Journal of Cellular Biochemistry 63:199–206 (1996)

Potent Gene Regulatory and Antiproliferative Activities of 20-Methyl Analogues of 1,25 Dihydroxyvitamin D_3

Carina Danielsson, Sepideh Nayeri, Herbert Wiesinger, Ruth Thieroff-Ekerdt, and Carsten Carlberg

Clinique de Dermatologie, Hôpital Cantonal Universitaire, CH-1211 Genève 14, Switzerland (C.D., S.N., C.C.) Schering Research Laboratories, D-13342 Berlin, Germany (H.W., R.T-E.)

Abstract The biological active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 (VD), regulates cellular growth and differentiation. This provides the hormone with an interesting therapeutic potential. However, hypercalcemia is a side effect, which is caused by VD's classical action, the regulation of calcium homeostasis. This made the need for VD analogues with selectively increased cell regulatory properties. Studies with 20-epi analogues pointed out the importance of the carbon-20 position and led to the development of 20-methyl derivatives of VD. In this report the biological properties of the compounds ZK161422 and ZK157202, which are 20-methyl- and 20-methyl-23-ene-analogues, respectively, have been analyzed in comparison with VD. Both compounds show about 2-fold lower affinity to the VD receptor (VDR) than VD. However, compared to VD, their antiproliferative effect is up to 30-fold higher on human peripheral blood mononuclear cells and even up to 300-fold higher on human breast cancer MCF-7 cells. Whereas the hypercalcemic effect for ZK157202 is also increased 10-fold, ZK161422 has the same calcium-mobilizing potency as VD. Moreover, ZK161422, but not ZK157202, showed preference for gene activation from a promoter carrying a VD response element with a palindromic arrangement of two hexameric receptor binding sites spaced by 9 nucleotides (IP9) rather than for activation from a response element formed by a direct repeat spaced by 3 nucleotides (DR3). This observation supports a model, in which promoter selectivity reflects the selectively increased antiproliferative effect of VD analogues.

Key words: regulation of transcription, control of proliferation, vitamin D_3 analogues, vitamin D_3 receptor, limited protease digestion assay, lymphocytes, breast cancer cells, promoter selectivity

INTRODUCTION

A very interesting property of the hormonal form of vitamin D_3, 1,25-dihydroxyvitamin D_3 (VD), is its ability to modulate the proliferation and the differentiation of several normal and malignant cell types [Frampton et al., 1983; Mangelsdorf et al., 1984]; moreover, VD has recently been shown to induce apoptosis [Elstner et al., 1995; James et al., 1995]. Thus VD has an promising therapeutical potential; however, one major side effect, the increase of the calcium level, has to be taken under control. Hypercalcemia, hypercalciuria and soft tissue calcification [Vieth, 1990] are caused by the "classical" function of the hormone, which is the regulation of calcium and phosphate transport in the intestine and their mobilization from the bone [for review see Walters, 1992; Bikle & Pillai, 1993]. This made the need for the development of analogues that show a prominent antiproliferative effect, but have reduced potency on calcium homeostasis; i.e. the two main functions of VD have to be separated.

One promising key for such functional dissection is the understanding of VD's molecular action, which drastically increased during the last few years. VD can easily pass biological membranes and binds with high affinity to a nuclear receptor [Pike, 1991; Walters, 1992], which is a transcription factor that belongs to the nuclear receptor superfamily [Mangelsdorf et al., 1995]. All primary VD responding genes contain within their promoter region a binding site for VDR, referred to as VD response element, which is formed by two hexameric core binding sites in either directly repeated or inverted palindromic orientation. These core binding sites are rather divergent in their sequence and in the number of intervening nucleotides, so that various different natural and synthetic VD response elements have been observed [for re-

Address reprint requests to Dr. Carsten Carlberg, Clinique de Dermatologie, Hôpital Cantonal Universitaire, CH-1211 Genève 14, Switzerland. E-mail:carlberg-carsten@diogenes.hcuge.ch.

view see Carlberg, 1995]. However, the majority of the known natural VD response elements are formed by a direct repeat spaced by 3 nucleotides (DR3) and are bound by a heterodimeric complex formed by VDR and the retinoid X receptor (RXR). VDR-RXR heterodimers do also bind with high affinity to an inverted palindrome spaced by 9 nucleotides (IP9) [Schräder et al., 1994a] and recently two natural VD response elements of this structure have been identified [Schräder et al., 1995].

Recently, it was observed that an IP9-type VD response element was activated at 15-fold lower concentrations of the highly anti-proliferative VD analogue EB1089 than a DR3-type VD response element [Nayeri et al., 1995]. The observation of promoter-selectivity led to the hypothesis that primary VD responding genes that are involved in the control of the cell cycle may contain an IP9-type response element in their promoter region, whereas the regulation of calcium homeostasis may be related to genes that carry a DR3-type VD response element. This hypothesis also implies the prediction that in a given cell line the half-maximal growth inhibition (IC_{50}) value should correlate with the half-maximal gene activation (EC_{50}) value on an IP9-type VD response element rather than with the EC_{50}-value on a DR3-type element. This idea was very recently supported by a study of structurally related VD analogues [Mørk Hansen et al., 1996a].

