Pergamon

0960-0760(94)E0081-U

J. Steroid Biochem. Molec. Biol. Vol. 50, No. 3/4, pp. 145–150, 1994
Copyright © 1994 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0960-0760/94 $7.00 + 0.00

Vitamin D Receptor Binding and Biological Effects of Cholecalciferol Analogues in Rat Thyroid Cells

Jens P. Berg,[1,2]* Kjellaug M. Liane,[1] Siri B. Bjørhovde,[1] Trine Bjøro,[1] Peter A. Torjesen[1] and Egil Haug[1]

[1]*Hormone Laboratory, Aker University Hospital, Trondheimsveien 235, N-0514 Oslo, Norway and* [2]*Department of Tissue Culture, Institute for Cancer Research, The Norwegian Radium Hospital, N-0310, Oslo, Norway*

The cholecalciferol analogues 1(S),3(R)-dihydroxy-20(R)-[3′(S)-cyclopropyl-3′-hydroxyprop-1′(E)-enyl]-9,10-secopregna-5(Z),7(E),10(19)-triene (calcipotriol, MC 903), 1(S),3(R)-dihydroxy-20(R)-[3′-ethyl-3′-hydroxy-pentoxy]-9,10-secopregna-5(Z),7(E),10(19)-triene (KH 1060) and 1(S),3(R)-dihydroxy - 20(R) - [5′ - ethyl - 5′ - hydroxy - hepta - 1′(E),3′(E) - diene - 1′ - yl] - 9,10 - secopregna-5(Z),7(E),10(19)-triene (EB 1089) have been modified in the side chain to increase their effects on cell differentiation and proliferation and to reduce the risk of inducing hypercalcemia. The effects of these analogues were tested on FRTL-5 cells, a strain of continuously growing and well-differentiated rat thyroid cells. FRTL-5 cells express a normal vitamin D receptor (VDR), and 1,25-$(OH)_2D_3$ potently attenuates the thyrotropin (TSH) stimulated production of the intracellular signalling molecule 3′,5′-cyclic adenosine monophosphate (cAMP), iodide uptake and cell growth of these cells. These effects were also induced by the cholecalciferol analogues after 4 days of incubation. KH 1060 was the biologically most potent of the analogues and, compared to KH 1060, the IC_{50} values were 1.2-, 2.7- and 14-fold higher when 1,25-$(OH)_2D_3$, EB 1089 and MC 903, respectively, were used for the displacement of receptor bound [^{3}H]1,25-$(OH)_2D_3$. As indicated by their VDR binding, 1,25-$(OH)_2D_3$ and EB 1089 were equipotent inhibitors of the TSH stimulated adenylyl cyclase activity, iodide uptake and FRTL-5 cell growth. The analogue MC 903 was the second most potent inhibitor of cell growth in spite of expressing the lowest affinity for the VDR and the weakest inhibition of TSH-stimulated adenylyl cyclase activity and iodide uptake. In conclusion, the biological effects of these cholecalciferol analogues in rat thyroid FRTL-5 cells seem to be mainly determined by their binding affinity for the VDR, although non-genomic effects can not be excluded.

J. Steroid Biochem. Molec. Biol., Vol. 50, No. 3/4, pp. 145–150, 1994

INTRODUCTION

It is well known that vitamin D plays an important role in regulating the calcium metabolism. Recently, it has become evident that vitamin D is also a potent regulator of proliferation and differentiation in a wide range of cells [1, 2]. Systemic administration of 1,25-$(OH)_2D_3$ activates the intracellular vitamin D receptor (VDR), a nuclear transcription factor, and regulates gene transcription in every cell expressing the receptor [3]. The potential use of vitamin D as a differentiation factor in the treatment of proliferative disorders has been hampered by the development of hypercalcemia. This has led to the search for cholecalciferol analogues with augmented effects on cell proliferation and differentiation and reduced effects on calcium homeostasis compared to 1,25-$(OH)_2D_3$ [4]. Topical administration of the cholecalciferol analogue MC 903 (calcipotriol) has been introduced in the treatment of psoriasis [5]. The analogue EB 1089 inhibited tumor cell growth both in a human breast cancer cell line (MCF-7) and in rats with breast cancer [6]. KH 1060, another cholecalciferol analogue, regulated cell growth and differentiation much more potently than 1,25-$(OH)_2D_3$ in a human histiocytic lymphoma cell line [7]. It also inhibited T lymphocyte proliferation and showed an additive immunosuppressive effect in combination with

*Correspondence to J. P. Berg.
Received 10 Nov. 1993; accepted 31 Mar. 1994.

cyclosporin A [8]. Most of the studies with cholecalciferol analogues have been performed in cancer cell lines, leukocytes/lymphocytes and in the classical vitamin D target tissues.

