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Characterization of five 19-nor-analogs of 1α,25(OH)$_2$-Vitamin D$_3$ with 20-cyclopropyl-modified side-chains: implications for ligand binding and calcemic properties☆

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Abstract

The steroid hormone 1α,25(OH)$_2$-Vitamin D$_3$ [1α,25(OH)$_2$D$_3$] exerts a wide variety of biological actions through one or more receptors/binding proteins. The nuclear Vitamin D receptor (VDR) when bound to its natural ligand, 1α,25(OH)$_2$D$_3$, can stimulate transcription of a wide variety of genes. The synthesis of 1α,25(OH)$_2$D$_3$ analogs allows the study of structure–function relationships between ligand and the VDR. 1α,25(OH)$_2$D$_3$ is a conformationally flexible molecule; specifically the side-chain of the hormone can display a large variety of shapes for its receptor. Here, we describe and analyze the properties of 10 1α,25(OH)$_2$D$_3$ analogs modified at the side-chain of which five lack carbon-19 (19-nor) but have a novel 20-cyclopropyl functionality. Analog NG [20,21-methylene-23-yne-26,27-F$_6$-19-nor-1α,25(OH)$_2$D$_3$] possesses a respectable binding affinity for the VDR and exhibits a high transcriptional activity (EC$_{50}$ ∼10 pM), while retaining low induction of hypercalcemia in vivo in the mouse, making it a primary candidate for further analyses of its anti-proliferative and/or cell differentiating properties.

Keywords: Structure–function studies; Vitamin D; 1α,25(OH)$_2$D$_3$ Analog; Steroid hormone; Nuclear receptor; VDR

1. Introduction

1α,25(OH)$_2$-Vitamin D$_3$ [1α,25(OH)$_2$D$_3$], the biologically active form of Vitamin D$_3$, exerts at least part of its biological actions through a well-studied nuclear receptor, the Vitamin D receptor (VDR) [1,2]. The classic endocrine system of 1α,25(OH)$_2$D$_3$ includes effects upon calcium homeostasis [3], the immune system [4–6], insulin secretion by the pancreas [7,8], and inhibition of proliferation of several cell lines [9,10]. Hence, there is a large interest in 1α,25(OH)$_2$D$_3$ with respect to its potential clinical applications [11–13].

An important focus of structure–function relationships is the analysis and synthesis of novel ligands, followed by the study of their affinity for a receptor, and the consequent biological response(s) [10,11]. Several analogs have been designed with the purpose of eliminating some of the less-desirable effects of a ligand, particularly the onset of hypercalcemia, when used at their active concentration [10,14–17]. Analogs of 1α,25(OH)$_2$D$_3$ have been implicated in many processes, however its significance lies upon its therapeutic properties related to renal osteodystrophy [18], psoriasis [19], osteoporosis [20], and autoimmune diabetes [21]. Other analogs have also been proposed to treat leukemia [22], breast [23] and prostate cancer [24–27], and selected immune disorders [28,29]. Thus, structure–function studies may aid design of new therapeutic drug forms of 1α,25(OH)$_2$D$_3$.

Binding of a ligand or analog to the VDR leads to a conformational change in the receptor [30,31] that promotes its heterodimerization, as observed similarly for other nuclear receptors [32,33]. This in turn enhances their interaction with specific DNA response elements to initiate transcription and exert biological responses [34]. It is generally accepted that the ligand affinity for a receptor is directly proportional to its transcriptional activity, although exceptions do exist [9,35].

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Explanation for these differences may lie with different interactions of ligands with their receptors that may lead to a different conformational state of the receptor.

We have been analyzing the mechanism of action of analogs of 1α,25(OH)$_2$D$_3$ that possess similar or greater transcriptional activities (transactivation) than the natural hormone. That is, we sought more effective analogs eliciting these responses at the same concentration or lower than 1α,25(OH)$_2$D$_3$ [10]; in some cases, their affinity for the VDR may be lower than that of 1α,25(OH)$_2$D$_3$. Another factor to take into consideration is an analog's affinity for the Vitamin D binding protein (DBP) [36]. DBP affects analogs in vivo since they must bind to this protein in the blood in order for them to be delivered to target tissues. Hence, when considering delivery of therapeutic analogs, it is necessary to consider an affinity for DBP that is comparable to that of 1α,25(OH)$_2$D$_3$.