To date more than 1000 VD analogues have been synthesized by different academic and industrial institutions, which have in majority modifications in the side-chain [Bouillon et al., 1995]. However, only a limited number of compounds showed an outstanding functional profile as, e.g., KH1060 [Binderup et al., 1991] and EB1089 [Mathiasen et al., 1993], which are 20-epi-22-oxa- and 22,24-diene-analogues, respectively. The carbon-20 atom has a central role for the orientation of the side chain in relation to the seco-steroid body. In this way, a 20-epimerization, as with KH1060, creates a rather drastic change in the space filling of the whole molecule [Binderup et al., 1991]. An other possibility is to replace a hydrogen atom a the C-20 position with a methyl group, which results in the elimination of the center of chirality at this crucial position. 20-epi VD analogues have already been investigated in various experimental systems [Binderup et al., 1991; Elstner et al., 1995] including their function in transcriptional regulation [Carlberg et al., 1994; Nayeri et al., 1996]. In contrast, studies on 20-methyl analogues have so far only be reported in a conference abstract [Neef et al., 1995].

In this report, the compounds ZK161422 and ZK157202 (Fig. 1), which are 20-methyl- and 20-methyl-23-ene-analogues, respectively, have been analyzed for their antiproliferative effect on human peripheral blood monocytes and on human breast cancer MCF-7 cells, for their functional affinity to VDR and for their ability to induce transactivation from DR3- and IP9-type VD response elements.

METHODS

Compounds. VD, ZK161422 and ZK157202 were synthesized in the Institute of Medical Chemistry (Schering, AG, Germany). The compounds were dissolved in ethanol at 10 mM and dilutions were performed in ethanol (final concentration of ethanol in the cell culture medium: 0.1% or less).

Proliferation assay of peripheral blood mononuclear cells. Peripheral blood mononuclear cells were prepared by density gradient

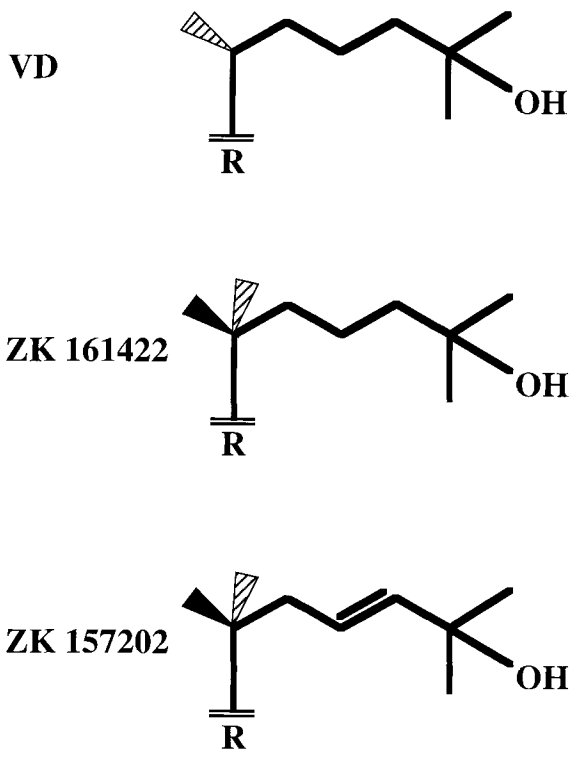

Fig. 1. The structure of VD in comparison with ZK161422 and ZK157202. The structure of the side-chain of the compounds is shown; R represents the rest of the VD molecule. ZK161422 is 20-methyl VD and ZK157202 has an additional double bond at carbon-23.

centrifugation from human blood. The cells were seeded into 96-well microtiter plates (50,000 cells per well) and grown in RPMI (Life Technologies) supplemented with 10% fetal calf serum (FCS) and 5 μg/ml phytohemagglutinin (Sigma). The cells were treated once with graded concentrations of the test compounds starting at the time of seeding. Control cultures were treated with 0.03% ethanol. After 96 h 0.2 μCi [^{3}H]-thymidine was added to the cells and incubation was continued for 4 h. Cells were then harvested on glassfiber filters and incorporated [^{3}H]-thymidine was measured with a scintillation counter. Five determinations were performed for each group.

Proliferation assay of MCF-7 cells. MCF-7 human breast cancer cells were seeded into 96-well microtiter plates (1,000 cells per well) and grown in RPMI supplemented with 2.5% charcoal-treated FCS. The cells were treated with graded concentrations of the test compounds every 2 days with a change of medium; control cultures were given 0.1% ethanol. After 8 days 5-bromo-2'-desoxy-uridine (BrdU) was added for 2 h and its incorporation was measured by colorimetric immunoassay following the instructions of the supplier (Boehringer Mannheim). Eight determinations were performed for each group.

