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Synthesis and biological activities of 2α-chloro-1-epicalcitriol and 1-epicalcitriol

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*Anomalous diequatorial epoxide ring opening of 1β,2β-oxido-cholesta-5,7-diene-3β,25-diol **1** produces the 1β-hydroxy-2α-chloro-provitamin **2** and its corresponding 1β-hydroxy-provitamin **3**. The provitamins **2** and **3** are transformed by irradiation and thermal isomerization to 2α-chloro-1-epicalcitriol **NS3 (4)** and 1-epicalcitriol **NS8 (5)**, respectively. These two A-ring derivatives were tested for their in vitro biological activity in the mesenchymal, murine cell line C3H10T½, and their effects were compared with those of the native vitamin D_3 derivatives 25(OH)D_3 and 1,25(OH)$_2D_3$. **NS3** and **NS8** showed marked differences in their affinity for the vitamin D binding protein (DBP) and in their ability to inhibit cell proliferation. **NS8** has the ability to bind to a high-affinity DBP-binding site for which 25(OH)D_3 has none affinity. The 2α-chloro-substitution (**NS3**) prevents binding to the postulated noncompetitive, **NS8**-specific DBP-binding site and diminishes the affinity to the vitamin D receptor (VDR) and therefore diminishing **NS3**'s biological abilities. The elucidation of the structure-function relationships at the DBP-binding-sites could have major impact on the development of new vitamin D_3 derivatives with extended serum half-life.* (*Steroids* **63**:28–36, 1998) *© 1998 by Elsevier Science Inc.*

Keywords: vitamin D; A-ring derivatives; diequatorial 1β,2β-epoxide cleavage; vitamin D responsive element; vitamin D binding protein; affinity studies

Introduction

The biologically active vitamin D_3 metabolite, 1,25(OH)$_2D_3$ is of pivotal importance in mediating calcium and phosphorous homeostasis as well as in controlling the differentiation and proliferation of several cell types.[1–4] This has augmented the efforts to develop vitamin D_3 derivatives with highly specific effects in contrast to the pluripotent activity of 1,25(OH)$_2D_3$. The interest is centered on the development of vitamin D_3 derivatives with low calcemic activity and with the ability to inhibit proliferation and to modulate differentiation.[5–7] Most of these substances were side chain derivatives of 1,25(OH)$_2D_3$ with unchanged A-ring structure and configuration. In this study, we report the synthesis and the biological capabilities of 1-epicalcitriol and 2α-chloro-1-epicalcitriol, both derived from 1β,2β-oxido-cholesta-5,7-dien-3β,25-diol **1.** The 1β-hydroxy derivative **NS8** (1-epicalcitriol) has been previously characterized as a potent antagonist of 1,25(OH)$_2D_3$-induced intestinal calcium transport (transcaltachia).[8] 2α-Chloro-1-epicalcitriol (**NS3**) is a new derivative and to our knowledge the first member of the 2α-substituted 1-epicalcitriols. Recently, 2β-substituted 1-epicalcitriols with interesting biological activities have been described.[9]

For the cell culture experiments, we used the murine fibroblastic cell line C3H10T½. After stable transfection with the human bone morphogenetic-4 (BMP-4) gene, this cell line has the ability to differentiate into chondrocytes, adipocytes, and cells of the osteogenic lineage by addition of ascorbate and β-glycerophosphate.[10] It is possible to cultivate this cell line for up to four weeks in the absence of fetal calf serum (FCS) so that the in vitro cellular effects of vitamin D_3 derivatives can be separated from their DBP-mediated actions (FCS contains significant amounts of DBP). We used this highly vitamin D-sensitive cell line to test the ability of vitamin D_3 derivatives to inhibit cell proliferation and modulate adipocyte differentiation.[11] C3H10T½-cells were also stably transfected with a construct containing the vitamin D responsive element (VDRE) of the human osteocalcin gene coupled to a chloramphenicolacetyltransferase (CAT)-reporter gene in order to evalu-

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ate the ability of the vitamin D_3 derivatives to activate gene transcription at the VDRE.

Experimental

Chemistry

1β,2β-oxido-cholesta-5,7-dien-3β,25-diol **1** was synthesized from (20S)-20-(*p*-toluene-sulfonyloxymethyl)-pregna-1,5-dien-3β-ol[12] in 8 steps, as previously described.[13,14] The 1β,2β-epoxy group of compound **1** reacts with hydrochloric acid in *N,N*-dimethyl-formamide via an anomalous trans-diequatorial intermediate, giving the 1β-hydroxy-2α-chloro compound **2** (^{1}H NMR data). By reductive cleavage of the 1β,2β-epoxy group of **1** with lithium aluminum hydride, the hitherto unknown 1β-hydroxy compound **3** (provitamin of 1-epicalcitriol) was formed, in contrast to the 2β-hydroxy compound we previously specified.[13]

For the transformation of the provitamins into the previtamins, simultaneous irradiation at <-40°C with light in the range of 285 to 300 nm and >330 nm was carried out (filter solution), as described for the synthesis of precalcitriol.[15] Irradiation was terminated after nearly 50% of the provitamins was consumed in order to avoid the production of undesired side products. HPLC and flash chromatography[16] furnished the desired previtamins, starting materials, and mixtures of the other photoisomers. The starting materials (provitamins) and the other photoisomers were recycled to improve the yields of previtamins.[15]

After chromatographic purification, thermal isomerization of the previtamins gave vitamins **NS3** and **NS8**. The lower yields in comparison to the synthesis of calcitriol[15] are due to the lack of optimized reaction conditions (irradiation and isomerization). In this way, 1-epicalcitriol (**NS8**) was synthesized for the first time by UV irradiation and thermal isomerization.

The trans-diequatorial epoxide opening of compound **1** is surprising. Normally, 1β,2β-epoxides with a saturated A-ring give the trans-diaxial 1α-substituted 2β-hydroxy compounds.[17–19] The reasons for the anomalous epoxide cleavage of compound **1**, which has an unsaturated B-ring, are under investigation. Through this anomalous cleavage, a route was developed to the production of a new class of provitamins and vitamins possessing a 1β-hydroxy group and a substituent in the 2α-position.