In this communication, we report the characterization of a total of 10 analogs modified at the side-chain, 5 of these being novel 19-nor analogs of 1α,25(OH)$_2$D$_3$ with a 20-cyclopropyl functionality. Initial characterization of these analogs reveals a significantly stronger activity than that of 1α,25(OH)$_2$D$_3$; in particular, analog NG [20,21-methylene-23-yne-26,27-F$_6$-19-nor-1α,25(OH)$_2$D$_3$] displays a 60–110-fold increase in transactivation potency over that of 1α,25(OH)$_2$D$_3$ while having a significantly lower calcemic index (1/100). Thus, analog NG and the analog family are excellent targets for further analysis and development.

2. Materials and methods

2.1. Reagents

[^{3}H]-1α,25(OH)$_2$D$_3$ (1α,25-dihydroxy[23,24(*n*)-^{3}H] cholecalciferol) and [^{3}H]-25(OH)D$_3$ (25-hydroxy[26, 27-methyl-^{3}H] cholecalciferol) were obtained from Amersham Biosciences (Piscataway, NJ). 1α,25(OH)$_2$D$_3$, 25(OH)D$_3$ and the side-chain-modified analogs LZ, NA, NB, NC, NF, NG, NH, NI, NN and NO were provided by Dr. M.R. Uskokovic (Hofmann-La Roche, Nutley, NJ); 1α,25(OH)$_2$-20-epi-D$_3$ (analog IE) was kindly provided by Dr. Lise Binderup (Leo Pharmaceuticals, Ballerup, Denmark). The structural formulas for the key seco-steroids studied in this communication are shown in Fig. 1.

2.2. Cell culture and transfection assays

COS-1 monkey kidney cells were seeded at 8×10^5 cells per 150 mm culture dishes (Corning Inc. Corning, NY) in DMEM Nutrient Mixture-F12 Ham (Sigma, St. Louis, MO) with 10% Rehatuin FBS (Intergen, Purchase, NY). These cells were passed near confluency at 4×10^6 cells per 150 mm plate using Cellgro 0.25% trypsin, 0.1% EDTA solution (Mediatech Inc., Herndon, VA). For transfection, COS-1 cells were seeded at 1.2×10^5 cells per well on Costar 12-well plates (Corning Inc., Corning, NY). After 18 h incubation, phosphate-buffered saline (PBS)-washed cells at approximately 50% confluence were transfected using a 9 min pre-treatment with 1 mg/ml diethylaminoethyl (DEAE)-dextran (Sigma) in PBS. After being washed twice with PBS, pretreated cells were incubated for 24 min with 0.03 μg per well pGEM-4 VDR plasmid and 0.12 μg per well pCGH plasmid containing the Vitamin D response element from the osteocalcin gene (GGGTGACTCAC-CGGGTGA). This Vitamin D receptor element (VDRE) was attached to a human calcitonin promoter/human growth hormone fusion reporter gene [37]. Transfected cells were incubated in 80 mM chloroquine in DMEM Nutrient Mixture-F12 Ham with 4.5% charcoal-stripped FBS for 3 h followed by the same culture medium without chloroquine for 21 h. Twenty-four hours after transfection, the cell medium was replaced with the same medium containing 1α,25(OH)$_2$D$_3$ or its analogs with a final ethanol concentration of 0.1%. Twenty-four hours after treatment, the cell medium was harvested to measure hGH reporter using an ELISA kit from Monobind Inc. (Costa Mesa, CA). The data is presented as fold activation relative to 10 nM 1α,25(OH)$_2$D$_3$. All experiments were carried out on triplicate samples and the data are expressed as the mean ± S.E.M.

CV-1 cells were seeded at 0.4×10^4 cells per well on 24-well plates (Beckton Dickinson, Franklin Lakes, NJ). After 24 h incubation, phosphate-buffered saline-washed cells at approximately 60% confluence were transfected using a 10 min pre-treatment with 0.2 mg/ml DEAE-dextran (Sigma) in PBS. Pre-treated cells were washed in PBS and incubated for 30 min with PBS containing 0.1 μg per well activator pcDNA3.1(–)Nhe1(–)VDR or mutant plasmid and 0.5 μg per well reporter OC-pSEAP2 [38]. Transfected cells were incubated in 80 μM chloroquine in growth medium with only 5% charcoal stripped fetal bovine serum for 4 h followed by the same culture medium without chloroquine for 24 h. Twenty-eight hours after transfection, the cell medium was replaced with the same medium containing 1α,25(OH)$_2$D$_3$ or its analogs with a final ethanol concentration of 0.1%. At 22 h after analog treatment, an aliquot of the cell medium was harvested to measure secreted alkaline phosphatase using the Phospha-Light™ SEAP assay (Tropix, Bedford, MA). All experiments are carried in quadruplicate with the data expressed as the mean ± S.E.M.

