Biochemical Pharmacology, Vol. 40, No. 8, pp. 1827–1832, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

AFFINITY OF MC 903 FOR 1,25-DIHYDROXYVITAMIN D RECEPTOR AND ITS EFFECTS ON THE SYNTHESIS OF OSTEOCALCIN IN HUMAN OSTEOSARCOMA CELLS

TEIJA VALAJA, ANITTA MAHONEN, ASTA PIRSKANEN and PEKKA H. MÄENPÄÄ*
Department of Biochemistry and Biotechnology, University of Kuopio, P.O. Box 6, SF-70211 Kuopio, Finland

(*Received* 29 *March* 1990; *accepted* 6 *June* 1990)

Abstract—MC 903 is a new structural analog of the naturally occurring, biologically active 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. MC 903 and 1,25$(OH)_2D_3$ have shown similar receptor binding properties and comparable effects on leukemic cell differentiation. However, MC 903 is at least 100 times less potent in influencing calcium metabolism than 1,25$(OH)_2D_3$. We have therefore studied, how MC 903 competes for the binding sites of 1,25$(OH)_2D_3$, influences the 1,25$(OH)_2D_3$ induced synthesis of the most abundant bone non-collagenous protein, osteocalcin, and induces the activity of alkaline phosphatase in MG-63 human osteosarcoma cells. We found that the new compound binds to 1,25$(OH)_2D_3$ receptors and regulates receptor mRNA levels essentially like the natural ligand. Our results also indicate that MC 903 induces the synthesis of osteocalcin and the activity of alkaline phosphatase in MG-63 cells through a receptor-mediated process almost identically with 1,25$(OH)_2D_3$. Growth of the MG-63 cells was inhibited slightly more with MC 903 than with 1,25$(OH)_2D_3$.

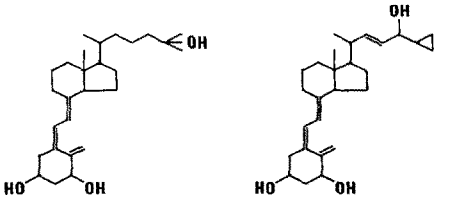
1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$†], the hormonal form of vitamin D_3, functions in its target cells through a receptor-mediated process [1]. It is an important factor in the regulation of bone and mineral metabolism [2]. 1,25$(OH)_2D_3$ also induces cell differentiation and inhibits cell proliferation [3, 4], which has led to efforts of using 1,25$(OH)_2D_3$ in the treatment of proliferative disorders, e.g. psoriasis [5, 6]. Difficulties have, however, been encountered in using highly active vitamin D_3 derivatives because of their potent effects on calcium metabolism [7, 8]. For example, hypercalcemia and hypercalciuria are often induced by systemic doses higher than a few micrograms per day [6].

MC 903 is a new synthetic analog of 1,25$(OH)_2D_3$ with a potent activity on differentiation without inducing hypercalcemia *in vivo* and *in vitro* [9, 10]. The secretion of bone non-collagenous protein, osteocalcin, by osteoblasts is induced by 1,25$(OH)_2D_3$ [11]. We have therefore studied how MC 903 influences the synthesis of osteocalcin in a human osteosarcoma cell line (MG-63). Alkaline phosphatase activity, which is also induced by 1,25$(OH)_2D_3$ [12], has been a further parameter when comparing the biological effects of MC 903 and the natural vitamin D metabolite. The ability of MC 903 to compete with 1,25$(OH)_2D_3$ in binding to the 1,25-dihydroxyvitamin D receptor [hVDR] was determined in uptake studies and the compound was also compared with the natural hormone in the regulation of hVDR and osteocalcin mRNA levels as well as cell growth.