We have studied the effects of 1,25-$(OH)_2D_3$ on FRTL-5 cells, a well differentiated, continuously growing, rat thyroid cell line that have retained the characteristics of normal thyroid follicular cells [9]. The cells express a functional thyrotropin (TSH) receptor and TSH stimulates the production of the intracellular messenger 3′,5′-cyclic adenosine monophosphate (cAMP) [10]. FRTL-5 cell proliferation [10], iodide uptake [11] and thyroglobulin synthesis [12] are all stimulated by TSH. These effects of TSH can be mimicked by cAMP analogues. The FRTL-5 cells express the VDR [13, 14], and 1,25-$(OH)_2D_3$ attenuates the TSH stimulated cAMP production in these cells [15]. Furthermore, both the TSH and cAMP analogue stimulated cell proliferation and iodide uptake are inhibited by 1,25-$(OH)_2D_3$ [16].

The FRTL-5 cells represent a well defined *in vitro* system for comparing the binding characteristics of the cholecalciferol analogues with their effects on cell proliferation and differentiation. We therefore compared the VDR binding affinity of the side chain modified cholecalciferol analogues MC 903, EB 1089, KH 1060 and 1,25-$(OH)_2D_3$ (Fig. 1) and their effects on the TSH stimulated adenylyl cyclase activity (ACA), iodide uptake and cell growth.

EXPERIMENTAL

Hormones and chemicals

1,25-$(OH)_2D_3$, and the synthetic analogues 1(S),3(R)-dihydroxy-20(R)-[3′(S)-cyclopropyl-3′-hydroxyprop-1′(E)-enyl]-9,10-secopregna-5(Z),7(E),10(19)-triene (calcipotriol, MC 903), 1(S),3(R)-dihydroxy-20(R)-[3′-ethyl-3′-hydroxy-pentoxy]-9,10-secopregna-5(Z),7(E),10(19)-triene (KH 1060) and 1(S),3(R)-dihydroxy-20(R)-[5′-ethyl-5′-hydroxy-hepta-1′(E),3′(E)-diene-1′-yl]-9,10-secopregna-5(Z),7(E),10(19)-triene (EB 1089) were gifts from Dr L. Binderup, Leo Pharmaceutical Products (Ballerup, Denmark). Coon's modified Ham's F-12 medium, bTSH, insulin, transferrin, MEM non-essential amino acids, bovine serum albumin (BSA), isobutymethylxanthine (IBMX), adenosine trisophosphate (ATP), creatine phosphate, creatine phosphokinase and potassium iodide were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Penicillin was brought from A/S Apothekernes Laboratorium (Oslo, Norway), streptomycin from Evans Medical (Langhurst, Horsham, U.K.), 1α,25-dihydroxy[26,27-methyl-^{3}H]cholecalciferol ([^{3}H]1,25-$(OH)_2D_3$), $Na^{125}I$ and [8-^{3}H]-3′,5′-cyclic adenosine monophosphate from