General remarks

Solvents were distilled and dried before use according to conventional methods. *N,N*-Dimethyl formamide was dried over molecular sieves 4Å and distilled in vacuo. All reactions were conducted under an atmosphere of dry argon and under exclusion of UV light. TLC: silicagel 60 F_{254} (MERCK) plates, layer thickness 0.2 mm, detection by UV ($\lambda_1 = 254$ nm; $\lambda_2 = 366$ nm) and spraying with a solution consisting of 80 mL conc. sulfuric acid and 20 mL methanol and by heating at 120°C. Flash column chromatography:[16] LiChroprep (MERCK, 25–40 μm) [eluents (v/v) are given in parentheses]. HPLC: SHIMADZU LC-8A equipped with a UV/VIS spectrophotometric detector SPD-10AV, stationary phases: LiChrosorb DIOL (MERCK, 150 × 3 mm, 5 μm) or LiChrosorb DIOL (MERCK, 250 × 25 mm, 7 μm) [eluents (v/v) are given in parentheses]. Melting points: Boetius micromelting point apparatus, uncorrected values. High-resolution mass spectra (HRMS): AMD 402 intectra with electron impact ionization at 70 eV. Nuclear magnetic resonance (NMR) spectra: BRUKER DRX 400 [chemical shifts (δ) are reported in ppm, relative to tetramethylsilane as the internal standard] Signals are assigned by TOCSY, selective TOCSY. UV spectra: CARL ZEISS JENA Specord M 500 [extinction coefficients (ε) are given in 1 mol^{-1} cm^{-1} in parentheses].

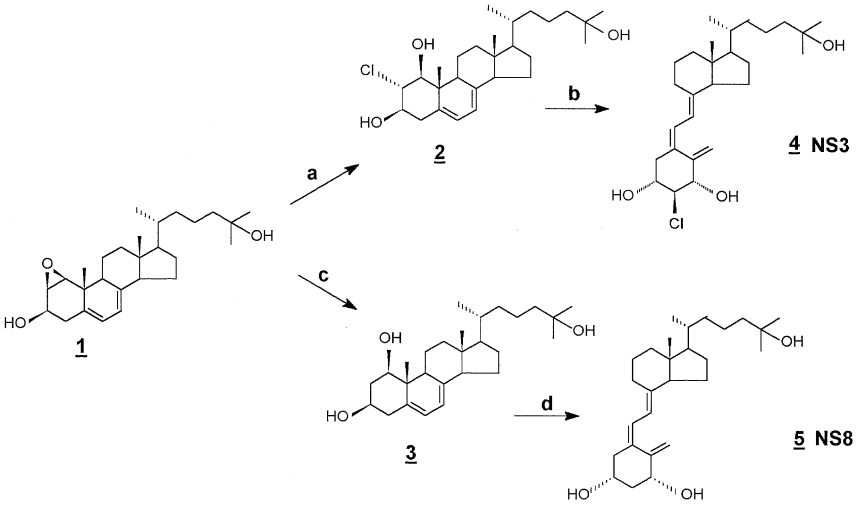
2α-Chloro-cholesta-5,7-dien-1β,3β,25-triol **2**

Epoxide **1** (1.25 g; 3.1 mmol) was dissolved in *N,N*-dimethyl formamide (80 mL) in absence of UV light. Conc. hydrochloric acid (3.3 mL; 4.2 mmol) in *N,N*-dimethyl formamide (20 mL) was added while stirring after 1 to 2 h, (TLC: ethyl acetate/isohexane 40/60 v/v) the reaction mixture was poured into chilled water. The mixture was extracted with diethyl ether, and the organic layer was dried over Na_2SO_4. After evaporation in vacuo, a white solid (1.23 g) was obtained, which was recrystallized from ethyl acetate/isohexane (75/25 v/v) giving white crystals of **2** (0.92 g). Flash chromatography of the mother liquor further gave crystalline **2** (0.13 g). Yield: 1.05 g (76%). m.p. 198–203°C. UV (CH_3OH) $\lambda_{max} = 284$ nm ($\epsilon = 11300$). ^{1}HNMR (400 MHz, $CDCl_3$) δ: 0.63 (s, 3H, 18-H); 0.97 (d, $^3J = 6.5$Hz, 3H, 21-H); 1.10 (s, 3H, 19-H); 1.23 (s, 6H, 26-H and 27-H); 3.62 (m, 2H, 1α-H and 3α-H); 3.96 (t, $J = 9.9$Hz, 1H, 2β-H); 5.33 (d, $^3J = 5.9$Hz, 1H, 7-H); 5.68 (dd, $^3J = 5.8$Hz, 1H, 6-H). Addition of trichloroacetyl isocyanate: δ: 1.60 (s, 6H, 26-H and 27-H); 4.27 (t, $^3J = 10.5$Hz, 1H, 2β-H); 4.90 (m, 1H, 3α-H); 5.25 (d, $^3J = 10.4$Hz, 1H, 1α-H); 8.18 (s, 1H, NH); 8.37 (s, 2H, NH). HRMS m/z found 450.2921 (calculated for $C_{27}H_{43}O_3Cl$: 450.2889)

(5Z,7E)-9,10-Seco-2α-chloro-cholesta-5,7,10(19)-trien-1β,3β,25-triol ***NS3* 4**

2α-Chloro-provitamin **2** (150 mg; 0.33 mmol) was dissolved in CH_3OH (100 mL) and *tert*-butyl methyl ether (350 mL), transferred to a photoreactor, and flushed with argon for 45 min. After cooling to −40°C, irradiation was carried out using a filter solution of 2,7-dimethyl-3,6-diaza-cyclohepta-1,6-diene tetrafluoroborate and biphenyl in ethanol and a high pressure mercury lamp TQ 150 Z1. After 140 min, the irradiation was stopped (HPLC control: LiChrospher DIOL, i-propanol/i-hexane (8/92 v/v, detection $\lambda_1 = 260$ nm, $\lambda_2 = 280$ nm), and the solvents were evaporated in vacuo. The residue was dissolved in ethyl acetate/i-hexane (20 mL, 70/30 v/v) and crystallized at −18°C (12 h). Unreacted provitamin **2** (55 mg; 37%) was obtained. The mother liqour was evaporated in vacuo, the residue was dissolved in i-propanol/i-hexane (5 mL, 10/90 v/v) and separated by HPLC (LiChrospher DIOL 7 μm 250 × 25 mm, i-propanol/i-hexane 8/92 v/v, 15 mL/min detection $\lambda_1 = 260$ nm, $\lambda_2 = 280$ nm) to give the expected previtamin (20 mg, 13%) as a colorless foam, further starting provitamin **2** (15 mg, 10%), and a mixture of **2** and other photoproducts (30 mg, 20%). After recycling of **2**, a total amount of 29 mg (19%) previtamin was obtained.