MATERIALS AND METHODS

Cell culture. MG-63 human osteosarcoma cell line was obtained from American Type Culture Collection (Rockville, MD, U.S.A.). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (irradiated), 2 mM L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 0.1 mg/mL kanamycin, and non-essential amino acids (Gibco, Paisley, U.K.) in a humidified 95% air:5% CO_2 incubator. The culture medium was replaced 24 hr before each study with a medium containing 2% charcoal-treated FCS. 1,25$(OH)_2D_3$ and MC 903 were also added in this medium.

Chemicals. 1,25$(OH)_2D_3$ was from Hoffmann-La Roche (Basel, Switzerland). MC 903 (Fig. 1) was a gift from Dr Lise Binderup, Leo Pharmaceutical Products Ltd, Ballerup, Denmark. The double-antibody radioimmunoassay kits for determination of



1,25$(OH)_2D_3$ MC 903

Fig. 1. The chemical structures of 1,25$(OH)_2D_3$ and MC 903.

* To whom reprint requests should be addressed.

† Abbreviations: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; FCS, fetal calf serum; hVDR, human 1,25-dihydroxyvitamin D receptor; RIA, radioimmunoassay.

osteocalcin were obtained from Immuno Nuclear Co. (Stillwater, MO. U.S.A.) [26,27-*methyl*-^{3}H]1,25(OH)$_2$D$_3$ (180 Ci/mmol), [α-^{32}P]dCTP (>3000 Ci/mmol), and [α-^{32}P]ddATP (>5000 Ci/mmol) were purchased from Amersham International (Amersham, U.K.). Nick-translation kits and 3'-end labelling kits were from Boehringer Mannheim GmbH (F.R.G.).

Effects of the hormones on growth of MG 63 cells. The MG-63 cells were seeded at 1×10^4 cells per well onto 4-well plates and incubated in DME containing 10% FCS for 24 hr. The medium was changed into a medium containing 2% charcoal-treated FCS plus the hormone, and the cells were cultured for the times indicated. Cell numbers after each treatment were determined by a hemocytometer.

Induction of osteocalcin synthesis and secretion. Culture plates (60-mm) were treated with a medium containing increasing concentrations of 1,25(OH)$_2$D$_3$ or MC 903 (1 pM to 1 μM) up to 48 hr or with a constant concentration (10 nM) up to 96 hr. After each treatment, the secreted osteocalcin was measured by RIA.

Determination of 1,25(OH)$_2$D$_3$ uptake. The uptake studies were performed as described previously [13]. The confluent plates were incubated in culture conditions for 2 hr at 37° in a medium containing 0.8 nM [26,27-*methyl*-^{3}H]1,25(OH)$_2$D$_3$ with or without unlabelled 1,25(OH)$_2$D$_3$ or MC 903 (concentrations 0.1 μM, 1 nM or 1 pM). After incubation, unbound and bound radioactivity were determined.

Determination of alkaline phosphatase activity. For determination of alkaline phosphatase activity, the cells were treated up to 72 hr with a medium containing 10 nM of 1,25(OH)$_2$D$_3$ or MC 903. The activity was measured as described by Murray *et al.* [14] by determining spectrophotometrically (410 nm) the release of *p*-nitrophenol from *p*-nitrophenyl-phosphate. Total protein was measured with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Table 1. Growth inhibitory activity of 1,25(OH)$_2$D$_3$ and MC 903 in MG-63 cells

Time	1,25(OH)$_2$D$_3$ vs control	MC 903 vs control
72 hr	0.85 ± 0.16	0.74 ± 0.06
120 hr	0.59 ± 0.04	0.42 ± 0.06

The cells were treated for the indicated times with 10^{-8} M 1,25(OH)$_2$D$_3$ or MC 903 before determination of cell numbers. Data are expressed as means ± SE (N = 4).

Slot blot hybridization analysis. For the RNA experiments, the cells were treated for 48 hr with a medium containing 10 nM of 1,25(OH)$_2$D$_3$ or MC 903. Total RNA was prepared according to the method of Anderson *et al.* [15] and the RNA samples were applied onto nylon membranes. Filters were prehybridized, hybridized with probes, and washed as described previously [13]. The probe for hVDR was a cDNA clone (hVDR 1/3) containing the entire coding sequence of the hVDR. The probe for osteocalcin was a synthetic oligonucleotide complementary to the human mRNA sequence coding for amino acids 20–32 of the mature protein.