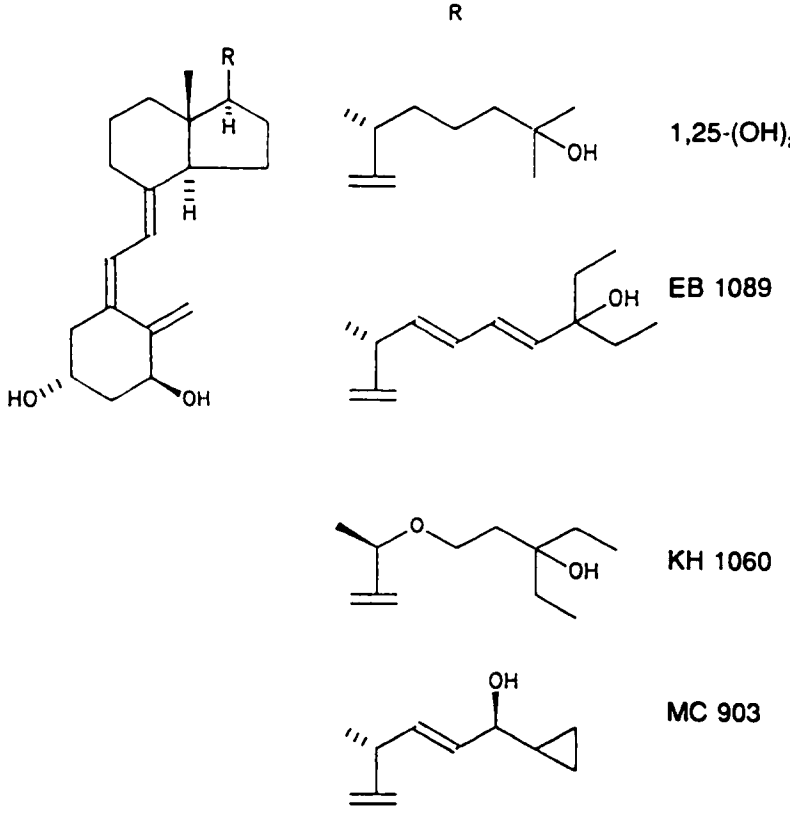

Fig. 1. Structural formulas for the cholecalciferol analogues, 1,25-$(OH)_2D_3$, EB 1089, KH 1060 and MC 903.

Amersham International (Buckinghamshire, U.K.), Dextran T-70 from Pharmacia (Uppsala, Sweden), Norit A from Amend Drug and Chemical (Irvington, U.S.A.), dithiotreitol from Calbiochem-Behring Corp. (La Jolla, U.S.A.) and heat-inactivated newborn calf serum from Flow Laboratories (Irvine, Scotland, U.K.).

Cell culture

The FRTL-5 cells were kindly provided by Dr Ulla Feldt-Rasmussen (Copenhagen, Denmark) through Interthyr Research Foundation Inc. and cultured, as previously described [16], in modified Ham's F-12 medium supplemented with 5% heat-inactivated newborn calf serum, bTSH (1 U/l), insulin (1.7 μmol/l), transferrin (60 nmol/l), penicillin (10,000 U/l) and streptomycin (10 mg/l).

1,25-$(OH)_2D_3$ receptor binding

The receptor binding experiments were performed essentially as previously described [13]. Cell cytosol was prepared by sonicating the cells in ice-cold hypertonic TEKMD buffer (Tris 10 mmol/l, EDTA 1.5 mmol/l, KCl 300 mmol/l, Na_2MoO_4 10 mmol/l, dithiotreitol 1 mmol/l, pH 7.4) and centrifugation for 60 min (100,000 *g*, 4°C). Protein concentration was measured in the supernatant, which was used for the binding studies. Cytosol (100 μl, 3 mg protein/ml), [^{3}H]1,25-$(OH)_2D_3$ (100 μl, 4 nmol/l) without or with increasing concentrations of unlabelled 1,25-$(OH)_2D_3$, MC 903, KH 1060 or EB 1089 (100 μl) were incubated over night at 4°C. Bound and free ligand were separated by charcoal adsorption and bound [^{3}H]1,25-$(OH)_2D_3$ was measured in a RackBeta 1219 liquid scintillation counter (LKB Wallac, Turku, Finland).

Adenylyl cyclase activity

FRTL-5 cell homogenates were made by disrupting the cells in a hypotonical buffer (Pipes 25 mmol/l, $MgCl_2$ 5 mmol/l, IBMX 1.0 mmol/l, EDTA 1.0 mmol/l and ATP 3 mmol/l, pH 7.6), to which was added an ATP regenerating system (creatine phosphate 20 mmol/l and creatine phosphokinase 33 IU/l) [17]. The homogenates were treated with TSH (10 U/l) for 5 min at 30°C, and cAMP measured using a competitive protein binding assay [18]. The results were expressed per mg cell protein.

Iodide uptake

The cells were grown in Costar (Cambridge, MA, U.S.A.) tissue culture dishes (24 wells) in the presence of cholecalciferol analogue or vehicle as indicated. The assay was performed essentially as described by Weiss *et al.* [11]. Briefly, the cells were incubated in Hanks balanced salt solution (HBSS) supplemented with KI (10 μmol/l), known amounts of Na^{125}I and Hepes (10 mmol/l) at pH 7.4 for 45 min at 37°C. The cells were washed in ice-cold HBSS and finally incubated