2.3. Ligand binding assays

Comparison of the relative affinity of 1α,25(OH)$_2$D$_3$ and the analogs to VDR and DBP in vitro was determined by a relative competition index (RCI) assay according to standard procedures [39]. Using the Vitamin D nuclear receptor or the Vitamin D binding protein as binding proteins with a constant amount of [^{3}H]-1α,25(OH)$_2$D$_3$ or [^{3}H]-25(OH)D$_3$ (0.2 pmol), respectively, increasing amounts of non-radioactive competitor

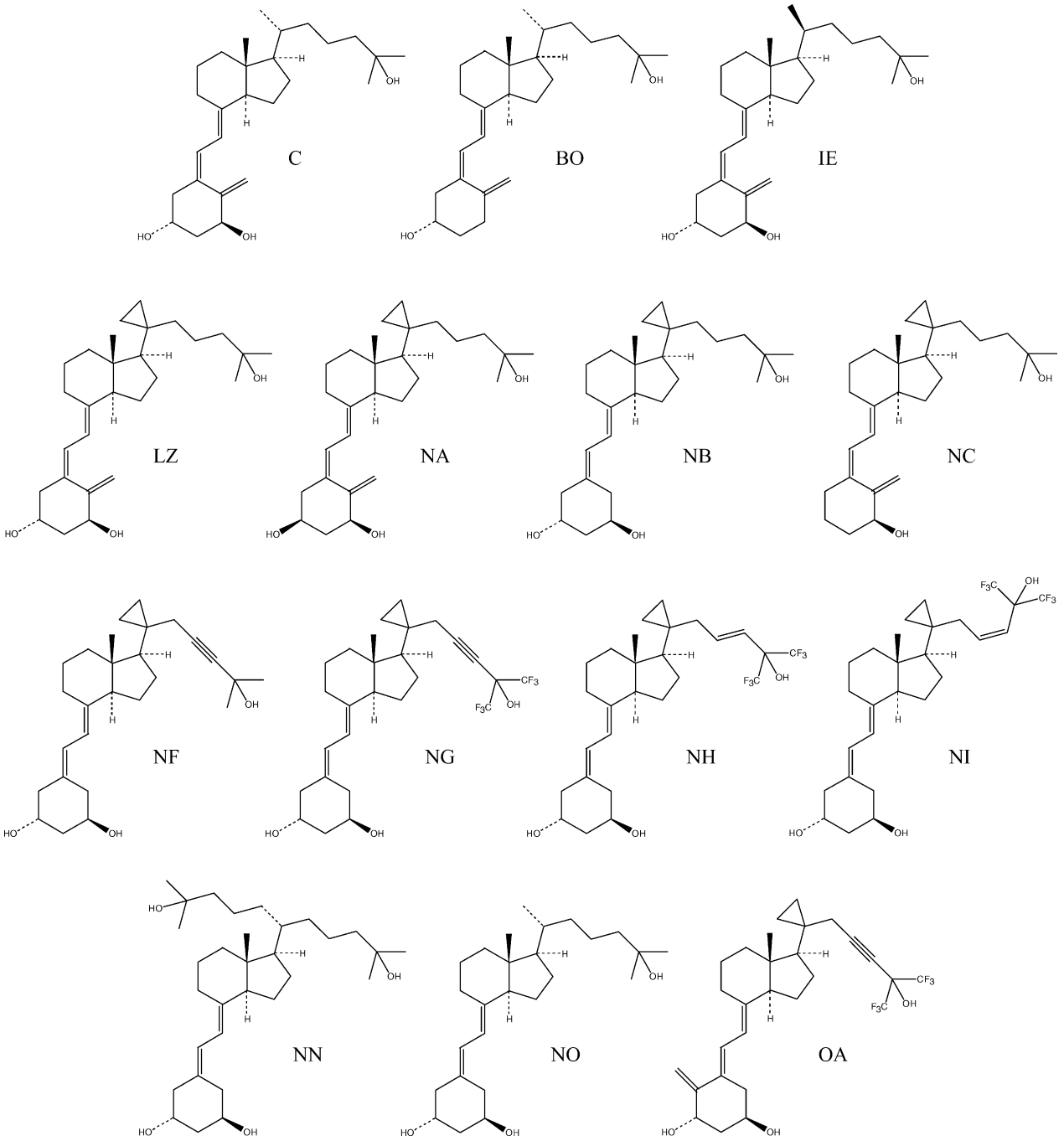

Fig. 1. Structural formulas of 1α,25(OH)$_2$D$_3$ and analogs. Structures of 1α,25(OH)$_2$D$_3$ (C), its precursor 25(OH)D$_3$ (BO), and of its analogs: 20-epi-1α,25(OH)$_2$D$_3$ (IE); 20,21-methylene-1α,25(OH)$_2$D$_3$ (LZ), 20,21-methylene-3-epi-1α,25(OH)$_2$D$_3$ (NA), 20,21-methylene-19-nor-1α,25(OH)$_2$-D$_3$ (NB), 20,21-methylene-3-deoxy-1α,25(OH)$_2$D$_3$ (NC), 20,21-methylene-23-yne-19-nor-1α,25(OH)$_2$D$_3$ (NF), 20,21-methylene-23-yne-26,27-F$_6$-19-nor-1α,25(OH)$_2$D$_3$ (NG), 20,21-methylene-23E-ene-26,27-F$_6$-19-nor-1α,25(OH)$_2$D$_3$ (NH), 20,21-methylene-23Z-ene-26,27-F$_6$-19-nor-lα,25(OH)$_2$D$_3$ (NI), 21-(3-OH-3-methyl-butyl)-19-nor-1α,25(OH)$_2$D$_3$ (NN), 19-nor-1α,25(OH)$_2$D$_3$ (NO), and 20,21-methylene-23-yne-26,27-F$_6$-5,6-trans-1α,25(OH)$_2$D$_3$ (OA).

were added and incubated for 16 h at 4 °C. Protein-bound [^{3}H]-1α,25(OH)$_2$D$_3$ or [^{3}H]-25(OH)D$_3$ was separated from free [^{3}H]-1α,25(OH)$_2$D$_3$ or [^{3}H]-25(OH)D$_3$, using the hydroxylapatite procedure [39]. The data is plotted as competitor/[^{3}H]-ligand ratio versus the inverse of percent remaining maximally bound [^{3}H]-1α,25(OH)$_2$D$_3$ or [^{3}H]-25(OH)D$_3$ (100/[% Max Bd]). The relative competitive index is a measure of the affinity of a ligand to a binding protein (VDR or DBP) in comparison to 1α,25(OH)$_2$D$_3$ or 25(OH)D$_3$. This is calculated as the ratio: [competitor slope/1α,25(OH)$_2$D$_3$ or 25(OH)D$_3$ slope] × 100. DBP was purchased from Sigma; the VDR solution is a chick intesti-