Limited protease digestion assay. Linearized cDNA for human VDR was used for in vitro transcription as recommended by the supplier (Promega). Ten μl of in vitro transcribed VDR RNA were mixed with 175 μl rabbit reticulocyte lysate (Promega), 100 U RNasin, 20 μl [^{35}S]-methionine (1000 Ci/mmol) and 20 μM amino acid mixture (minus methionine) in a total volume of 250 μl and incubated at 30°C for 1 h. For the determination of the functional dissociation constant (K_{df}), 1–2 μl of this in vitro translated VDR were preincubated for 30 min with graded ligand concentrations in a total volume of 7.5 μl. Then 2.5 μl of trypsin (Promega) were added to a final concentration of 27 μg/ml and the mixtures were incubated for 15–30 min at room temperature. The digestion reactions were stopped by adding 10 μl protein gel loading buffer (0.25 M Tris, pH 6.8, 20% glycerol, 5% mercaptoethanol, 2% SDS, 0.025% bromophenol blue) and the samples were denatured at 95°C for 5 min, electrophoresed through a 15% SDS-polyacrylamide gel (acrylamide/N,N'-methylene-bisacrylamide weight ratio 33:1), electrotransferred to a nitrocellulose filter, air-dried and autoradiographed overnight. The protease-sensitive VDR fragment was localized, excised from the filter and radioactivity was measured in a scintillation counter.

DNA constructs. The fusion of the DR3-type VD response element of the pig osteopontin gene promoter and of the IP9-type element of the human calbindin D_{9k} promoter with the thymidine kinase (tk) promoter in front of the chloramphenicol acetyl transferase (CAT) reporter gene has been described previously [Nayeri et al., 1995; Schräder et al., 1995]. The core sequences of the elements are given in Fig. 5. The cDNA for human VDR had been subcloned into pSG5 (Stratagene) [Carlberg et al., 1993].

Transfection and CAT assays. MCF-7 cells are an appropriate model system for the analysis of VD signalling pathways, since it endogenously expresses VDR and various other nuclear receptors [Carlberg et al., 1993; Schräder et al., 1994b, 1995]. The cells were seeded into 6-well plates (2×10^5 cells per well) and grown overnight in phenol red-free RPMI supplemented with 10% charcoal-treated FCS. Liposomes were formed by incubating 2 μg of the reporter plasmid and 1 μg of the reference plasmid pCH110 (Pharmacia) with 15 μg N-[1-(2,3-Dioleoxyloxy-)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP, Boehringer Mannheim) for 15 min at room temperature in a total volume of 100 μl. After dilution with 0.9 ml phenol red-free RPMI, the liposomes were added to the cells. 500 μl phenol red-free RPMI supplemented with 30% charcoal-treated FCS were added 4–8 h after transfection. At this time VD, VD analogues or their solvent ethanol were also added. The cells were harvested 40 h after onset of the stimulation and CAT-assays were performed as described [Pothier et al., 1992]. The CAT activities were normalized to β-galactosidase activity and induction factors were calculated as the ratio of CAT activity of ligand-stimulated cells to that of solvent controls.

RESULTS

The compounds ZK161422 and ZK157202 are 20-methyl and 20-methyl-23-ene analogues of VD, the structure of their side chains are given in Fig. 1. The anti-proliferative effect of VD and the two analogues was tested on human peripheral blood mononuclear cells. The cells were incubated for 4 days with graded concentrations of VD, ZK161422 and ZK157202 before assessment of cell proliferation by [^{3}H]-thymidine in-

corporation. All three compounds exhibited dose-dependent inhibition of cell growth down to about 20% of control (Fig. 2), but showed a clear difference in their potency. The concentration needed for the half-maximal inhibitory effect (IC_{50}) was for ZK157202 with 0.12 nM about 29-fold lower than that of VD (3.5 nM); ZK161422 showed with an IC_{50}-value of 0.3 nM an about 12-fold higher potency than VD.

The potent anti-proliferative effect of 20-methyl analogues was even more pronounced in MCF-7 cells. The cells were incubated for 8 days with graded concentrations of ZK161422 and ZK157202 before assessment of BrdU incorporation. VD is known to have in this cellular system with 18 nM a relative high IC_{50}-value [Nayeri et al., 1995; Mørk Hansen et al., 1996a], so that with 0.06 and 0.2 nM the IC_{50}-values of ZK157202 and ZK161422, respectively, are 300- and 90-fold more potent than the natural hormone (Fig. 3).

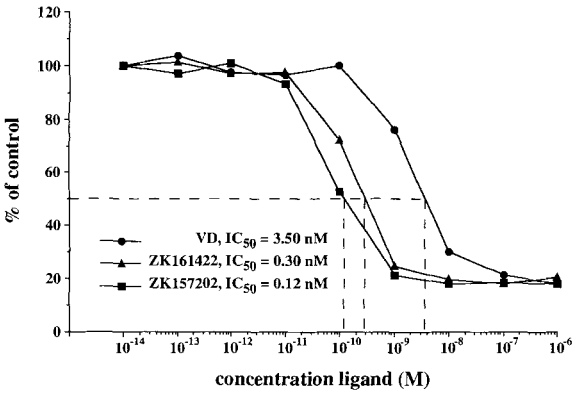

Fig. 2. Antiproliferative effects of VD, ZK161422, and ZK157202 on human peripheral blood lymphocytes. Proliferation was assessed by [^{3}H]-thymidine incorporation after 4 days in culture with graded concentrations of VD, ZK161422, or ZK157202. Results are expressed as the % mean of control and are based on 5 determinations.