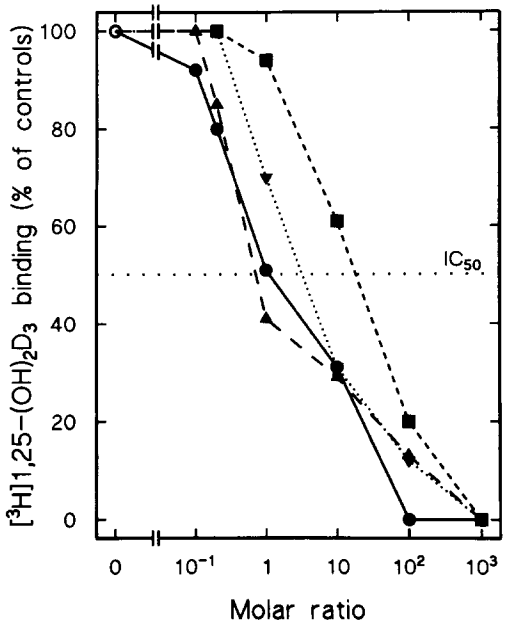

Fig. 2. Effects of unlabelled cholecalciferol analogues on the binding of [^{3}H]1,25-$(OH)_2D_3$ to FRTL-5 cell cytosol. Aliquots of cytosol were incubated with [^{3}H]1,25-$(OH)_2D_3$ in the absence or presence of increasing concentrations of either 1,25-$(OH)_2D_3$ (●), MC 903 (■), KH 1060 (▲) or EB 1089 (▼). The results are expressed as the percentage of specifically bound [^{3}H]1,25-$(OH)_2D_3$ in the absence of competitor (○).

for 60 min in HBSS to which was added potassium perchlorate (20 μmol/l) to release the intracellularly accumulated $^{125}I^-$ by blocking the re-uptake [19]. The amount of $^{125}I^-$ in the buffer was measured in a 1260 Multigamma gammacounter (LKB Wallac, Turku, Finland). The cells in the corresponding wells were harvested by trypsination and counted using a VBA 140 cell counter (Analys Instrument, Stockholm, Sweden). The amount of accumulated I^- was expressed as fmol per cell.

Measurement of cell growth

FRTL-5 cells were cultured in Costar tissue culture dishes. Cells in 3 dishes were harvested by trypsination and the cell numbers determined (VBA 140 cell counter). The other dishes got fresh growth medium to which was added increasing amounts of the cholecalciferol analogues. The cells were harvested by trypsination after 4 days and the increase in cell numbers during the incubation period determined.

Protein measurements

Protein was measured by the Coomassie blue dye method with BSA as standard, using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Statistics

The TSH stimulated adenylyl cyclase activity, iodide uptake and cell growth in the presence of

cholecalciferol analogues are presented as a percentage of the corresponding control cell values. The concentration of analogue needed to displace the radioligand binding by 50% (IC_{50}) and B_{max} was calculated using the computer program LIGAND (Elsevier-Biosoft, Cambridge, U.K.). The analogues are compared by analysing the differences in the inhibition at every concentration tested. The sum of these differences is expressed as the mean difference and the statistical analyses are performed using two-way ANOVA and the Student–Newman–Keuls test.

RESULTS

The binding affinity of the cholecalciferol analogues for the VDR was studied by measuring the displacement of [^{3}H]1,25-$(OH)_2D_3$ specifically bound to FRTL-5 cell cytosol (Fig. 2). B_{max} for [^{3}H]1,25-$(OH)_2D_3$ was estimated to be 18 fmol/mg cytosol protein. Binding studies were performed with 1.3 nmol/l [^{3}H]1,25-$(OH)_2D_3$, and specific binding was 12 fmol/mg protein. At this concentration the IC_{50}'s for KH 1060, 1,25-$(OH)_2D_3$, EB 1089 and MC 903 were 1.6, 1.9, 4.3 and 22 nmol/l, respectively. Sucrose density gradient centrifugation demonstrated that all the cholecalciferol analogues displaced [^{3}H]1,25-$(OH)_2D_3$ from a 3.7 S receptor in the FRTL-5 cell cytosol [13] (data not shown).

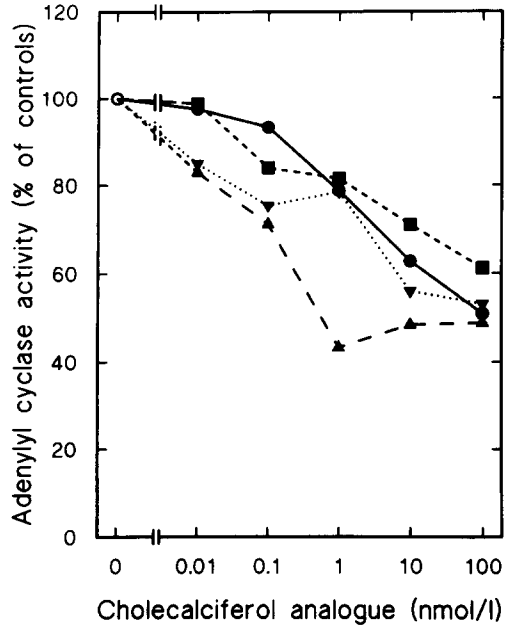

Fig. 3. Effects of increasing concentrations of cholecalciferol analogues on the TSH stimulated adenylyl cyclase activity in FRTL-5 cells. The cells were incubated for 4 days in 2H medium with increasing concentrations of either 1,25-$(OH)_2D_3$ (●), MC 903 (■), KH 1060 (▲) or EB 1089 (▼). The adenylyl cyclase activity was measured after 5 min incubation with 30 U/l TSH. The results (mean, $n = 6$) are expressed as a percentage of the adenylyl cyclase activity in vehicle treated controls (○) (mean: 68.0 pmol cAMP mg protein^{-1} min^{-1}). The following rank order of potency was obtained: KH 1060 > EB 1089 > 1,25-$(OH)_2D_3$ = MC 903 (>denotes a statistically significant difference ($P < 0.05$); two-way ANOVA and the Student–Newman–Keuls test).