nal nuclear/cytosol fraction that is highly enriched with the nuclear receptor for 1α,25(OH)$_2$D$_3$ [40]. Tritium activity was measured by liquid scintillation spectrometry; each sample was counted until 5000 dpm (2% error) had been accumulated.

2.4. Determination of maximum tolerated dose (MTD)

Eight week-old female C 57 BL/6 mice (three to four mice per group) were dosed orally (0.1 ml per mouse) with various concentrations of 1α,25(OH)$_2$D$_3$ or analogs daily for 4 days. Analogs were formulated in miglyol for a final concentration of 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10.0, 30.0, 100.0 and 300.0 μg/kg when given at 0.1 ml per mouse p.o. daily. Blood for serum calcium assay was drawn by tail bleed on day 5. Serum calcium levels were determined using a colorimetric assay (Sigma Diagnostics, procedure no. 597). The highest dose of analog tolerated without inducing hypercalcemia (serum calcium >10.7 mg/100 ml) was defined as the maximum tolerated dose.

3. Results

This communication reports the biological profiles of 1α,25(OH)$_2$D$_3$ and of 11 analogs of which 5 are novel 19-nor compounds, possessing a 20-cyclopropyl-modified side-chain. Fig. 1 presents the structures and code names of these seco-steroids as well as two reference compounds; the precursor 25(OH)D$_3$ and analog 20-epi-1α,25(OH)$_2$D.

Fig. 2 compares, for four new 19-nor analogs, their affinity for the Vitamin D receptor and the serum Vitamin D binding protein relative to 1α,25(OH)$_2$D$_3$ and the reference compounds BO and IE. By incubation of increasing amounts of analog competitor in the presence of a constant amount of tritiated 1α,25(OH)$_2$D$_3$, the relative competitive index of the analogs for VDR and DBP is determined. By definition, the RCI of 1α,25(OH)$_2$D$_3$ for VDR and DBP is set to 100%. The relative affinity strength for binding to the VDR for analogs with an RCI greater than 10% is NG ≈ NH ≈ NI > LZ > NF > OA > NB in comparison to 1α,25(OH)$_2$D$_3$ and is summarized in Table 1. In contrast, only analogs LZ, NA, and NC had a good affinity for DBP relative to 1α,25(OH)$_2$D$_3$.