The limited protease digestion method enables the direct visualization and quantification of VDR-VD analogue interactions [Nayeri et al., 1995; Nayeri et al., 1996] and has therefore a great advantage compared to the traditional indirect measurement by displacement of [^{3}H]-labelled VD [Peleg et al., 1995; Wiberg et al., 1995; Mørk Hansen et al., 1996a]. In particular, for analogues that have higher affinity to VDR than VD, the latter assay may provide misleading results. The principle of the limited protease digestion assay is that the digestion of a VDR-ligand complex with a limited amount of a protease as trypsin results in a protein fragment that is resistant to digestion in contrast to unliganded receptor. In the presence of a specific ligand, VDR is in a conformation that prevents the exposure of a cutting side to the protease; therefore, the relative amount of resistant protein fragment is directly proportional to the saturation of the receptor with ligand. The li-

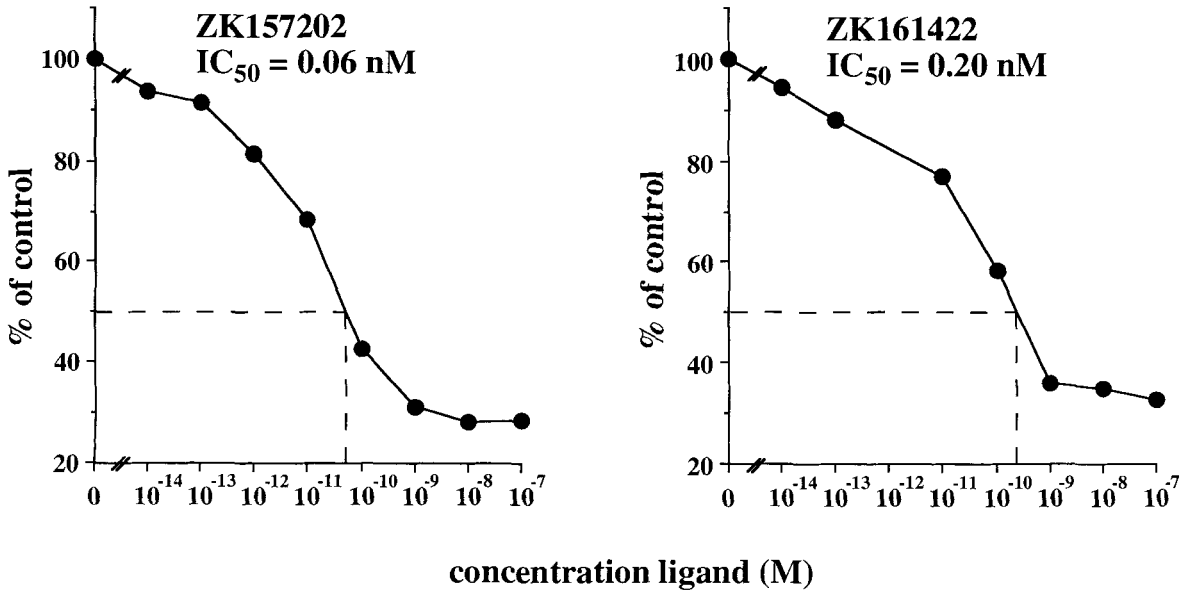

Fig. 3. Antiproliferative effects of ZK161422 and ZK157202 on human breast cancer MCF-7 cells. Proliferation was assessed by BrdU incorporation after 8 days in culture, in the presence or absence of increasing amounts of ZK161422 or ZK157202. Results are expressed as the % mean BrdU incorporation of control and are based on 8 to 10 determinations.

gand concentration that provides 50% of protease-resistant VDR fragment at saturating ligand concentration is defined as K_{df}-value. The assay was performed with in vitro translated, [^{35}S]-methionine labelled VDR and graded concentrations of ZK161422 and ZK157202. The relative amounts of the protease-resistant VDR fragments were obtained by scintillation counting of the respective excised bands and normalization by the background of the solvent control. Scatchard plots of the data are shown in Fig. 4 and provide K_{df}-values of 2.25 nM for ZK161422 and of 1.46 nM for ZK157202. The K_{df}-value for VD has already been determined as 0.9 nM [Nayeri et al., 1995].