RESULTS

Table 1 shows the effects of 10 nM 1,25(OH)$_2$D$_3$ and MC 903 on the growth of MG-63 cells. Both hormones inhibited cell growth significantly at 72 and 120 hr. The magnitude of the inhibition at 72 hr was −15% and −26%, respectively, after 1,25(OH)$_2$D$_3$- and MC 903-treatment. At 120 hr, the decrease in cell numbers was −41% after 1,25(OH)$_2$D$_3$-treatment and −58% after MC 903-

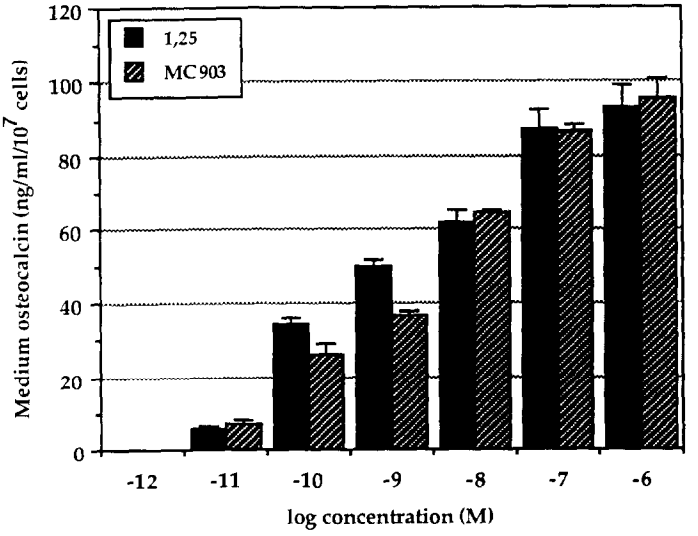


Fig. 2. Secretion of osteocalcin by MG-63 cells during a 48 hr-treatment with 1,25(OH)$_2$D$_3$ or MC 903 at increasing hormone concentrations (1 pM to 1 μM). Vertical bars indicate means + SE from three experiments.

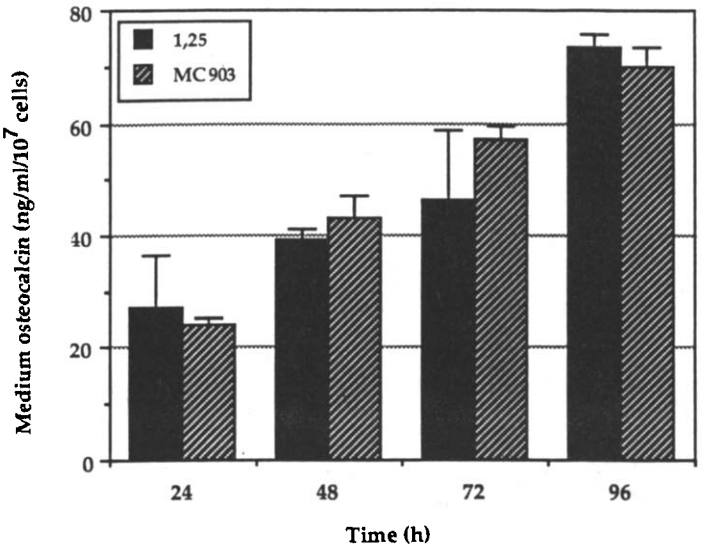


Fig. 3. Secretion of osteocalcin up to 96 hr by MG-63 cells treated with $1,25(OH)_2D_3$ or MC 903 at a constant concentration (10 nM). Vertical bars indicate means + SE from three experiments.

treatment. The differences between the treatment groups were not significant.