The transcriptional activity of 1α,25(OH)$_2$D$_3$ is reported in Fig. 3. The transcription assay was performed using fusion genes driven by a human calcitonin promoter containing either an osteocalcin Vitamin D receptor element regulating the transcription of the human growth hormone gene or a VDRE regulating the transcription of alkaline phosphatase (secreted), as well as a VDR construct to observe the effects of 1α,25(OH)$_2$D$_3$ and its analogs in COS-1 or CV-1 cells, respectively. Fig. 3 presents a typical dose–response curve in COS-1 cells used to establish the concentration of 1α,25(OH)$_2$D$_3$ required to elicit the 50% maximal response, or EC$_{50}$ for any given system. A typical comparison of

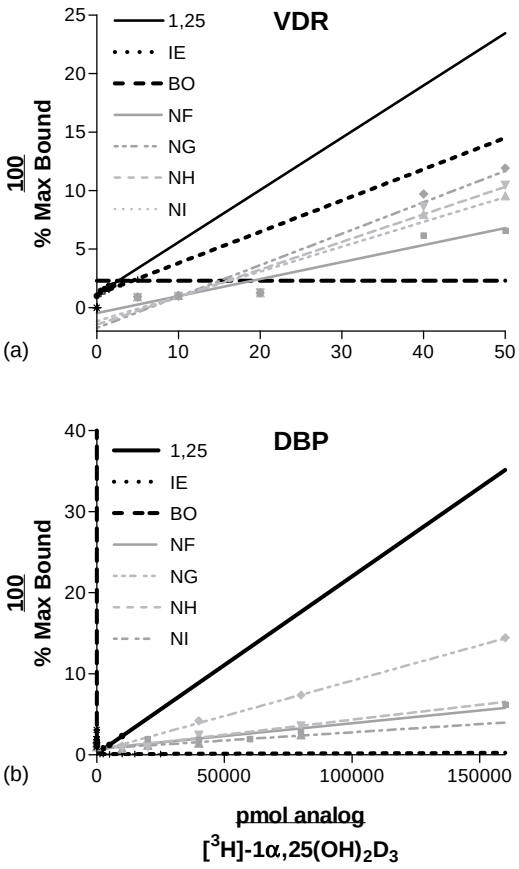

Fig. 2. Ligand binding competition assay of 1α,25(OH)$_2$D$_3$ and its analogs for VDR and DBP. (a) Relative competition index (RCI) of 1α,25(OH)$_2$D$_3$ analogs for the VDR and (b) DBP. The data is plotted as competitor/[^{3}H]-1α,25(OH)$_2$D$_3$ ratio vs. the inverse of percent remaining maximally bound [^{3}H]-1α,25(OH)$_2$D$_3$ (100/[% Max Bd]). By definition 1α,25(OH)$_2$D$_3$ has an RCI of 100% when bound to VDR or DBP. This graph illustrates one of four representative replicate RCI assays for each analog and each point represents the average of triplicate determinations for that experiment. The resulting VDR and DBP values for each analog are summarized in Table 1.

EC$_{50}$ curves is illustrated in Fig. 4. The four representative 19-nor-20-cyclopropyl analogs and the respective positions of their curves are offset by approximately 100-fold to the left of the 1α,25(OH)$_2$D$_3$ curve (bold) which emphasizes their potency in transactivation in this cell line.

A summary of the transactivational effectiveness EC$_{50}$ of all analogs evaluated is reported for the CV-1 cells in Table 1 and for the COS-1 cells in Table 2. The order of potency of the transcriptional activities (lowest EC$_{50}$ value) for the five novel analogs in the CV-1 cells is NH ≈ NG > NI ≈ NF > NB > 1α, 25(OH)$_2$D$_3$ while the order of effectiveness in the COS-1 cells is NI > NG > NH > NF > 1α, 25(OH)$_2$D$_3$ The two most potent analogs, NG and NI have EC$_{50}$ 110-140 (COS-1) and 56–70 (CV-1) fold higher than 1α,25(OH)$_2$D$_3$.

Additionally summarized in Table 1 are the results from a bioassay which defines the serum calcemic properties of the analogs in mice. After four consecutive daily oral doses

Table 1
Biological activities of analogs of 1α,25(OH)$_2$D$_3$ including transactivation in transfected CV-1 cells