Consequently, the potency of the two 20-methyl analogues in gene activation was tested. Heterologous promoter/reporter gene constructs, formed by either the DR3-type VD response element of the pig osteopontin gene [Zhang et al., 1992] or the IP9-type VD response element of the human calbindin D_{9k} gene [Schräder et al., 1995] fused with the tk minimal promoter driving the CAT reporter gene, were transfected into MCF-7 cells and then stimulated with graded concentrations of ZK161422 or ZK157202. The plot of CAT reporter gene activity over ligand concentration provided typical sigmodial shaped dose response curves, from which the concentrations of half-maximal gene activity (EC_{50}-values) were calculated: for ZK161422 these are 0.65 and 0.16 nM on the DR3-type and the IP9-type VD response element, respectively, and for ZK157202 0.058 and 0.088 nM (Fig. 5). In the same experimental setup the EC_{50}-values for VD had already been determined as 0.35 nM on the DR3-type and of 0.53 nM on the IP9-type element [Nayeri et al., 1995]. The efficacy of reporter gene activity at saturating ligand concentration did not showed significant variation; it was on both types of VD response elements and with both analogues as well as with VD [Nayeri et al., 1995] between 4- and 5-fold.

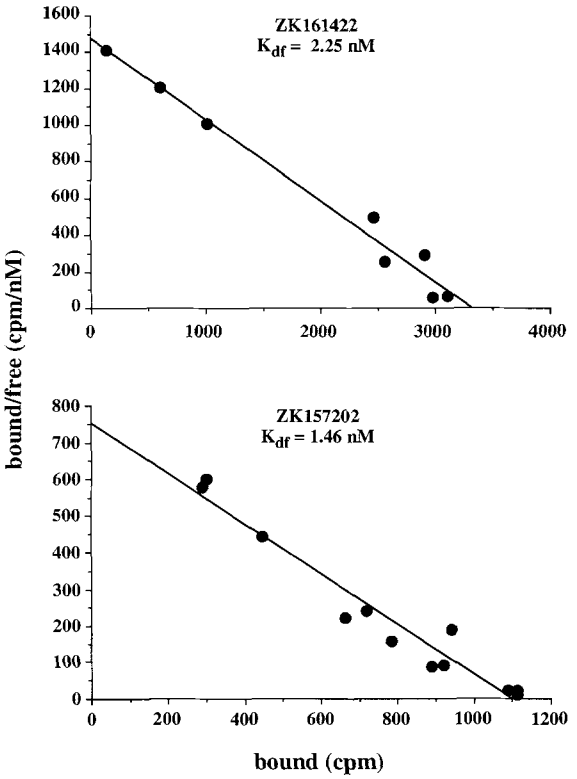

Fig. 4. Binding of 20-methyl VD analogues to in vitro translated VDR. In vitro synthesized [^{35}S]-methionine-labelled VDR was preincubated with graded concentrations of ZK161422 or ZK157202. Trypsin to a final concentration of 27 µg/ml was added and the mixtures were incubated for 30 min at room temperature. Samples were electrophoresed through a 15% SDS-polyacrylamide gel, electrotransferred to a nitrocellulose filter, air-dried and autoradiographed. The respective protease resistant VDR fragments (in a size between 30 and 34 kDa) were localized, cut out from the filter, and their radioactivity was measured in a scintillation counter. Scatchard plots of representative experiments are shown.

DISCUSSION

In this report the biological profile of 20-methyl VD analogues has been presented; beside a recent abstract [Neef et al., 1995] this is the only available information concerning this novel class of VD analogues. Tab. 1 shows a summary of the results in comparison with VD. Although both analogues have an about 2-fold lower affinity than VD to VDR, their potency in the inhibition of proliferation of peripheral blood mononuclear cells and of MCF-7 breast cancer cells and in the induction of differentiation of HL-60 promyelocytic cells is considerably higher than that of VD. Interestingly, both analogues have about the same VDR affinity, but in its cell regulatory properties ZK157202 appears to be about 3 times more potent than ZK161422. The differences among the analogues and in comparison with VD might be related to a different cellular uptake and metabolism, but this has not been investigated so far. The low affinity of the 20-methyl VD analogues to the serum vitamin D binding protein (DBP) may contribute to the enhanced in vitro activity [Bouillon et al., 1991] but could not explain the difference between both analogues.