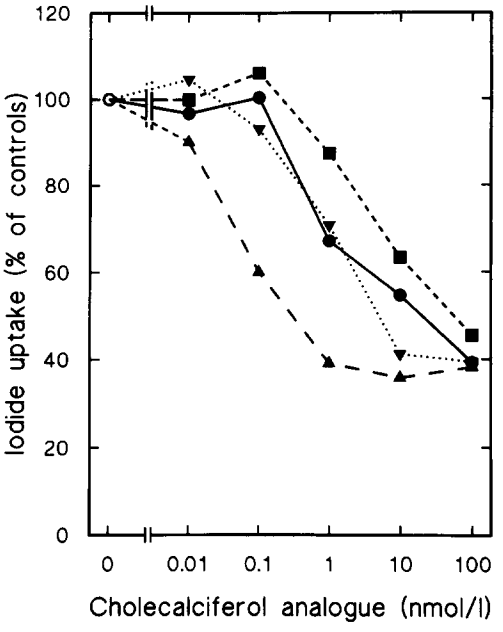

Fig. 4. Effects of increasing concentrations of cholecalciferol analogues on the TSH stimulated iodide uptake in FRTL-5 cells. The cells were incubated for 4 days in 3H medium in the presence of increasing concentrations of either 1,25-$(OH)_2D_3$ (●), MC 903 (■), KH 1060 (▲) or EB 1089 (▼). The results (mean, $n = 12$) are expressed as a percentage of the iodide uptake in vehicle treated controls (○) (mean: 0.87 fmol I^- cell). The following rank order of potency was obtained: KH 1060 > EB 1089 = 1,25-$(OH)_2D_3$ > MC 903 [>denotes a statistically significant difference ($P < 0.05$); two-way ANOVA and the Student–Newman–Keuls test].

It has previously been shown that the ability of TSH to stimulate cAMP production was augmented when FRTL-5 cells were precultured in the absence of TSH [10], giving a maximally inhibitory effect of 1,25-$(OH)_2D_3$ after 4 days [15]. Since the maximal effects of the cholecalciferol analogues were also obtained after 4 days (data not shown), the cells were incubated with increasing concentrations of the cholecalciferol analogues for 4 days in culture medium without TSH (Fig. 3). KH 1060 was the most potent inhibitor of the TSH stimulated ACA.

The TSH stimulated iodide uptake by FRTL-5 cells was maximally attenuated after 4 days of incubation with both 1,25-$(OH)_2D_3$ [16] and the synthetic cholecalciferol analogues (data not shown). When increasing amounts of the analogues were added to the growth medium, KH 1060 attenuated the TSH stimulated iodide uptake most potently (Fig. 4). EB 1089 and 1,25-$(OH)_2D_3$ were almost equipotent and MC 903 was significantly the least potent inhibitor of iodide uptake.

Although the analogues induced almost the same maximal inhibition of cell proliferation, KH 1060 was also the most potent inhibitor of cell growth (Fig. 5).