Analog name	Code	VDR RCI	DBP RCI	MTD (μg/kg)	EC_{50} in CV-1 (nM)	1,25D-EC_{50}/analog-EC_{50}
1α,25(OH)$_2$D$_3$	C	100 ± 0	100 ± 0	1	0.56 ± 0.23	1
20,21-Methylene-1α,25(OH)$_2$D$_3$	LZ	47 ± 8	44 ± 4	10	0.15 ± 0.04	4
20,21-Methylene-3-epi-1α,25(OH)$_2$D$_3$	NA	3 ± 1	23 ± 14	100	7.00 ± 2.80	0.1
20,21-Methylene-19-nor-1α,25(OH)$_2$D$_3$	NB	12 ± 3	8 ± 4	10	0.50 ± 0.40	1
20,21-Methylene-3-deoxy-1α,25(OH)$_2$D$_3$	NC	3 ± 2	31 ± 19	30	1.30 ± 0.70	0.5
20,21-Methylene-23-yne-19-nor-1α,25(OH)$_2$D$_3$	NF	39 ± 10	3 ± 1	10	0.08 ± 0.05	7
20,21-Methylene-23-yne-26,27-F$_6$-19-nor-1α,25(OH)$_2$D$_3$	NG	67 ± 6	11 ± 6	100	0.01 ± 0.00	56
20,21-Methylene-23E-ene-26,27-F$_6$-19-nor-1α,25(OH)$_2$D$_3$	NH	56 ± 14	8 ± 3	0.3	0.008 ± 0.00	70
20,21-Methylene-23Z-ene-26,27-F$_6$-19-nor-lα,25(OH)$_2$D$_3$	NI	53 ± 14	6 ± 3	10	0.03 ± 0.03	16
21-(3-OH-3-methyl-butyl)-19-nor-1α,25(OH)$_2$D$_3$	NN	6 ± 3	0.7 ± 0.4	10	2.90 ± 2.30	0.2
19-Nor-1α,25(OH)$_2$D$_3$	NO	9 ± 2	8 ± 2	10	0.28 ± 0.13	2
20,21-Methylene-23-yne-26,27-F$_6$-5,6-trans-1α,25(OH)$_2$D$_3$	OA	20 ± 2	10 ± 2	3	0.27 ± 0.20	2

Analog activity is summarized by tabulating the chick intestinal VDR relative competitive index (RCI), the Vitamin D binding protein (DBP) RCI, the maximum tolerated dose (MTD) of an analog without inducing hypercalcemia in mice, and the 50% maximal transactivation response (EC_{50}) in CV-1 cells. A comparison of activity relative to 1α,25(OH)$_2$D$_3$ for fold transactivation of the osteocalcin promoter is also shown. By definition, the VDR and DBP RCIs of 1α,25(OH)$_2$D$_3$ are set to 100%. Transactivation values are the average ± S.E.M. of four individual replicates per experiment ($n = 3$). MTD: maximum tolerated dose of analog or 1α,25(OH)$_2$D$_3$ administered to mice (3–4) over a 4-day period that did not result in the onset of hypercalcemia (serum $Ca^{2+} > 10.7$ mg/100 ml; see Section 2 for details).

Table 2
VDR transactivation activities of analogs of 1α,25(OH)$_2$D$_3$ in transfected COS-1 cells

Analog name	Code	EC_{50} (nM)	1,25D-EC_{50}/analog-EC_{50}
1α,25(OH)$_2$D$_3$	C	1.25 ± 0.30	1
20,21-Methylene-23-yne-19-nor-1α,25(OH)$_2$D$_3$	NF	0.03 ± 0.00	42
20,21-Methylene-23-yne-26,27-F$_6$-19-nor-1α,25(OH)$_2$D$_3$	NG	0.011 ± 0.00	114
20,21-Methylene-23E-ene-26,27-F$_6$-19-nor-1α,25(OH)$_2$D$_3$	NH	0.014 ± 0.01	89
20,21-Methylene-23Z-ene-26,27-F$_6$-19-nor-lα,25(OH)$_2$D$_3$	NI	0.009 ± 0.00	139

COS-1 cells were transfected with a VDR plasmid containing a human calcitonin promoter/human growth hormone reporter fusion gene. The EC_{50} (concentration of analog required to achieve 50% maximum transactivation) was determined as described in Section 2. Each experiment was carried out in triplicate with the data expressed as the mean ± S.E.M. ($n = 3$).

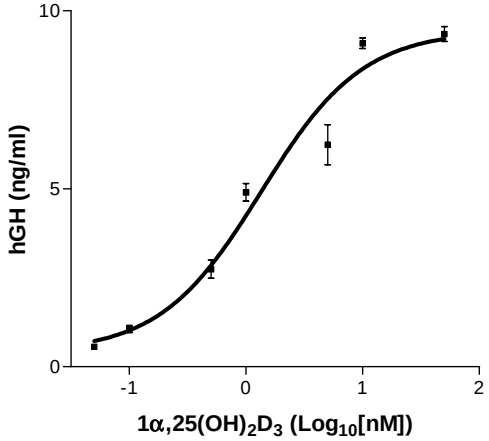

Fig. 3. Transcription assay of 1α,25(OH)$_2$D$_3$ in COS-1 cells. COS-1 cells were transfected with a VDR plasmid in conjunction with a human calcitonin promoter/human growth hormone reporter fusion gene (see Section 2). Each point on the graph represents an average of three independent replicates within one experiment ($n = 10$). EC_{50} curve transformation of a typical 1α,25(OH)$_2$D$_3$ dose–response experiment.