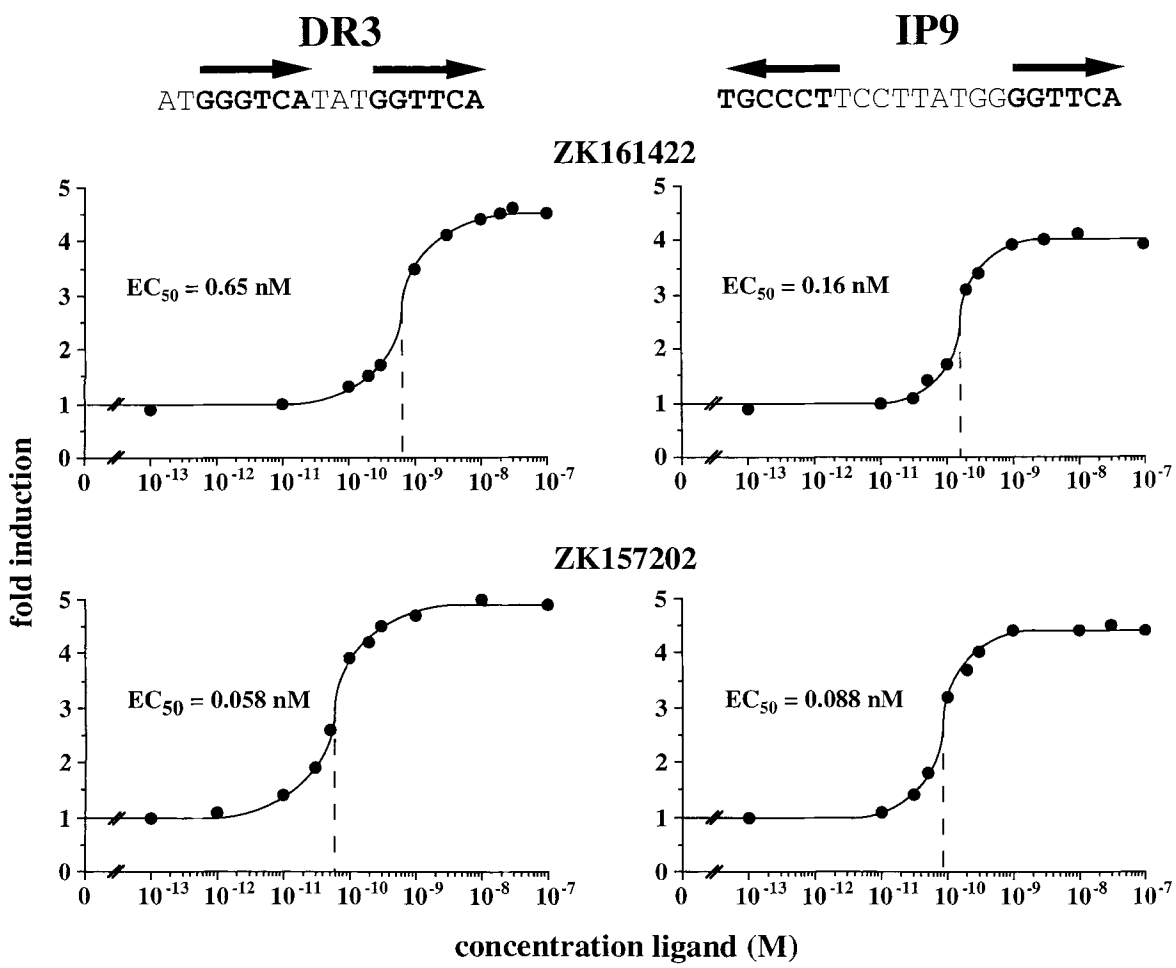

Fig. 5. Selectivity of ZK161422 and ZK157202 for gene activation from different VD response element structures. MCF-7 cells were transfected with tk promoter/CAT reporter gene constructs containing either the DR3-type response element of pig osteopontin or the IP9-type response element of human calbindin D_{9k} (core sequences indicated above). The cells were treated for 40 h with graded concentrations of ZK161422 or ZK157202. Stimulation of CAT activity was calculated in comparison to solvent-induced controls. Each data point represents the mean of triplicates; the standard deviation was always < 10%.

Cell regulatory properties of nuclear hormones are in part related to their anti-AP-1 activity [Fanjul et al., 1994], but in contrast to retinoids, VD and its analogues do not show this activity [Nayeri et al., 1995]. Therefore, for VD analogues it appears to be sufficient to analyze their potency on primary VD responding genes or on representative heterologous promoter/reporter gene constructs. ZK161422 shows on the DR3-type VD response element about 2-fold lower potency than VD, but is on the IP9-type VD response element 3-fold more potent than the natural hormone, whereas ZK157202 shows 6- and 2-fold higher potency on DR3- and IP9-type VD response elements, respectively. It is an interesting correlation that ZK157202 also shows increased potency on both main functions of VD; it is 10 times more calcemic [Neef et al., 1995] and up to 300-fold more antiproliferative than VD. Thus ZK157202 has to be regarded as a non-selective VD analogue. In contrast, ZK161422 shows selectivity in its cell regulatory over its calcemic effect and also selectivity for IP9- over DR3-type VD response elements. The ratio between the EC_{50}-values of DR3- and IP9-type response elements is defined as promoter selectivity and the ratio of VD (0.35 nM/0.53 nM = 0.66) is taken as standard. This provides for ZK161422 a promoter selectivity of 6.2. In this respect ZK161422 resembles EB1089 [Mathiasen et al., 1993], which has an outstanding promoter selectivity of 22.8 [Nayeri et al., 1995] and a very potent antiproliferative, but rather low calcemic activity. Interestingly,

TABLE I. Summary of Biological Properties of ZK161422 and ZK157202 in Relation to VD