The ability of MC 903 to induce the synthesis of osteocalcin was compared with that obtained with $1,25(OH)_2D_3$. The secretion of osteocalcin was induced dose-dependently with $1,25(OH)_2D_3$ and MC 903 (from 1 pM to 1 μM) (Fig. 2). The secretion was first detected at 10 pM hormone concentration and it reached a maximum at 0.1 μM. At a constant concentration (10 nM), the stimulation was first shown at about 24 hr and it continued up to 96 hr (Fig. 3). The results indicate that there are no significant differences between $1,25(OH)_2D_3$ and MC 903 in inducing the synthesis and secretion of osteocalcin.

The results in Fig. 4 demonstrate that $1,25(OH)_2D_3$ and MC 903 increase alkaline phosphatase activity almost identically in MG-63 cells. The activity raised to a 2.4-fold and 2.1-fold level 48 hr after the administration of 10 nM $1,25(OH)_2D_3$ or MC 903, respectively. At 72 hr, the increase was 2.8-fold and 2.5-fold, respectively.

In binding studies, a half maximal displacement from the hVDR was obtained with $1,25(OH)_2D_3$ at 0.25×10^{-10} M and with MC 903 at 0.40×10^{-10} M

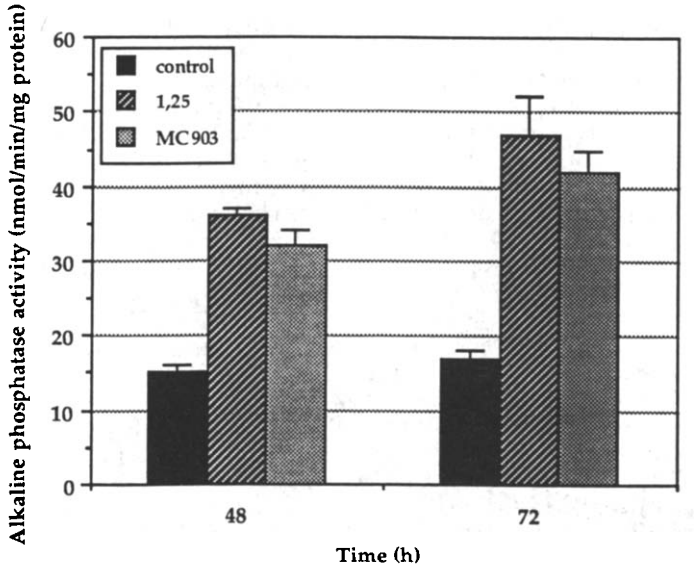


Fig. 4. Alkaline phosphatase activity in MG-63 cells during a 48 hr- or 72 hr-treatment with $1,25(OH)_2D_3$ or MC 903 (10 nM). Vertical bars indicate means + SE from triplicate determinations.

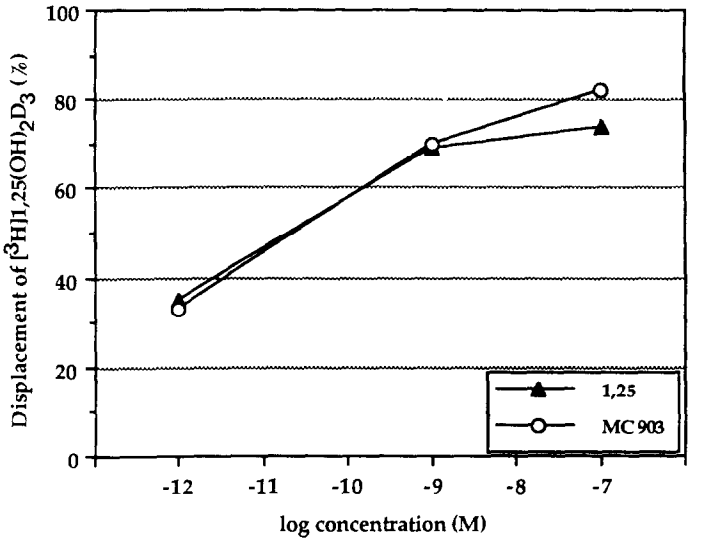


Fig. 5. Displacement of [^{3}H]1,25(OH)$_2$D$_3$ by unlabelled 1,25(OH)$_2$D$_3$ or MC 903 (1 pM, 1 nM, or 0.1 μM). The values represent means of two to three experiments.

hormone concentration (Fig. 5). Thus, the binding of MC 903 to the hVDR is almost as effective as that of 1,25(OH)$_2$D$_3$.