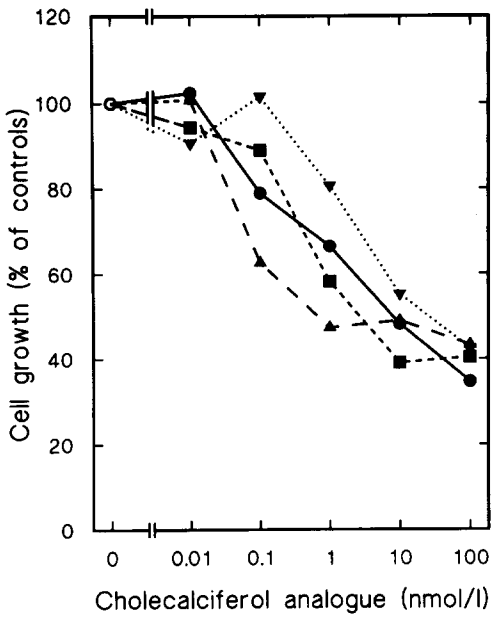

Fig. 5. Effects of increasing concentrations of cholecalciferol analogues on the TSH stimulated FRTL-5 cell growth. The cells were incubated for 4 days in 3H medium in the presence of increasing concentrations of either 1,25-$(OH)_2D_3$ (●), MC 903 (■), KH 1060 (▲) or EB 1089 (▼). The increase in cell numbers during the incubation period was measured and expressed as a percentage (mean, $n = 6$) of the increment found in vehicle treated controls (○) (mean: 1.59×10^6 cells/dish). The following rank order of potency was obtained: KH 1060 = MC 903 = 1,25-$(OH)_2D_3$ > EB 1089 (> denotes a statistically significant difference ($P < 0.05$); two-way ANOVA and the Student–Newman–Keuls test).

However, the effect was not significantly different when compared to the effects of MC 903 and 1,25-$(OH)_2D_3$ EB 1089 was the least potent inhibitor of FRTL-5 cell growth.

DISCUSSION

The synthetic cholecalciferol analogues MC 903, EB 1089 and KH 1060 showed high VDR affinity and they were also potent inhibitors of FRTL-5 cell growth and function. KH 1060 had the highest affinity for the VDR and was also the most potent inhibitor of TSH stimulated ACA, iodide uptake and cell growth. In agreement with our results, Binderup *et al.* [7] have shown the VDR affinity for KH 1060 to be marginally higher than for 1,25-$(OH)_2D_3$. In their study they found that KH 1060 inhibited cell proliferation and induced cell differentiation at concentrations several orders of magnitude lower than 1,25-$(OH)_2D_3$ [7]. The other cholecalciferol analogues were always significantly less potent than KH 1060, except for the MC 903 and 1,25-$(OH)_2D_3$ induced inhibition of cell proliferation. In contrast, MC 903 was the least potent inhibitor of the TSH stimulated ACA and iodide uptake. EB 1089 and 1,25-$(OH)_2D_3$ were almost equipotent inhibitors of FRTL-5 cell function and growth. Similar biologic effects of EB 1089 and 1,25-$(OH)_2D_3$ were also demonstrated in a human breast cancer cell line (MCF-7) [6].

Norman *et al.* [4] have compared the binding characteristics and biological activity of 228 vitamin D analogues and found that several analogues selectively activated specific components of the vitamin D endocrine system. Differences in the availability of the analogues to the cells or non-genomic responses induced by the analogues were postulated to explain these differences. The VDR binding assay was performed by incubating FRTL-5 cell cytosol in TEKMD buffer with cholecalciferol analogues, while the biological effects were studied in cells grown in culture medium supplemented with 5% newborn calf serum. Thus, the medium contained vitamin D binding proteins (DBP) which reduce the free and presumably biologically active concentration of the analogues [20]. It has been shown that some cholecalciferol analogues have a relatively low affinity to the DBP and the VDR binding of the analogues are therefore less affected by the presence of DBP in the cell culture medium [21]. The side-chain modified analogues, such as KH 1060, EB 1089 and MC 903, have, in general, low affinity to DBP [22] and are accordingly less protein bound than 1,25-$(OH)_2D_3$.

MC 903 was a surprisingly potent inhibitor of FRTL-5 cell proliferation compared to its VDR affinity and other biological effects. The MC 903 induced inhibition of cell growth was not significantly different from the effect of 1,25-$(OH)_2D_3$, as demonstrated in other cell lines [6, 22, 23], but the VDR affinity of MC 903 was lower in FRTL-5 cells. Non-genomic effects of 1,25-$(OH)_2D_3$ have been demonstrated on the cAMP pathway [24], phosphoinositide metabolism [25] and intracellular Ca^{2+} concentration [26] (reviewed in [27]). Furthermore, covalent modifications of intracellular proteins by 1,25-$(OH)_2D_3$ have been suggested [28] and may be related to these non-genomic effects. No correlation was observed between the effectiveness of a cholecalciferol analogue to cause rapid Ca^{2+} influx in the rat osteosarcoma cell line ROS 17/2.8 and the VDR affinity and receptor dependent biological effects of the analogue [29]. In FRTL-5 cells, Törnquist *et al.* showed that 1,25-$(OH)_2D_3$ after 2 days of incubation did not change the resting intracellular Ca^{2+} concentration [30]. Differences in the receptor binding and the biological effects might be explained by possible non-genomic effects of an analogue and a specific membrane receptor for vitamin D has been suggested [31]. However, non-genomic responses of 1,25-$(OH)_2D_3$ in FRTL-5 cells have not been reported. A complex regulated process like cell replication would probably be more susceptible to inhibition by possible non-VDR mediated non-genomic effects of MC 903 than the TSH stimulated ACA and iodide uptake.