	ZK161422	ZK157202	Reference
Relative functional affinity to VDR	0.40	0.62	this paper
Relative affinity to pig intestinal VDR	1	0.8	(Neef et al., 1995)
Relative affinity to DBP	0.08	0.06	(Neef et al., 1995)
Antiproliferative potency on human peripheral blood lymphocytes	12	29	this paper
Antiproliferative potency on human MCF-7 cells	90	300	this paper
Potency in induction of HL-60 cell differentiation	7.1	20	(Neef et al., 1995)
Potency on DR3-type VD response element activation	0.54	6.0	this paper
Potency on IP9-type VD response element activation	3.3	6.0	this paper
Promoter selectivity	6.2	1	this paper
Relative calcemic activity	1	10	(Neef et al., 1995)

Numbers above 1 indicate that the potency of the 20-methyl analogue is higher than that of VD.

EB1089 has with a K_{df}-value of 3.4 nM [Nayeri et al., 1995] a lower VDR affinity than ZK161422 and ZK157202.

The observation of promoter selectivity for ZK161422 and of non-selectivity for ZK157202, supports the concept that promoter selectivity may reflect the desired functional selectivity of VD analogues. In this way the determination of promoter selectivity would allow a preselection in a functional screening of novel VD analogues. However, the molecular mechanisms of promoter selectivity are not fully understood. For both ZK161422 and EB1089 there is a discrepancy between reduced VDR binding affinity and potent activation of IP9-type VD response elements. It has to be investigated whether the heterodimerization with RXR and the consequent complex formation with either DR3- or IP9-type response elements both differentially and selectively enhances the ligand binding affinity of VDR. It is well established that the majority of the primary VD responding genes that are involved the regulation of calcium homeostasis carry a DR3-type VD response element, but it remains to be shown that some of the key regulators of the cell cycle, e.g., like cyclins and cyclin kinase inhibitors, are regulated by VD and do contain IP9-type rather than DR3-type VD response elements in their promoter regions. A good candidate that may support this hypothesis is $p21^{WAF1/CIP1}$, an inhibitor of cyclin dependent kinases, which has been shown to be a primary VD responding gene [Jiang et al., 1994], and which contains a IP9-type VD response element that is bound specifically by VDR-RXR heterodimers (C.C., unpublished results).

Taken together, a rather simple modification of the VD molecule, the addition of a methyl group at C-20 position, provides an analogue (ZK161422) with a promising biological profile. A comparable analogue, 20-epi VD (MC1288) (Binderup et al., 1991), was tested in the same experimental system [Nayeri et al., 1996]. Compared to ZK161422 MC1288 shows with a K_{df}-value of 0.41 nM higher affinity to VDR and with EC_{50}-values of 0.22 and 0.062 nM on DR3- and IP9-type VD response elements, respectively, higher potency in gene activation, but MC1288 is also 2.5 times more calcemic than ZK161422. However, both analogues have identical IC_{50}-values in the MCF-7 system and their promoter selectivity is also nearly the same [Mørk Hansen et al., 1996a; Nayeri et al., 1996]. Thus ZK161422 provides a promising basis for the development of further selective 20-methyl analogues as MC1288 did for the series of 20-epi analogues.

ACKNOWLEDGMENTS

We would like to thank A. Steinmeyer and J.-H. Saurat for discussions. This work was supported by the Swiss National Foundation (No. 3100-040314.94) and the Cancer Research Switzerland.

REFERENCES

Bikle DD, Pillai S (1993): Vitamin D, calcium, and epidermal differentiation. Endocr Rev 14:3–19.

Binderup L, Latini S, Binderup E, Bretting C, Calverley M, Hansen K (1991): 20-epi-vitamin D_3 analogues: a novel class of potent regulators of cell growth and immune responses. Biochem Pharmacol 42:1569–1575.

Bouillon R, Allewaert K, Xiang DZ, Tan BK, van Baelen H (1991): Vitamin D analogs with low affinity for the vitamin D binding protein: enhanced in vitro and decreased in vivo activity. J Bone Mineral Res 6:1051–1057.

Bouillon R, Okamura WH, Norman AW (1995): Structure-function relationships in the vitamin D endocrine system. Endocr Rev 16:200–257.

Carlberg C (1995): Mechanisms of nuclear signalling by vitamin D_3: interplay with retinoid and thyroid hormone signalling. Eur J Biochem 231:517–527.

Carlberg C, Bendik I, Wyss A, Meier E, Sturzenbecker LJ, Grippo JF, Hunziker W (1993): Two nuclear signalling pathways for vitamin D. Nature 361:657–660.

Carlberg C, Mathiasen I, Saurat J-H, Binderup L (1994): The 1,25-dihydroxyvitamin D_3 analogues MC903, EB1089 and KH1060 function as transcriptional activators: vitamin D receptor homodimers show higher ligand sensitivity than heterodimers with retinoid X receptors. J Steroid Biochem Molec Biol 51:137–142.