This previtamin (UV $\lambda_{max} = 259$ nm) was immediately dissolved in i-propanol/i-hexane (10 mL, 10/90 v/v) in the dark under argon and heated to 40 to 50°C for 5 h (HPLC control: LiChrospher DIOL, i-propanol/i-hexane 8/92 v/v, detection $\lambda = 260$ nm). The solution was evaporated in vacuo, the residue was dissolved in i-propanol/i-hexane (2 mL, 10/90 v/v) and separated by HPLC (LiChrosorb DIOL 7 μm 250 × 25 mm, i-propanol/i-hexane 10/90 v/v, 17 mL/min, detection $\lambda_1 = 260$ nm, $\lambda_2 = 230$ nm). After evaporation and drying in vacuo, the following products were obtained: 1.) 15 mg previtamin (53%) 2.) 9 mg 2α-chlorovitamin **4** (31%). The unreacted previtamin was thermically isomerized once more giving additional vitamin **4** as white needles. m.p. 173–179°C (CH_3OH). UV (CH_3OH) $\lambda_{max} = 264$ nm ($\epsilon = 15300$). ^{1}H NMR (400MHz, $CDCl_3$) δ: 0.50 (s, 3H, 18-H); 0.91 (d, $^3J = 6.3$Hz, 3H, 21-H); 1.19 (s, 6H, 26-H and 27-H); 2.25 (m, 1H, 4β-H); 2.54 (t, $^3J = 9.7$Hz, 4α-H); 2.71 (m, 1H, 9β-H); 3.81–3.83 (m, 2H, 2β-H and 3α-H); 4.12 (m, 1H, 1α-H); 5.11 and 5.56 (2 × t, $^4J = 2$Hz, 2H, 19-H); 5.94 and 6.38 (2 × d, $^3J = 11,3$Hz, 2H, 7- and 6-H). Addition of trichloroacetyl isocyanate: δ: 1.49 (s, 6H, 26-H and 27-H); 4.13 (t, $^3J = 7.1$Hz, 1H, 2β-H); 5.22 (m, 1H, 3α-H); 5.51 (d, $^3J = 7.3$Hz 1H, 1α-H); 8.17 (s, 1H, NH);



Scheme

Scheme a: HCl/DMF, r.t.; **b:** *i* photoisomerization: *tert*-butyl methyl ether/CH_3OH (80/20 v/v); photoreactor, Hg high pressure lamp (TQ 150 Z1), filter solution (2,7-dimethyl-3,6-diaza-cyclohepta-1,6-diene-tetrafluoroborate/biphenyl), 140 min; *ii* HPLC: diol gel 7 μm, propan-2-ol/i-hexane (8/92 v/v); *iii* thermal isomerization: propan-2-ol/i-hexane (10/90 v/v); 40–50°C, 5 h; *iv* HPLC: diol gel 7 μm, propan-2-ol/i-hexane (10/90 v/v); **c:** $LiAlH_4$/ether, r.t.; **d:** *i* photoisomerization: *tert*-butyl methyl ether/CH_3OH (90/10 v/v); photoreactor, Hg high pressure lamp (TQ 150 Z1), filter solution (2,7-dimethyl-3,6-diaza-cyclohepta-1,6-diene-tetrafluoroborate/biphenyl), 45 min; *ii* flash chromatography: Ag^+-modified silica gel 25–40 μm, ethyl acetate/hexane (70/30 v/v); *iii* thermal isomerization: ethyl acetate/hexane (75/25 v/v); 55–60°C, 5 h; *iv* flash chromatography: Ag^+-modified silica gel 25–40 μm, ethyl acetate/hexane (75/25 v/v).

8.58 (s, 2H, NH). HRMS m/z found 450.2917 (calculated for $C_{27}H_{43}O_3Cl$: 450.2889).

1β,3β,25-Trihydroxy-cholesta-5,7-dien 3

Epoxide **1** (250 mg; 0.6 mmol) was dissolved in THF (20 mL). A solution of $LiAlH_4$ (76 mg; 2 mmol) in THF (25 mL) was added at 0°C with stirring, and the mixture was refluxed (about 30 min) until starting material was no longer detectable by TLC (TLC: ethyl acetate/hexane 75/25 v/v; R_f: **1**: 0.45; **3**: 0.28). Diethylether (100 mL) was added, and the reaction mixture was poured into chilled water. The organic layer was separated, and the $A1(OH)_3$ residue was extracted several times with methanol (50 mL). The combined extracts were dried over Na_2SO_4, and the solvent was removed under reduced pressure. The yellowish crude product was recrystallized from methanol giving white needles of **3** (165 mg, 65.7%), and the mother liquor was purified by flash chromatography (ethyl acetate/hexane 75/25 v/v) giving more **3** (48 mg, 19.1%). UV (CH_3OH) λ_{max} = 284 nm (ϵ = 11400). ^{1}H NMR (400MHz, $CDCl_3$) δ: 0.61 (s, 3H, 18-H); 0.94 (d, $^3J \approx$ 6.3Hz, 3H, 21-H); 1.03 (s, 3H, 19-H); 1.22 (s, 6H, 26-H and 27-H); 3.70 (m, 2H, 1α-H and 3α-H); 5.25 (m, 1H, 7-H); 5.64 (dd, $^3J \approx$ 6.5Hz, $^4J \approx$ 1.5Hz, 1H, 6-H). Addition of trichloroacetyl isocyanate: δ: 0.60 (s, 3H, 18-H); 0.92 (d, $^3J \approx$ 6.1 Hz, 3H, 21-H); 1.16 (s, 3H, 19-H); 1.53 (s, 6H, 26-H and 27-H); 5.39 (dd, $^3J \approx$ 6.5Hz, $^4J \approx$ 1.5Hz, 1H, 7-H); 5.73 (m, 1H, 6-H); 8.20, 8.34 and 8.40 (3 × s, 3 × 1H, 3 × NH) HRMS m/z found 416.3279 (calculated for $C_{27}H_{44}O_3$; 416.3290).