Although the apparent discrepancy between the effect of MC 903 on FRTL-5 cell growth and its VDR

affinity indicate the possibility of non-genomic effects, we conclude that the biological potency of the cholecalciferol analogues in these rat thyroid cells seem to be determined by their affinity for the VDR.

Acknowledgements—We thank Ms Anne Nærby, Vigdis Enge and Nina Gjerlaugsen for skilled technical assistance. We gratefully acknowledge the substantial financial support from the Norwegian Cancer Society and the Nordic Insulin Foundation. Dr Jens P. Berg is a research fellow of the Norwegian Cancer Society.

REFERENCES

1. Walter M. R.: Newly identified actions of the vitamin D endocrine system. *Endocrine Rev.* **13** (1992) 719–764.
2. Bikle D. D.: Clinical counterpoint: vitamin D: new actions, new analogs, new therapeutic potential. *Endocrine Rev.* **13** (1992) 765–784.
3. Pike J. W.: Vitamin D_3 receptors: structure and function in transcription. *Annu. Rev. Nutr.* **11** (1991) 189–216.
4. Norman A. W., Koeffler H. P., Bishop E., Collins E. D., Sergeev I., Zhou L.-X., Nemere I., Zhou J., Henry H. L. and Okamura W. H.: Structure–function relationships in the vitamin D endocrine system for 1,25-$(OH)_2D_3$ analogs. In *Vitamin D. Gene Regulation, Structure Function Analysis and Clinical Application. Proceedings of the Eighth Workshop on Vitamin D* (Edited by A. W. Norman, R. Bouillon and M. Thomasset.). Walter de Gruyter, Berlin (1991) pp. 146-154.
5. Kragballe K.: Treatment of psoriasis by the topical application of the novel cholecalciferol analogue calcipotriol (MC 903). *Arch. Dermatol. Res.* **125** (1989) 1647–1652.
6. Colston K. W., Mackay A. G., James S. Y., Binderup L., Chander S. and Coombes R. C.: EB 1089: a new vitamin D analogue that inhibits the growth of breast cancer cells *in vivo* and *in vitro*. *Biochem. Pharmac.* **44** (1992) 2273–2280.
7. Binderup L., Latini S., Binderup E., Bretting C., Calverley M. and Hansen K.: 20-epi-vitamin D_3 analogues: a novel class of potent regulators of cell growth and immune responses. *Biochem. Pharmac.* **42** (1991) 1569–1575.
8. Lillevang S. T., Rosenkvist J., Anderson C. B., Larsen S., Kemp E. and Kristensen T.: Single and combined effects of the vitamin D analogue KH 1060 and cyclosporin A on mercuric-chloride-induced autoimmune disease in the BN rat. *Clin. Exp. Immun.* **86** (1992) 301–306.
9. Ambesi-Impiombato F. S., Parks L. A. M. and Coon H. G.: Culture of hormone-dependent functional epithelial cells from rat thyroids. *Proc. Natn. Acad. Sci. U.S.A.* **77** (1980) 3455–3459.
10. Valente W. A., Vitti P., Kohn L. D., Brandi M. L., Rotella C. M., Toccafondi R., Tramontano D., Aloj S. M. and Ambesi-Impiombato F. S.: The relationship of growth and adenylate cyclase activity in cultured thyroid cells separate bioeffects of thyrotropin. *Endocrinology* **112** (1983) 71-79.
11. Weiss S. J., Philp N. J. and Grollman E. F.: Iodide transport in a continuous line of cultured cells from rat thyroid. *Endocrinology* **114** (1984) 1090–1098.
12. Avvedimento V. E., Tramontano D., Ursini M. V., Monticelli A. and DiLauro R.: The level of thyroglobulin mRNA is regulated by TSH both *in vitro* and *in vivo*. *Biochem. Biophys. Res. Commun.* **122** (1984) 472–477.
13. Berg J. P., Torjesen P. A. and Haug E.: Calcitriol receptors in rat thyroid follicular cells (FRTL-5). *Acta Endocr. (Copenh.)* **125** (1991) 574–580.
14. Lamberg-Allardt C., Valtonen E., Polojärvi M. and Stewen P.: Characterization of a 1,25-dihydroxy-vitamin D_3 receptor in FRTL-5 cells. Evidence for inhibitory effect of 1,25-dihydroxy-vitamin D_3 on thyrotropin-induced iodide uptake. *Molec. Cell. Endocr* **81** (1991) 25–31.