Elstner E, Linker-Israeli M, Said J, Umiel J, de Vos S, Shintaku IP, Heber D, Binderup L, Uskokovic M, Koeffler HP (1995): 20-epi-vitamin D_3 analogues: a novel class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cell lines. Cancer Res 55:2822–2830.

Fanjul A, Dawson MI, Hobbs PD, Jong L, Cameron JF, Harlev E, Graupner G, Lu X-P, Pfahl M (1994): A new class of retinoids with selective inhibition of AP-1 inhibits proliferation. Nature 372:107–111.

Frampton RJ, Omond SA, Eisman JA (1983): Inhibition of human cancer cell growth by 1,25-dihydroxyvitamin D_3 metabolites. Cancer Res 43:4443–4447.

James SY, Mackay AG, Colston KW (1995): Vitamin D derivatives in combination with 9-cis retinoic acid promote active cell death in breast cancer cells. J Mol Endocrinol 14:391–394.

Jiang H, Lin J, Su Z-z, Collart FR, Huberman E, Fisher PB (1994): Induction of differentiation in human promyelotic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53. Oncogene 9:3397–3406.

Mangelsdorf DJ, Koeffler HP, Donaldson CA, Pike JW, Haussler MR (1984): 1,25-dihydroxyvitamin D_3 induced differentiation in a human promyelocytic leukemia cell line HL-60: receptor-mediated maturation to macrophage-like cells. J Cell Biol 98:391–398.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM (1995): The nuclear receptor superfamily: the second decade. Cell 83:835–839.

Mathiasen IS, Colston KW, Binderup L (1993): EB1089, a novel vitamin D analogue, has strong antiproliferative and differentiation inducing effects on cancer cells. J Steroid Biochem Molec Biol 46:365–371.

Mørk Hansen C, Danielsson C, Carlberg C (1996a): The potent anti-proliferative effect of 20-epi analogues of 1,25 dihydroxyvitamin D_3 in human breast cancer MCF-7 cells is related to promoter selectivity. Int J Cancer: in press.

Mørk Hansen C, Mathiasen IS, Binderup L (1996b): The antiproliferative and differentiation-inducing effects of vitamin D analogues are not determined by the binding affinity for the vitamin D receptor alone. J Invest Dermatol 106: S44–S48.

Nayeri S, Danielsson C, Kahlen J-P, Schräder M, Mathiasen IS, Binderup L, Carlberg C (1995): The anti-proliferative effect of vitamin D_3 analogues is not related to the AP-1 pathway, but related to promoter selectivity. Oncogene 11:1853–1858.

Nayeri S, Mathiasen IS, Binderup L, Carlberg C (1996): High affinity nuclear receptor binding of 20-epi analogues of 1,25 dihydroxyvitamin D_3 correlates well with gene activation. J Cell Biochem 61: in press.

Neef G, Kirsch G, Schwarz K, Wiesinger H, Menrad A, Fähnrich M, Thieroff-Ekerdt R, Steinmeyer A (1995): 20-methyl vitamin D analogues. Proceedings of the 9th workshop on vitamin D. Norman AW, Bouillon R, Thomasset M (eds) de Gruyter, Berlin: 97–98.

Peleg S, Sastry M, Collins ED, Bishop JE, Norman AW (1995): Distinct conformational changes induced by 20-epi analogues of 1α,25-dihydroxyvitamin D_3 are associated with enhanced activation of the vitamin D receptor. J Biol Chem 270:10551–10558.

Pike JW (1991): Vitamin D_3 receptors: structure and function in transcription. Annu Rev Nutr 11:189–216.

Pothier F, Ouellet M, Julien J-P, Guérin SL (1992): An improved CAT assay for promoter analysis in either transgenic mice or tissue culture cells. DNA Cell Biol 11:83–90.

Schräder M, Müller KM, Becker-André M, Carlberg C (1994a): Response element selectivity for heterodimerization of vitamin D receptors with retinoic acid and retinoid X receptors. J Mol Endocrinol 12:327–339.

Schräder M, Müller KM, Nayeri S, Kahlen J-P, Carlberg C (1994b): VDR-T_3R heterodimer polarity directs ligand sensitivity of transactivation. Nature 370:382–386.

Schräder M, Nayeri S, Kahlen J-P, Müller KM, Carlberg C (1995): Natural vitamin D_3 response elements formed by inverted palindromes: polarity-directed ligand sensitivity of VDR-RXR heterodimer-mediated transactivation. Mol Cell Biol 15:1154–1161.

Vieth R (1990): The mechanisms of vitamin D toxicity. Bone Mineral 11:267–272.

Walters MR (1992): Newly identified actions of the vitamin D endocrine system. Endocr Rev 13:719–764.

Wiberg K, Ljunghall S, Binderup L, Ljunggren O (1995): Studies on two new vitamin D analogs, EB1089 and KH1060: effects on bone resorption and osteoclast recruitment in vitro. Bone 17:391–395.

Zhang Q, Wrana JL, Sodek J (1992): Characterization of the promoter region of the porcine opn (osteopontin, secreted phosphoprotein 1) gene. Eur J Biochem 207:649–659.