*(5Z,7E)-9,10-Seco-cholesta-5,7,10(19)-trien-1β,3β,25-triol **NS8 5***

Provitamin **3** (150 mg, 0.36 mmol) was dissolved in methanol (100 mL), and *tert*-butyl methyl ether (350 mL) was added. The solution was transferred into a 450 mL photoreactor, and the photoreaction was carried out as described above. After 45 min of irradiation time, about 60% of **3** was consumed (TLC on Ag^+-modified plates: ethyl acetate/hexane 75/25 v/v), the irradiation was stopped, and the solvent was evaporated under reduced pressure. The residue (yellowish oil) was dissolved in ethyl acetate/hexane and allowed to crystallize the unreacted **3** at −18°C (48 mg, 32%). The mother liqour was separated by flash chromatography (Ag^+-modified silica gel, ethyl acetate/hexane 75/25 v/v). The following photoisomers were eluted in this order: previtamin (44 mg, 29.3%, colourless foam, UV (Ethanol): λ_{max} = 259 nm), tachysterol (11 mg, 7.3%, colorless foam, UV (Ethanol): λ_{max} = 284 nm), and unreacted **3** (8 mg, 5%). After irradiation recycling of **3** (56 mg), a total amount of 61 mg (40.7%) previtamin was obtained.

The corresponding tachysterol (10 mg in 40 mL *tert*-butyl methyl ether) was converted to the previtamin by sensitized photoisomerization using fluorenone (16 mg) as the sensitizer.[20] TLC and flash-chromomatographic separation of the reaction mixture as described above gave further previtamin (11 mg, 68.7% from tachysterol). Previtamin (70 mg) was dissolved in ethylacetate/hexane (50 mL, 75/25 v/v) under argon and was heated to 60°C in the dark for 5 h (TLC on Ag^+-modified plates: ethyl acetate/hexane 75/25 v/v). The solvent was evaporated under reduced pressure, and the mixture was separated by flash-chromatography (Ag^+-modified silica gel, ethyl acetate/hexane 75/25 v/v). After recycling of the unreacted previtamin (21 mg), a total amount of 56 mg **5** (37, 3% from **3**) was obtained as white needles. m.p. 210–12°C. UV (C_2H_5OH) λ_{max} = 265 nm (ϵ = 16 600). -^{1}H NMR (400MHz, $CDCl_3$): δ: 0.53 (s, 3H, 18-H); 0.94 (d, $^3J \approx$ 6.4Hz, 3H, 21-H); 1.20 (s, 6H, 26-H and 27-H); 4.09 (m, 1H, 3α-H), 4.34 (t, 1H, 1α-H), 4.99 (d, $^4J \approx$ 1.9Hz, 1H, 19-H); 5.27 (s, 1H, 19-H), 6.03 and 6.45 (2 × d, AB pattern, $^3J \approx$ 11.3Hz, 2H, 6-H and 7-H). Addition of trichloroacetyl isocyanate: δ: 0.50 (s, 3H, 18-H); 0,90 (d, $^3J \approx$ 6.4Hz, 3H, 21-H); 1.51 (s, 6H, 26-H and 27-H); 5.19 (s, 1H, 19-H); 5.20 (m, 1H, 3α-H); 5.48 (t, 1H, 1α-H); 5.51 (s, 1H, 19-H); 5.87 and 6.37 (d, $^3J \approx$ 10.8Hz, 2H, 6-H and 7-H); 8.12, 8.59 and 8.69 (3 × s, 3 × 1H, 3 × NH). These physical data are in agreement with data in the literature.[21]

Biological methods and materials

Cell culture and transfection experiments

C3H10T½ cells were obtained from the European Collection of Animal Cell Cultures (Porton Down) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2mM glutamine, 60 mg/L penicillin, and 100 mg/L streptomycin at 37°C under a humidified atmosphere (95% humidity) with 5% CO_2. All cell culture flasks were precoated prior to cell culture for 1 h at 37°C with a solution of gelatin (10 mg/mL). The cell culture

medium of the stably transfected cell lines C3H10T½(BMP-4) and C3H10T½(4xVDRE-CAT) were additionally supplemented with 5 μg/mL puromycin during the entire cultivation period.

C3H10T½-cells stably transfected with the eukaryotic expression vector pMBC-2T-f1, containing the entire coding sequence of the human version of BMP-4, were used for the DNA-synthesis and the adipogenesis inhibition experiments. To create a vitamin D_3-specific cellular gene activation test system, four direct repeats of the 32-base-pair DNA-fragment from the human osteocalcin-vitamin D responsive element (osteocalcin-VDRE) with the sequence 5′-CTTGGTGACTCACCGGGTGAACGGGGGCA-TTA-3′ were cloned into the *Bam*H1 site of the chloramphenicolacetyltransferase(CAT)-expression vector pBLCAT2. The resulting expression vector pBLCAT2(4×VDRE) was stably transfected into C3H10T½-cells. Stable transfections were produced using the calcium-phosphate precipition method. Puromycin resistance of the stable transfectants was mediated by cotransfection with the plasmid pB SpacΔp, which codes for the puromycin-acetyltransferase-gene.