Our previous study has suggested that 1,25(OH)$_2$D$_3$ is able to regulate mRNA levels of its own receptors and those of osteocalcin in human osteosarcoma cells [13]. The ability of MC 903 to affect the mRNA levels was determined by treating the MG-63 cells with 10 nM hormone analog for 48 hr. The hVDR and osteocalcin mRNA levels were elevated to a 1.7-fold and 1.6-fold level, respectively, after the MC 903-treatment (Fig. 6). With 1,25(OH)$_2$D$_3$-treatment, the cells responded with a 2.1-fold increase in hVDR mRNA levels and a 1.6-fold increase in osteocalcin mRNA levels.

DISCUSSION

Efforts of using the natural metabolite of vitamin D$_3$, 1,25(OH)$_2$D$_3$, in the treatment of disease states characterized by excessive cell proliferation and incomplete cell differentiation have often failed because of the compound's profound influence on calcium homeostasis [7]. Alterations in the structure of the vitamin D$_3$ molecule have yielded important information on the structural features of the sterol required for biological activity. This has stimulated efforts of synthesizing analogs with potent effects on cell proliferation and differentiation, while having a weak effect on calcium metabolism [16]. MC 903 is a cyclopropyl derivative, which differs from 1,25(OH)$_2$D$_3$ in having a 25-hydroxyl group at position 24 and a cycloalkane ring consisting of carbons 25, 26 and 27 [17]. This structure is thought to be responsible for the ability described above [17]. Previous studies by Kragbelle [18] have demonstrated that topical application of MC 903 provides an effective and well-tolerated treatment of psoriasis vulgaris. In view of those results, we were interested in studying the effects of MC 903 and 1,25(OH)$_2$D$_3$ in cultured osteoblast-derived human cells. As a biochemical marker related to calcium metabolism in osteoblast-like cells, we studied the synthesis and secretion of the bone calcium-binding protein, osteocalcin. The growth inhibitory activity of 1,25(OH)$_2$D$_3$ and MC 903 revealed that MC 903 may have a slightly better differentiating effect compared with 1,25(OH)$_2$D$_3$ in human osteosarcoma cells. This agrees with the result of Zhou *et al.* [16], who showed that MC 903 is more potent than 1,25(OH)$_2$D$_3$ in modulating leukemic cell growth and differentiation.

The effects of MC 903 on osteocalcin secretion are essentially similar to those produced by 1,25(OH)$_2$D$_3$. MC 903 and 1,25(OH)$_2$D$_3$ caused an identical dose-dependent increase in osteocalcin production reaching saturation at 0.1 μM hormone concentration and being about 70-fold at 96 hr. The values were similar to those found in human bone cell cultures after 1,25(OH)$_2$D$_3$-treatment [19]. The response of the cells to both hormones was also seen in similarly increased levels of osteocalcin mRNA. Franceschi *et al.* [20] and Mulkins *et al.* [21] have shown that treatment of human osteoblast-like osteogenic sarcoma cells with 1,25(OH)$_2$D$_3$ increases the specific activity of alkaline phosphatase. We observed similar elevations in alkaline phosphatase activities in MG-63 cells treated either with MC 903 or 1,25(OH)$_2$D$_3$. Therefore, the effects of MC 903 on osteocalcin synthesis and alkaline phosphatase activity seem to be almost identical with those obtained by 1,25(OH)$_2$D$_3$.

