins consisting of glutathione-S-transferase (GST) attached to the coactivator glucocorticoid receptor-interacting protein (GRIP), the VDR-interacting protein (DRIP), or the dimerization partner retinoid X receptor (RXR) [Rachez and Freedman, 2000]. The receptor bound to the GST-fusion protein was eluted and detected by gel electrophoresis and Western blot analysis. Figure 3 shows that VDR-$1,25D_3$ complexes from Caco-2 cells strongly interacted with GST-GRIP, but VDR-analog complexes from these cells did not. On the other hand, both $1,25D_3$ and the analog induced a strong interaction between GST-GRIP and VDR from hFOB. That the VDR-analog complexes from Caco-2 cells did not interact with GST-GRIP was not due to an absence of immunoreactive VDR, because both $1,25D_3$- and analog-treated cells have similar amount of immunoreactive VDR (data not shown). It was also not due to poor uptake of the analog by Caco-2 cells, because binding assays showed that 90% of the VDR are occupied by either $1,25D_3$ or the analog within 1 h of incubation of the cells with these compounds. These results strongly suggested that VDR bound with the analog in Caco-2 cells could not assume a conformation that supports interaction with GST-GRIP, whereas the VDR-analog complexes in hFOB cells can.

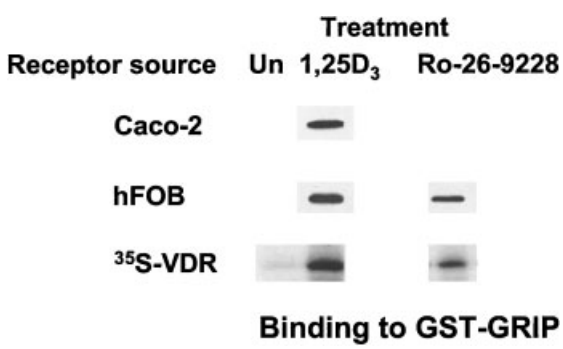

Fig. 3. Cell-specific activation of VDR. Whole-cell extracts were prepared from hFOB and Caco-2 cells incubated either with $1,25D_3$ or Ro-26-9228. VDR from the cell extracts or in vitro synthesized ^{35}S-VDR was incubated with GST-GRIP, and the complexes were isolated by affinity chromatography using glutathione-sepharose. The receptor complexes were eluted, separated by gel electrophoresis, and detected by Western blotting (cellular VDR) or by autoradiography (^{35}S-VDR).

To determine whether the cellular environment in Caco-2 cells represses the interaction of the VDR-analog complexes with the coactivator or the osteoblast environment facilitates this interaction, we repeated the pull-down assays with GST-GRIP using in vitro synthesized ^{35}S-VDR incubated with either $1,25D_3$ or with Ro-26-9228. Figure 3 shows that both $1,25D_3$ and Ro-26-9228 induce interaction of synthetic VDR with the coactivator, though the analog is less effective than $1,25D_3$ in that respect. Similar results were observed with the GST-DRIP and GST-RXR fusion proteins (data not shown). These results suggest that the cellular environment in Caco-2 cells represses the interaction of VDR-analog complexes with coactivators and dimerization partners. This repression mechanism depends on the type of ligand bound, because VDR-$1,25D_3$ complexes are not repressed in these cells.

CONCLUSION

Pharmaceutical research on nuclear receptors has revealed numerous examples of synthetic compounds that can activate their respective receptors in a selective fashion [Bentel et al., 1999; Wagner et al., 1999; Hammond et al., 2001]. One of the best examples is the SERMs, which are synthetic analogs of estrogen used for treatment of bone disease and breast cancer [Lufkin et al., 1998; Love et al., 2002]. The SERMs act as agonists in certain tissues or cells and as antagonists in others [Shang and Brown, 2002]. The tools used to study the mechanisms of action of SERMs include animal models [Fitts et al., 2001], cell culture [Terakawa et al., 1988], and molecular analyses [Shiau et al., 1998]. Furthermore, structural studies by X-ray crystallography have revealed the conformations of receptor-agonist and receptor-antagonist complexes [Brzozowski et al., 1997] and thus explained their different actions. The multifaceted pharmacological actions of $1,25D_3$ suggest it should be possible to selectively enhance or decrease some of these actions by modifying the structures of VDR ligands. Investigative approaches similar to those used to investigate the SERMs must be used to identify and characterize vitamin D analogs that may be

either antagonists or selective VDR modulators before they can be safely used to treat clinical conditions. The mechanisms of action and modes of interaction of the VDR with several antagonists and superagonists (but not selective agonists) have been reported recently [Miura et al., 1999; Ozono et al., 1999; Ishizuka et al., 2001; Tocchini et al., 2001].