Measurement of DNA synthesis

C3H10T½(BMP-4)-cells were precultured to near-confluence, diluted to 30,000 cells/mL, and transferred into 96-well plates. After cultivation to confluence, the culture medium was substituted with DMEM without FCS supplementation, and the cells were treated with 10^{-13} M to 10^{-5} M vitamin D_3 derivatives or solvent (ethanol). Five days later, the cells were labeled with 1 μCi/mL [methyl-^{3}H]-thymidine (47 Ci/mmol). After 4.5 h of incubation and washing with PBS, 50 μL of 0.4 M NaOH were added to each well, and each plate was sealed with a plate sealer sheet. The cells were lysed for 30 min at 80°C, and after the plates were cooled, 200 μL of 2% perchloro acetic acid were added to each well. Following incubation for 2 h at 4°C, the radioactivity was harvested on glass fiber filters (XTalScint, Beckman) with a semi-automatic cell harvester (Bibby Dunn, 2800 Series). The radioactivity on each filter was counted in a Beckman LS 6000 IC scintillation counter. Six parallel experiments were conducted with each vitamin D derivative concentration.

Determination of CAT-activity

C3H10T½(4×VDRE)-cells were precultured to near-confluence, diluted to 30,000 cells/mL, and transferred into petri dishes (96 mm diameter) in DMEM containing 10% FCS. The cells were subsequently washed with PBS and further cultured in DMEM without FCS and in the presence of various amounts of vitamin D_3 derivative or solvent. The solvent concentration in the medium never exceeded 0.1%. After an incubation period of 60 h, the cells were washed three times with PBS. The washed cells were lysed, and the CAT-concentration of each sample was determined using a CAT-enzyme linked immunosorbent assay (ELISA) kit (Boehringer Mannheim).

Determination of the affinity of vitamin D_3 derivatives for the vitamin D receptor (VDR)

A 1,25$(OH)_2D_3$-assay system (Amersham-Buchler, Braunschweig, Germany) was modified to determine the affinity of vitamin D_3 derivatives for the VDR. Lyophilized intestinal VDR from rachitic chicken was reconstituted with 31 mL of 0.05 M potassium phosphate buffer, pH 7.4, containing 0.05 M potassium chloride, 1 mM dithiothreitol, and 0.1% (w/v) bovine γ globulin. VDR solution (500 μL), 1,25-dihydroxy[26,27-methyl-^{3}H]vitamin D_3 (25 μL, 0.5 ng/mL, 0.24 μCi/mL), and a sample of vitamin D_3

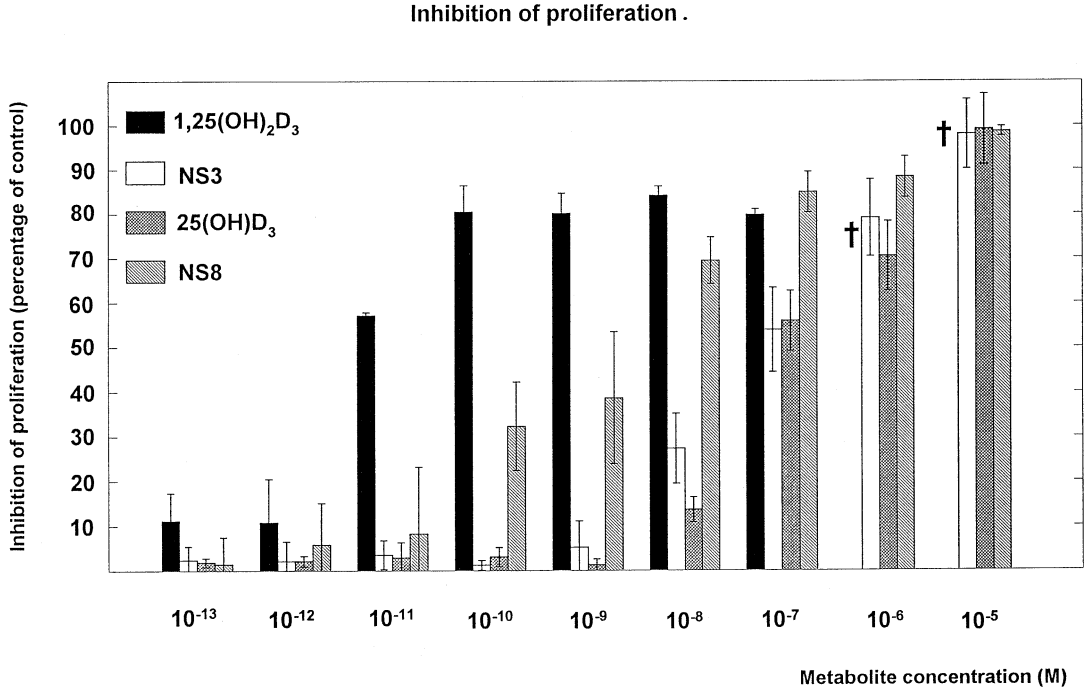


Figure 1 Inhibition of cell proliferation of C3H10T1/2(BMP-4)-cells by 1,25$(OH)_2D_3$, 25$(OH)D_3$, NS3 and NS8. C3H10T1/2(BMP-4)-cells were grown in 96-well plates and treated in serum-free medium with various concentrations of vitamin D_3 derivatives or solvent (ethanol). Five days later the cells were labeled with 1 μCi/well [Methyl-^{3}H]-thymidine (47 Ci/mmol) for 4.5 h, washed with PBS and lysed and [^{3}H]-Thymidine incorporation was determined. †, 1,25$(OH)_2D_3$-induced cell lysis. Each point of the dose-response curve is the average of six wells. The results shown are representative of two to three experiments.

derivative (50 μL) were combined and incubated for 1 h at 22°C. Immediately after this incubation period, free and bound vitamin D_3 were separated by adding 200 μL of a dextran-coated charcoal suspension (0.5% w/v charcoal, 0.05% w/v dextran). Displacement of 1,25(OH)$_2$D$_3$-tracer from VDR by the vitamin D_3 derivatives was determined by assessing the radioactivity with a liquid scintillation counter.