1,25(OH)$_2$D$_3$ functions by binding to specific nuclear receptor proteins in target cells [1]. The specificity of various derivatives of vitamin D in inducing differentiation is correlated with their association with the receptor [22]. MC 903 is similar to 1,25(OH)$_2$D$_3$ when tested in the MG-63 cell line displaying receptors for 1,25(OH)$_2$D$_3$ [20]. According to our studies, MC 903 binds to the hVDR at least as effectively as 1,25(OH)$_2$D$_3$ and elevates

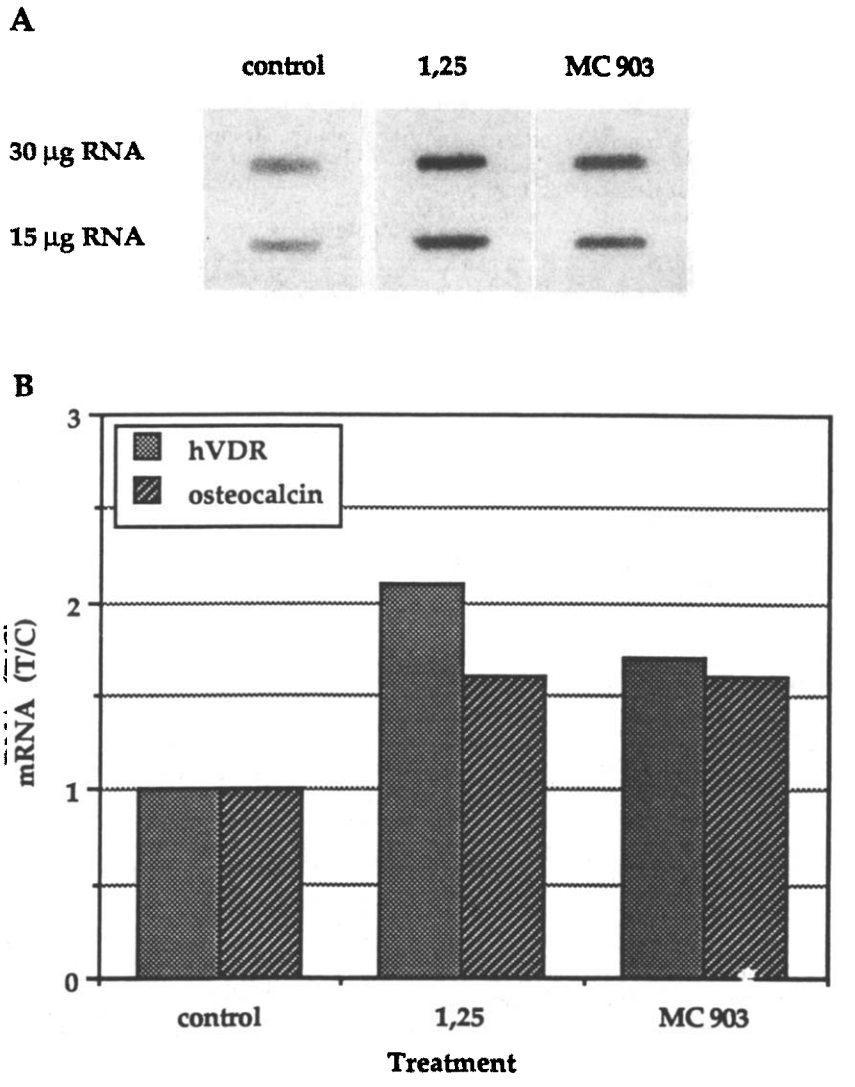


Fig. 6. Elevation of hVDR and osteocalcin mRNA levels after a 48 hr-treatment with $1,25(OH)_2D_3$ or MC 903 (10 nM). (A) Autoradiogram of a slot blot hybridization with hVDR cDNA. (B) Mean values of autoradiograms of hVDR and osteocalcin mRNA levels quantified by densitometric scanning.

mRNA levels of hVDR essentially like the natural ligand.