15. Berg J. P., Sørnes G., Torjesen P. A. and Haug E.: Cholecalciferol metabolites attenuate cAMP production in rat thyroid cells (FRTL-5). *Molec. Cell. Endocr.* **76** (1991) 201–206.
16. Berg J. P., Torjesen P. A. and Haug E.: 1,25-dihydroxyvitamin D_3 attenuates TSH and 8-(4-chlorophenylthio)-cAMP-stimulated growth and iodide uptake by rat thyroid cells (FRTL-5). *Thyroid* **3** (1993) 245–251.
17. Torjesen P. A. and Aakvaag A.: Characterization of the adenylate cyclase of the rat corpus luteum during luteolysis by a prostaglandin $F_{2\alpha}$ analogue. *Molec. Cell. Endocr.* **44** (1986) 237–242.
18. Brown B. L., Albano D. M., Ekins R. P. and Sgherzi A. M. A simple and sensitive saturation assay method for the measurement of adenosine 3′:5′-cyclic monophosphate. *Biochem. J.* **121** (1971) 561–562.
19. Bidey S. P. and Tomlinson S.: Differential modulation by Ca^{2+} of iodide transport processes in a cultured rat thyroid cell strain. *J. Endocr.* **112** (1987) 51–56.
20. Bikle D. D. and Gee E.: Free, and not total 1,25-dihydroxyvitamin D regulates 25-hydroxyvitamin D metabolism by keratinocytes. *Endocrinology* **124** (1988) 649–654.
21. Bouillon R., Allewaert K., Xiang D. Z., Tan B. K. and van Baelen H.: Vitamin D analogs with low affinity for the vitamin D binding protein: enhanced *in vitro* and *in vivo* activity. *J. Bone Mineral Res.* **6** (1991) 1051–1057.
22. Bouillon R., Allewaert K., Tan B. K. van Baelen H., de Clercq P. and Vanderwalle M.: Biological activity of 1,25-dihydroxyvitamin D analogs: influence of relative affinities for DBP and vitamin D receptor. In *Vitamin D. Gene Regulation, Structure Function Analysis and Clinical Application. Proceedings of the Eighth Workshop on Vitamin D* (Edited by A. W. Norman, R. Bouillon and M. Thomasset.). Walter de Gruyter, Berlin, (1991) pp. 131–138.
23. Trydal T., Lillehaug J. R., Aksnes L. and Aarskog D.: Regulation of cell growth, *c-myc* mRNA, and 1,25-$(OH)_2$ vitamin D_3 receptor in C3H/10T1/2 mouse embryo fibroblast by calcipotriol and 1,25-$(OH)_2D_3$. *Acta Endocr. (Copenh.)* **126** (1992) 75–79.
24. Selles J. and Boland R.: Evidence on the participation of the 3′,5′-cyclic AMP pathway in the nongenomic action of 1,25-dihydroxy-vitamin D_3 in cardiac muscle. *Molec. Cell. Endocr.* **82** (1991) 229-235.
25. Baran D. T., Sorensen A. M. and Honeyman T. W.: Rapid action of 1,25-dihydroxyvitamin D_3 on hepatocyte phospholipids. *J. Bone Mineral Res.* **3** (1989) 593–600.
26. Lieberherr M.: Effects of vitamin D_3 metabolites on cytosolic free calcium in confluent mouse osteoblasts. *J. Biol. Chem.* **262** (1987) 13,168–13,173.
27. Nemere I. and Norman A. W.: Steroid hormone actions at the plasma membrane: induced calcium uptake and exocytotic events. *Molec. Cell. Endocr.* **80** (1991) C165–C169.
28. Takahashi N. and Breitman T. R.: Covalent modification of proteins by ligands of steroid hormone receptors. *Proc. Natn. Acad. Sci. U.S.A.* **89** (1992) 10,807–10,811.
29. Civitelli R., Kim Y. S., Gunsten S. L., Fujimori A., Huskey M., Avioli L. V. and Hruska K. A.: Nongenomic activation of the calcium message system by vitamin D metabolites in osteoblast like cells. *Endocrinology* **127** (1990) 2253–2262.
30. Törnquist K., Stewen P. and Lamberg-Allardt C.: Regulatory effects of 1,25-dihydroxycholecalciferol on calcium fluxes in thyroid FRTL-5 cell. *Molec. Cell. Endocr.* **86** (1992) 21–27.
31. Lieberherr M., Grosse B. and Bourdeau A.: Non-gene-mediated effects of calcitriol: a possible involvement of membrane receptor. In *Vitamin D. Gene Regulation, Structure Function Analysis and Clinical Application. Proceedings of the Eighth Workshop on Vitamin D* (Edited by A. W. Norman, R. Bouillon and M. Thomasset.). Walter de Gruyer, Berlin, (1991) pp. 368–375.