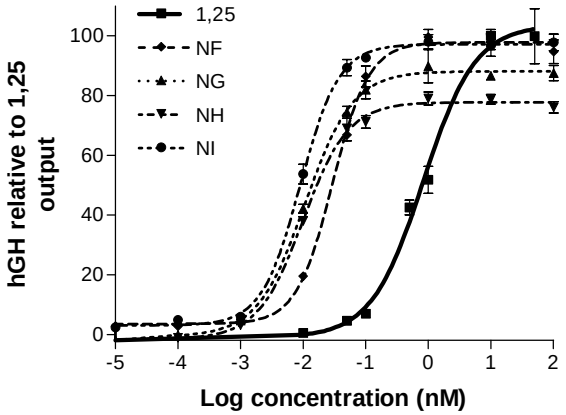

Fig. 4. Relative transactivation of 1α,25(OH)$_2$D$_3$ and analogs in COS-1 cells. Representative dose–response curves of transactivation in COS-1 cells by 1α,25(OH)$_2$D$_3$ and analogs NF, NG, NH, and NI. Each point on the graph represents an average of three independent replicates within one experiment ($n = 3$). The EC_{50} values for each analog are summarized in Table 1.

of individual analogs to groups of mice, the maximum tolerated dose of an analog without inducing hypercalcemia (serum Ca^{2+} not greater than 10.7 mg/100 ml of serum) was established (MTD). The MTD observed for the 11 analogs evaluated was highest for analogs NA and NG (100 μg/kg) and lowest for analog NH (0.3 μg/kg).

4. Discussion

The widespread biological effects of 1α,25(OH)$_2$D$_3$ include the genomic responses and recently a growing body of evidence supporting more rapid, non-genomic responses [41,42]. In light of this spectrum, there has been an ever-growing quest for analogs more suitable for clinical purposes (see review [11–13]). Numerous analogs have been synthesized in an attempt to improve therapy for psoriasis, osteoporosis, and malignancies such as leukemia and breast cancer, without inducing hypercalcemia. Some of these analogs are now used as tools for understanding structure–function relationships of Vitamin D analogs [11,43], particularly for analogs IE [20-epi-1α,25(OH)$_2$D$_3$] [38] and KH [1α,25(OH)$_2$-21-(3-hydroxy-3-methylbutyl)-D$_3$] [44] and their conformational changes elicited in VDR upon binding. Advances in this field have occurred through the synthesis of a multitude of novel compounds modified at the hydroxyl positions, by introducing double and triple bonds at different positions, and by modification of the side-chain [10]. This report highlights the analysis of five novel analogs where carbon 19 has been removed and the side-chain have been modified by the addition of a cyclopropyl ring at carbon 20 and with the introduction of double or triple bonds, as well as fluorination of carbons 26 and 27.

We have evaluated these analogs with respect to their effectiveness of transactivation in two cell lines, their in vitro binding to the VDR and DBP, and the determination of their maximum in vivo tolerated dose before onset of hypercalcemia. Transfection of the osteocalcin VDRE into COS-1 or CV-1 cells in the presence of its natural ligand or analog demonstrated up to a 140-fold or 70-fold reduction (respectively) in EC_{50} compared to 1α,25(OH)$_2$D$_3$ by these novel compounds (see Tables 1 and 2); analogs NG and NI were the most potent in both cell lines. From Fig. 4, we observe a leftward shift of the EC_{50} for all four analogs in comparison to 1α,25(OH)$_2$D$_3$ ($EC_{50(COS\text{-}1)} = 1.25$ nM). Contrary to what might be expected, the EC_{50} values do not correlate with the affinities for the VDR (Tables 1 and 2). However, this has been previously observed for other strong agonists for the VDR, like analog IE [30,38], as well as the double side-chain analog KH [44].

The analog affinities ranged from approximately 2–70% of that of 1α,25(OH)$_2$D$_3$ for the VDR, with analog NG having the highest affinity (RCI = 67). The four highlighted analogs NF, NG, NH, and NI displayed an overall stronger RCI than the remaining analogs (∼53%). Hence, their unusual transactivational activity is not directly explained by their affinity to VDR. Studies, however have shown that the carbon 20 of 1α,25(OH)$_2$D$_3$ possesses unique contacts within the VDR allow a more efficient heterodimerization with the retinoic X receptor [45].