In view of its strong effects on cell proliferation, cell differentiation, osteocalcin production, alkaline phosphatase activity, and its affinity for the hVDR, the low activity of MC 903 in calcium mobilization [10] is somewhat surprising. MC 903 binds, however, less efficiently than $1,25(OH)_2D_3$ to serum proteins [9], suggesting that the affinity of MC 903 for the vitamin D binding protein in serum is lower than that of $1,25(OH)_2D_3$ [10]. In our cell culture experiments, we used charcoal-stripping of serum, which nonspecifically may remove, e.g., binding proteins. These conditions may explain, why we did not see differences in the effects of $1,25(OH)_2D_3$ and MC 903, which would be caused by different affinity of the hormones for the vitamin D binding protein in serum. Also, MC 903 has a shorter half-life in circulation than $1,25(OH)_2D_3$ being about 7 min (Lise Binderup, personal communication). This supports the view that MC 903 has a shorter time of influence in the human body. On the other hand, local application of MC 903 to the skin will assure an efficient contact with skin target cells, because the less efficient protein binding may result in decreased rate of clearance from the site of application into circulation. These observations thus support the conclusion that MC 903 is a new vitamin D metabolite, which may have value over the natural metabolite, e.g., in the treatment of proliferative disorders such as psoriasis [18].

Acknowledgements—This work was supported in part by grants from the Academy of Finland. We thank Dr Lise Binderup, Leo Pharmaceutical Products, Ltd, for kindly providing MC 903 and Drs Donald P. McDonnell and Bert W. O'Malley for the hVDR cDNA.