Determination of the affinity of vitamin D_3 derivatives for vitamin D binding protein (DBP)

A 25(OH)D_3-assay system (Amersham-Buchler, Braunschweig, Germany) was modified to determine the affinity of vitamin D_3 derivatives for serum DBP. Lyophilized DBP from sheep serum was dissolved in 0.05 M potassium phosphate buffer, pH 7.4 containing 0.01% (w/v) bovine immunoglobulin G and 0.01% (w/v) thimerosol. Fifty μL 25(OH)[26,27-methyl-^{3}H]vitamin D_3 (0.5 ng/mL, 0.19 μCi/mL) and 50 μL of the vitamin D derivative sample were added to 500 μL DPB solution, mixed, and incubated for 2 h at 4°C. Bound and free vitamin D_3 were separated by addition of 500 μL dextran-coated charcoal suspension (0.275% w/v charcoal, 0.0275% w/v dextran) immediately after the incubation period. Displacement of the 25(OH)D_3-tracer was measured with a liquid scintillation counter.

Determination of adipocyte differentiation and cell number

C3H10T½(BMP-4)-cells were precultured to near-confluence, diluted to 30,000 cells/mL, and transferred into 12-well plates. After culturing to confluence, the culture medium was replaced with DMEM supplemented with 10% FCS, 50 μg/mL ascorbate, and 10 mM β-glycerophosphate, and the cells were treated with 10^{-13} M to 10^{-5} M vitamin D_3 derivatives or solvent. Experiments were carried out in triplicate. Every 4–5 days, half of the media was replaced by fresh medium including the vitamin D_3 derivatives. After a culture period of 19 days, the cells were washed two times with PBS and 2 mL PBS per well were subsequently added. To each well, 20 μL of a solution of 100 μg/mL nile red in acetone were added. After an incubation period of 20 min at room temperature, the supernatant was discarded and 2 mL of PBS were added. The plates were scanned in a Cytofluor 2350 fluorescence photometer (excitation 485 nm/emission 530 nm) to determine the neutral lipid content of the cells. Subsequently, the cells were washed three times with PBS and fixed for 30 min with methanol, pH 8.5. Five hundred μL of a 0.1% (w/v) methylene blue solution in 10 mM borate buffer, pH 8.5, were added to each well. After 20 min, the cells were washed three times with 10 mM borate buffer, pH 8.5. For destaining, 4 mL of 0.1 M HCl containing 20% ethanol were added. After 20 min, the absorbance of the destaining solution was measured in a Multiskan photometer at 650 nm and correlated with a standard curve.

Results

The actions of 1,25(OH)$_2$D$_3$, 25(OH)D_3, **NS3**, and **NS8** on the murine fibroblastic cell line C3H10T½ were examined over a concentration range of 10^{-5}–10^{-13} M. The biologically active vitamin D_3 metabolite 1,25(OH)$_2$D$_3$ was the best inhibitor of proliferation of C3H10T½(BMP-4)-cells of all tested vitamin D_3 derivatives. Even at the low dose of 10^{-11} M inhibition of proliferation exceeds 50% (Figure 1). All other examined vitamin D_3 derivatives were significantly weaker. **NS3** and 25(OH)D_3 inhibited proliferation of C3H10T½(BMP-4)-cells in quite a similar way. In contrast to 1,25(OH)$_2$D$_3$, **NS3** and 25(OH)D_3 showed only a significant inhibitory effect at concentrations above 10^{-8} M. **NS8** was the best proliferation-inhibitor of the A-ring derivatives of 1,25(OH)$_2$D$_3$ with 30% inhibition at 10^{-10} M.

The most significant differences between 1,25(OH)$_2$D$_3$ and the synthetic vitamin D_3 derivatives were their gene activating properties at the VDRE-CAT-construct. 1,25(OH)$_2$D$_3$ induces the CAT-gene seventeen-fold at a concentration as low as 10^{-11} M (Figure 2). The gene activating activities of **NS3** and 25(OH)D_3 were nearly 10,000 times weaker. Both derivatives had a similar effect at all tested concentrations. **NS8** was slightly more active than NS3 and 25(OH)D_3 with nearly three times higher CAT-activity at 10^{-8} M.

The 1β-derivatives displayed a weak affinity for the VDR similar to that of the 1,25(OH)$_2$D$_3$-precursor 25(OH)D_3, which is about 20,000 times weaker than the VDR affinity of 1,25(OH)$_2$D$_3$ (Figure 3). As shown by the 50% displacement of **NS8** at a concentration of 9×10^{-7} M, this metabolite has nearly two times higher VDR affinity than 25(OH)D_3. The affinity of the 2α-chlor-derivative of **NS8**, **NS3,** was nearly ten times lower.

NS3 and **NS8** showed major differences in DBP affinity. At a concentration of 2×10^{-7} M, **NS3** displaced 50% of the 25(OH)D_3-tracer (Figure 4). **NS8** had a 50% displacement concentration of 9×10^{-9} M, which is nearly 22 times lower than that of **NS3** and only nearly six times higher than that of 25(OH)D_3, the main transportable form of vitamin D_3 in the blood. At concentrations of **NS8** below 10^{-8} M, the DBP binding curve in the competition experiment differed significantly from the ideal sigmoidal form. At these low concentrations, **NS8** showed an even higher affinity for DBP than 25(OH)D_3.

In contrast to all other tested substances, 1,25(OH)$_2$D$_3$ displayed a strong inhibitory effect on adipocyte differentiation. At all but the lowest concentration tested, 1,25(OH)$_2$D$_3$ diminished the neutral lipid concentration of C3H10T½(BMP-4)-cells to 20% of control. Even at a concentration of 10^{-13} M, the fat concentration barely exceeded the 60% mark. The microscopic control of cell morphology confirmed the fluorescence measurement because of the virtual absence of preadipocyte-like, fat droplets-containing, rounded cells at all concentrations with low fluorescence. The main reason for the inhibition of adipocyte differentiation is the proliferation-inhibitory activity of 1,25(OH)$_2$D$_3$. Its ID_{50} concentration was about ten times higher compared to its inhibition of proliferation in serum-free cell culture. The other three tested derivatives were weak inhibitors of adipocyte differentiation and cell proliferation at concentrations above 10^{-7} M. The significantly stronger proliferation-inhibitory effect of **NS8** on C3H10T½-cells in serum-free culture compared to both 25(OH)D_3 and **NS3** was not seen in the presence of FCS. Inhibition of adipogenesis and inhibition of cell proliferation of these three derivatives seems to be tightly coupled under these conditions.