These novel analogs possess a cyclopropyl ring on carbon 20 which may prove to have similar properties as the 20-epi side-chain of analog IE. The bulk of the cyclopropyl ring may act by reducing the freedom of rotation of the side-chain reducing the occupational volume accessible by the side-chain allowing a more efficient acquisition of the hydroxyl contacts, and increasing the overall stability of the ligand within the receptor. The double and triple bonds present on the side-chain may act to achieve a similar outcome. Hence, analog NG, with less rotational freedom, would support this hypothesis. Fluorine, being the most electronegative ion, may increase the overall negative charge on the surface of the receptor, although inside of the ligand binding domain, acting as a recruitment enhancer for coactivators. The difference in RCI values may lie in differences of the on and off rates of the ligand. Once the ligand is bound to the VDR, their stability within the ligand binding domain decreases the off-rate of the ligand from the receptor, allowing for increased transactivation. However, it may be the same decrease in rotational freedom in the side-chain which could prevent these more rigid compounds entering the ligand binding pocket, unlike the more flexible 1α,25(OH)$_2$D$_3$.

A major adverse effect of some analogs is their ability to induce hypercalcemia at higher doses, preventing clinical applications when used at such concentrations. To further characterize these analogs, we treated mice with 1α,25(OH)$_2$D$_3$ or analog orally at various concentrations daily for 4 days. Their blood serum calcium was determined and the maximum tolerated dose of 1α,25(OH)$_2$D$_3$ or analog before inducing hypercalcemia in mice was calculated. Hypercalcemia is defined here as having greater than 10.7 mg/dl of calcium present in serum. Noteworthy were analogs NA, NC, and NG, which have a 30–100-fold higher tolerated concentration limit than 1α,25(OH)$_2$D$_3$. Other analogs have a reasonable MTD (∼3–10 μg/kg), however NH on the other hand had a 70% lower MTD than 1α,25(OH)$_2$D$_3$. The removal of carbon 19 (analog NO) or the addition of the cyclopropyl ring at carbon 20 (analog LZ) on 1α,25(OH)$_2$D$_3$ reduced their binding affinity for VDR and DBP yet only slightly improved the transactivational activity of the analogs; additionally, the induction of hypercalcemia was lowered by 10-fold. The combination of 19-nor and the cyclopropyl (analog NB), however, did not exhibit an additive effect.

The modifications upon the A-ring with respect to carbon 3 (NN) did improve upon the onset of hypercalcemia index (30–100-fold higher concentration tolerated), nevertheless, both binding as well as transactivation were lowered with respect to 1α,25(OH)$_2$D$_3$. Further modification upon the side-chain, however, did improve both the transactivational activity as well as their hypercalcemic index when introducing sterical rigidity and bulk. The change from the

Z (same side) to E (opposite side) on the double bond in the side-chains of analogs NI and NH results in an apparent 30-fold decrease in stimulation of hypercalcemia, whereas no significant change is seen in their affinity for either VDR nor DBP, as well as their transactivational EC_{50} between the molecules (see Tables 1 and 2).

A large contribution of the synthesis of families of analogs is the ability to discern patterns and differences amongst the modifications present in the analog. In this case, the creation of pairs of modified side-chains reveals interesting properties amongst the modifications. Comparison of analog NF to NG (six fluorines added at carbons 26 and 27) shows that the presence of this electronegativity not only stabilized the binding of the receptor by a 70% increase, it also reduced the transactivational EC_{50} by approximately 100-fold. On the other hand, analogs NH and NI differ by orientation around the 22–23 double bond, and the *cis–trans* difference diminishes the analogs' hypercalcemic effect. By comparing analog NG with NH or NI, we see a more profound effect from the stabilization of the ligand within the receptor as a result of introducing a triple bond to further make rigid the side-chain. We therefore believe that the structural modifications reported for the 19-nor-20-cyclopropyl analogs could serve as basis for the synthesis of new analogs that can mimic the low calcemic effects while retaining the strong transactivation capability, and further our understanding of structure–function relationships within the Vitamin D endocrine system.

Analogs NH and NN were recently evaluated for their ability to stimulate hair growth in nude mice [46]; the concern in this animal model, however, is the onset of hypercalcemia despite the positive effects upon anti-proliferation and hair-growth. The syntheses of derivatives of NH have improved upon the desired properties within the analog. In time, analogs like NG or its derivatives, exhibiting high transactivational activities, in conjunction with a very low induction of hypercalcemia, could likely provide desirable outcomes in the clinical field.

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