REFERENCES

1. Minghetti PP and Norman AW, 1,25(OH)$_2$-vitamin D$_3$ receptors: gene regulation and genetic circuitry. *FASEB J* **2**: 3043–3053, 1988.
2. Cancela C, Nemere I and Norman AW, 1α,25(OH)$_2$ vitamin D$_3$: a steroid capable of producing pleiotropic receptor-mediated biological responses by both genomic and non genomic mechanisms. *J Steroid Biochem* **30**: 33–39, 1988.
3. Abe E, Miyaura C, Sakagami H, Takeda M, Konno K, Yamazaki T, Yoshiki S and Suda T, Differentiation of mouse myeloid leukemia cells induced by 1α,25-dihydroxyvitamin D$_3$. *Proc Natl Acad Sci USA* **78**: 4990–4994, 1981.
4. Dokoh S, Donaldson CA and Haussler MR, Influence of 1,25-dihydroxyvitamin D$_3$ on cultured osteogenic sarcoma cells: correlation with the 1,25-dihydroxyvitamin D receptor. *Cancer Res* **44**: 2103–2109, 1984.
5. Holick MF, McCarthy MF, Joyce-Otis D, Pochi P and Bhawan J, Long-term effects of orally and topically administered 1,25-dihydroxyvitamin D$_3$ on calcium and bone metabolism in healthy young and middle-aged male and female who suffer from psoriasis: 1,25-dihydroxyvitamin D$_3$ heralds an exciting new pharmacologic era for treating psoriasis. *J Bone Mineral Res* **4**: S298, 1988.
6. Holick MF, Smith E and Pincus S, Skin as the site of vitamin D synthesis and target tissue for 1,25-dihydroxyvitamin D$_3$. Use of calcitriol (1,25-dihydroxyvitamin D$_3$) for treatment of psoriasis. *Arch Dermatol* **123**: 1677–1683a, 1987.
7. Norman AW, Putkey JA and Nemere I, Intestinal calcium transport: pleiotropic effects mediated by vitamin D. *Fed Proc* **41**: 78–83, 1982.
8. Breslau NA, McGuire JL, Zerwekh JE, Frenkel EP and Pak CY, Hypercalcemia associated with increased serum calcitriol levels in three patients with lymphoma. *Ann Intern Med* **100**: 1–6, 1984.
9. Binderup L, MC 903—A novel vitamin D analogue with potent effects on cell proliferation and cell differentiation. In: *Vitamin D. Molecular, Cellular and Clinical Endocrinology* (Eds. Norman AW, Schaefer K, Grigoleit H-G and v. Herrath D), pp. 300–309. Walter de Gruyter & Co., Berlin, 1988.
10. Binderup L and Bramme E, Effects of a novel vitamin D analogue MC 903 on cell proliferation and differentiation *in vitro* and on calcium metabolism *in vivo*. *Biochem Pharmacol* **37**: 889–895, 1988.
11. Price P and Baukol SA, 1,25-dihydroxyvitamin D$_3$ increases synthesis of the vitamin K-dependent bone protein by osteosarcoma cells. *J Biol Chem* **255**: 11660–11663, 1980.
12. Manolagas SC, Spiess YH, Burton DW and Deftos LJ, Mechanism of action of 1,25-dihydroxyvitamin D$_3$-induced stimulation of alkaline phosphatase in cultured osteoblast-like cells. *Mol Cell Endocrinol* **33**: 27–36, 1983.
13. Mahonen A, Pirskanen A, Keinänen R and Mäenpää PH, Effect of 1,25(OH)$_2$D$_3$ on its receptor mRNA levels and osteocalcin synthesis in human osteosarcoma cells. *Biochim Biophys Acta* **1048**: 30–37, 1990.
14. Murray E, Provvedini D, Curran D, Catherwood D, Sussman H and Manolagas S, Characterization of a human osteoblastic osteosarcoma cell line (SaOs-2) with high bone alkaline phosphatase activity. *J Bone Mineral Res* **2**: 231–238, 1987.
15. Anderson CW, Lewis JB, Atkins JF and Gesteland RF, Cell-free synthesis of adenovirus 2 proteins programmed by fractionated messenger RNA: a comparison of polypeptide products and messenger RNA lengths. *Proc Natl Acad Sci USA* **71**: 2756–2760, 1974.
16. Zhou J-Y, Norman AW, Lübbert M, Collins ED, Uskokovic MR and Koeffler HP, Novel vitamin D analogs that modulate leukemic cell growth and differentiation with little effect on either intestinal calcium absorption or bone calcium mobilization. *Blood* **74**: 82–93, 1989.
17. Calverley M, Synthesis of MC 903, a biologically active vitamin D metabolite analogue. *Tetrahedron* **43**: 4609–4619, 1987.
18. Kragballe K, Treatment of psoriasis by topical application of the novel cholecalciferol analogue calcipotriol (MC 903). *Arch Dermatol* **125**: 1647–1652, 1989.
19. Beresford JN, Gallagher JA, Poser JW and Russell RGG, Production of osteocalcin by human bone cells in vitro. Effects of 1,25(OH)$_2$D$_3$, 24,25(OH)$_2$D$_3$, parathyroid hormone, and glucocorticoids. *Metab Bone Dis Rel Res* **5**: 229–234, 1984.
20. Franceschi RT, Wilbur MJ and Zerlauth G, 1α,25-Dihydroxyvitamin D$_3$ specific regulation of growth, morphology and fibronectin in a human osteosarcoma cell line. *J Cell Phys* **123**: 401–409, 1985.
21. Mulkins MA, Manolagas SC, Deftos LJ and Sussman HH, 1,25-dihydroxyvitamin D$_3$ increases bone alkaline phosphatase isoenzyme levels in human osteogenic sarcoma cells. *J Biol Chem* **258**: 6219–6225, 1983.
22. Yamada S, Yamamoto K, Naito H, Suzuki T, Ohmori M, Takayama H, Shiina Y, Miyaura C, Tanaka H, Abe E, Suda T, Matsunaga I and Nishii Y, Syntheses and differentiating action of vitamin D endoperoxides. Singlet oxygen adducts of vitamin D derivatives in human myeloid leukemia cells (HL-60). *J Med Chem* **28**: 1148–1153, 1985.