Our recent work on Ro-26-9228 suggested it might be the first vitamin D analog to selectively modulate the VDR. This hypothesis is supported by results obtained from in vivo studies and experiments in cell culture and in cell-free systems. However, unlike compounds such as tamoxifen and raloxifene, which act as antagonists of their natural hormone in certain tissues and as poor agonists in others, Ro-26-9228 appears to act as a selective agonist, i.e., it is a poor agonist of the VDR in some tissues (such as intestine (Figs. 2 and 3) and kidney (data not shown), but an effective agonist in bone. The selective actions of Ro-26-9228 in osteoblast cells compared with intestinal cells could be due to a number of factors. One proposed mechanism is that the VDR might be associated with an abundant corepressor in Caco-2 cells but not in osteoblasts (Fig. 4). Corepressors such as nuclear receptor corepressor (NcoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) [Liu et al., 2002] bind to the unoccupied steroid receptors. However, binding of $1,25D_3$ may be sufficient to change the conformation of the VDR in Caco-2 cells so that the corepressor is removed and a coactivator binds the complex. In contrast, the binding of Ro-26-9228 to the VDR in these cells may fail to release the VDR from the corepressor, thereby preventing the interaction of VDR-Ro-26-9228 complexes with coactivator proteins. A similar mechanism was recently proposed for the partial agonist/antagonist activity of various synthetic ligands

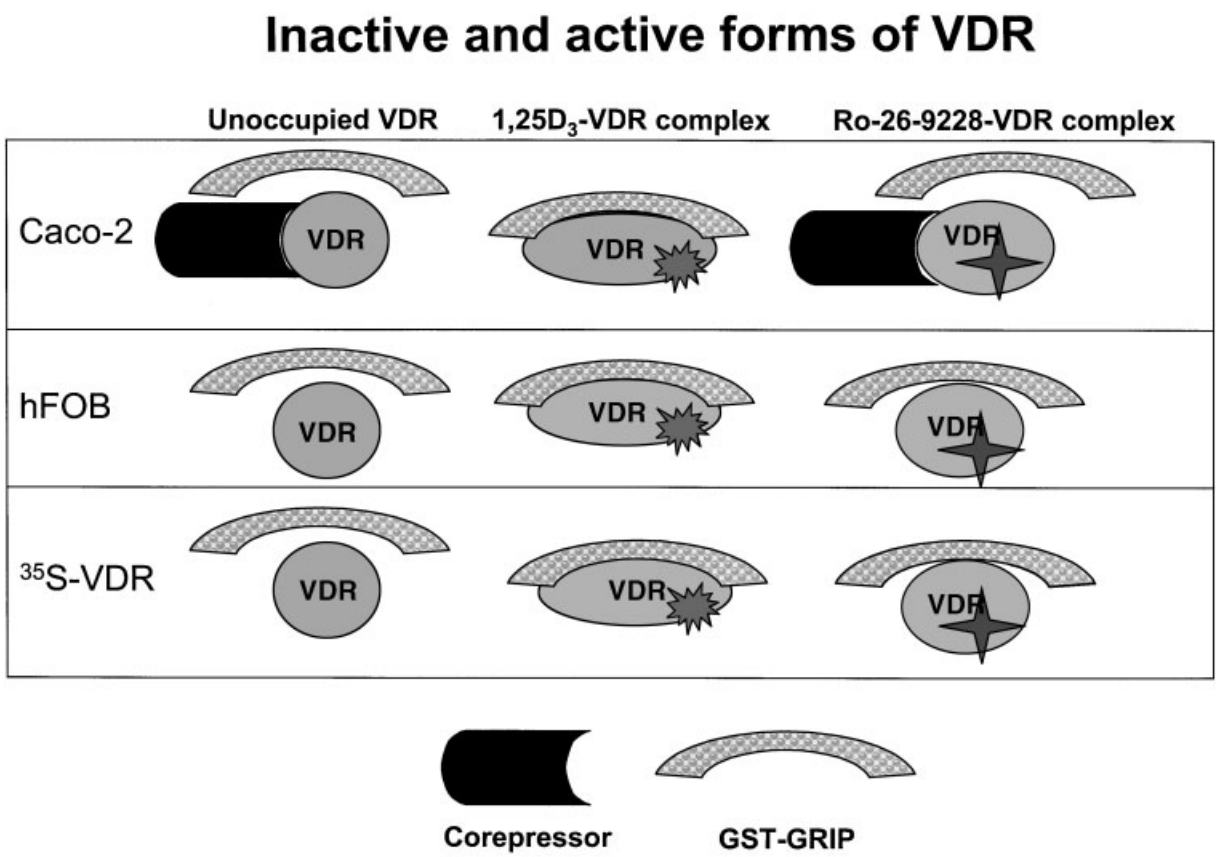

Fig. 4. A possible mechanism for the selective action of Ro-26-9228. VDR in Caco-2 cells is associated with a corepressor, and this repression is relieved in the presence of $1,25D_3$ but not when the VDR is bound to Ro-26-9228. On the other hand, in hFOB cells and in the cell-free system (rabbit reticulocyte lysate) there is no corepressor, which allows the VDR-Ro-26-9228 complexes to bind coactivator proteins.

of the progesterone receptor and the estrogen receptor [Liu et al., 2002; Shang and Brown, 2002]. These reports suggested that the ratio of coactivators and corepressors in a specific cell type may be a deciding factor in the selective actions of these ligands, whether they act as antagonists in cells rich with repressor proteins or agonists in cells rich with coactivator proteins. An alternative mechanism is that osteoblast-specific posttranslational modification of the VDR or an osteoblast-specific factor stabilizes the poor interaction of VDR-Ro-26-9228 complexes with coactivators of transcription. Studies are currently in progress to establish the precise molecular mechanism of the selective action of Ro-26-9228 and to determine its profile of action in a wider spectrum of cell types.

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