Discussion

We examined the in vitro biological activities of 1,25(OH)$_2$D$_3$, 25(OH)D3, and two 1β-derivatives on

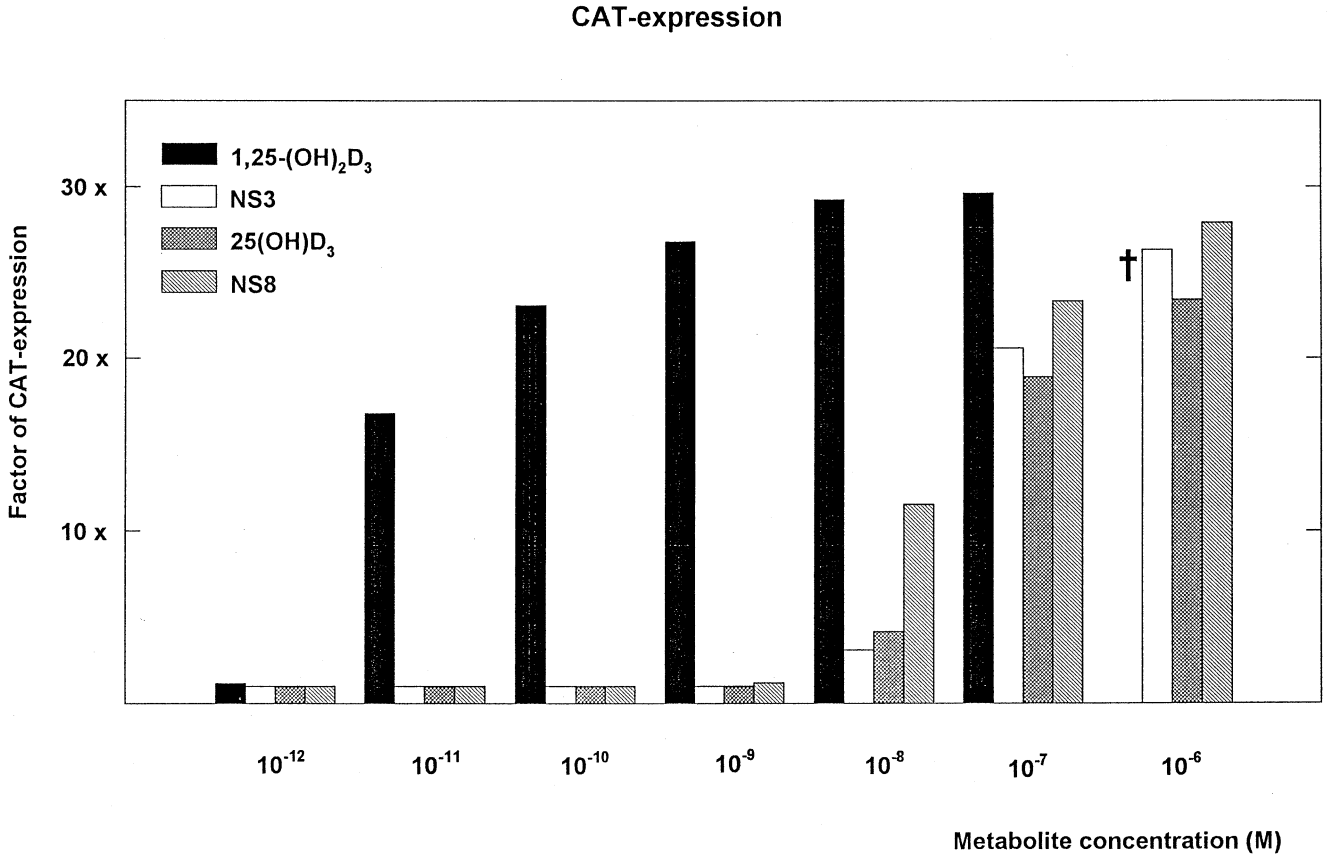


Figure 2 Dependence of CAT-production in C3H10T1/2(4xVDRE-CAT) cells on $1,25(OH)_2D_3$, $25(OH)D_3$, NS3 and NS8. Stable transfected C3H10T1/2 cells with chloramphenicol-acetyltransferase expression vector pBLCAT2 containing four direct repeats of the human osteocalcin VDRE were grown in 96 well plates and treated in serum-free medium with various concentrations of vitamin D_3 derivatives or solvent (ethanol) for 60 h. After washing the cells were lysed and the CAT concentration was determined using a CAT enzyme linked immunosorbent assay kit. †, $1,25(OH)_2D_3$-induced cell lysis. The experiments were carried out in triplicates. The results shown are representative of two to three experiments.

C3H10T½-cells and their binding affinities for VDR and DBP. We tested the inhibition of cell proliferation and the gene-activating activity of these seco-steroids in the absence of FCS. Therefore, it was possible to separate DBP-coupled processes from their direct cellular activities. The C3H10T½(BMP-4)-cells are highly sensitive for the native, biologically active vitamin D_3 metabolite $1,25(OH)_2D_3$: proliferation-inhibitory activity was clearly detectable at concentrations as low as 10^{-11} M. The same concentration was also sufficient to activate a VDRE-CAT-construct in C3H10T½(4×VDRE-CAT)-cells nearly 18-fold relative to the control. However, in the presence of FCS, a nearly ten times higher concentration of $1,25(OH)_2D_3$ was needed to reach half-maximal inhibition of cell proliferation due to the presence of DBP and lipoprotein in FCS to which binds a significant amount of $1,25(OH)_2D_3$. The effect of $1,25(OH)_2D_3$ on cell proliferation in the absence as well as in the presence of FCS was strictly dose dependent. Although mainly dependent on the strong proliferation-inhibitory activity of $1,25(OH)_2D_3$, the inhibitory effect of $1,25(OH)_2D_3$ on adipocyte differentiation seems to be regulated by an "on or off" mechanism. Further experiments with a lower concentration range are needed to elucidate the putative independency of adipocyte differentiation and inhibition of proliferation induced by $1,25(OH)_2D_3$.

The two examined 1β-derivatives of $1,25(OH)_2D_3$, **NS3** and **NS8**, both displayed a low affinity for VDR in the range of the weakly active $1,25(OH)_2D_3$-precursor $25(OH)D_3$. **NS8** showed a slightly higher VDR-affinity than $25(OH)D_3$, whereas **NS3** bound with the lowest affinity of all tested substances. This explains the low proliferation-inhibitory and CAT-activating activities of **NS3**. In both experiments, a concentration of more than 10^{-8} M was needed to see a significant effect. The slightly higher VDR-affinity of **NS8** compared to that of **NS3** led to a somewhat higher CAT-activity. However, the greater than hundred fold higher inhibition of cell proliferation is not fully explained by the VDR-affinity of **NS8**. Since the VDR-affinity of **NS8** is congruent with its CAT-activity and because gene-activating and cell proliferation experiments were both carried out in C3H10T½-cells,[22] different catabolic or "steroid-sorting" processes by membrane transporters during inhibition of cell proliferation are unlikely. One possibility is that the VDR needs a different dimerization partner for inhibition of cell proliferation than for VDRE-CAT activation. Since ligand binding and heterodimerization of

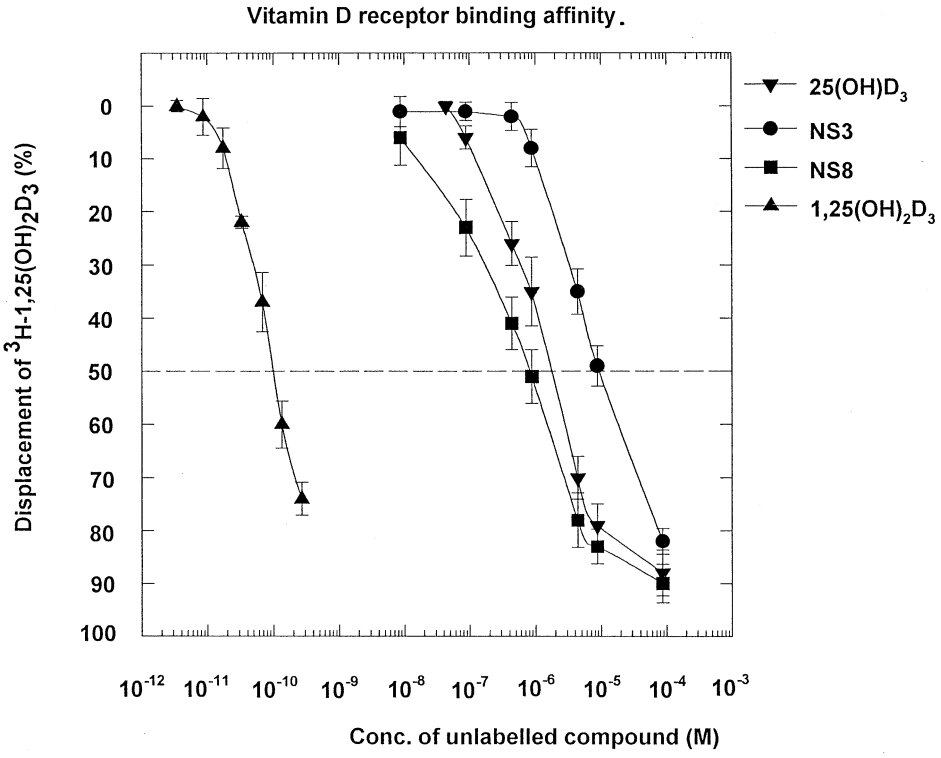


Figure 3 Displacement of [^{3}H]-1,25(OH)$_2$D$_3$ from chicken intestine vitamin D receptor by increasing concentrations of 1,25(OH)$_2$D$_3$, 25(OH)D$_3$, NS3 and NS8. An 1,25(OH)$_2$D$_3$ assay system was used to determine the affinity of vitamin D$_3$ derivatives to VDR. Intestinal VDR from rachitic chicken was incubated with 1,25-dihydroxy[26,27-methyl-^{3}H] vitamin D$_3$ and increasing concentrations of vitamin D$_3$ derivatives for 1 h at 22°C. Bound and free [^{3}H]-1,25(OH)$_2$D$_3$ were separated by the addition of dextran coated charcoal suspension. Displacement of [^{3}H]1,25(OH)$_2$D$_3$ was measured in a liquid scintillation counter. Experiments were carried out in duplicates.

members of the steroid hormone receptor superfamily are tightly coupled processes, the binding of **NS8** possibly favors the heterodimerization and subsequent transactivation events needed for effective inhibition of cell proliferation. **NS8** could be a vitamin D$_3$ derivative which shows a dissociation of the multiple vitamin D$_3$ effects at a transcriptional level.

All three derivatives display only weak effects on adipocyte differentiation (Figure 5) because of the high DBP-affinities and low VDR-affinities of 25(OH)D3 and **NS8**, and the extremely weak VDR-affinity of **NS3**.

The dose-response relationships of DBP-binding of the 1β-derivatives **NS3** and **NS8** differ significantly from each other. Bishop et al. have previously found that **NS8** has a higher affinity for DBP than 1,25(OH)$_2$D$_3$.[23] In Figure 4, **NS8** does not show the sigmoidal binding curve typical for equilibrium binding experiments. The higher affinity of **NS8** compared to that of 25(OH)D$_3$ for DBP in the low concentration range between 10^{-10} M and 10^{-9} M could only be explained by the existence of more than one population of binding sites on DBP.[24] At least one population of binding sites seems to have a higher affinity for **NS8** than for 25(OH)D$_3$. A 2α-chloro substituent led back to a sigmoidal monophasic binding curve as shown by **NS3**. This derivative seemed to have lost the ability to bind to high affinity binding site population(s) typical for **NS8** binding and had a lower affinity for the 25(OH)D$_3$-specific binding site than 25(OH)D$_3$. A possible explanation for this surprisingly different behavior could be differences in the A-ring conformation of these two A-ring derivatives of vitamin D$_3$. The 2α-chloro substituent and the 1β- and 3β-hydroxy functions in **NS3** stabilize a conformation with the three substituents in the equatorial position. Therefore, the whole

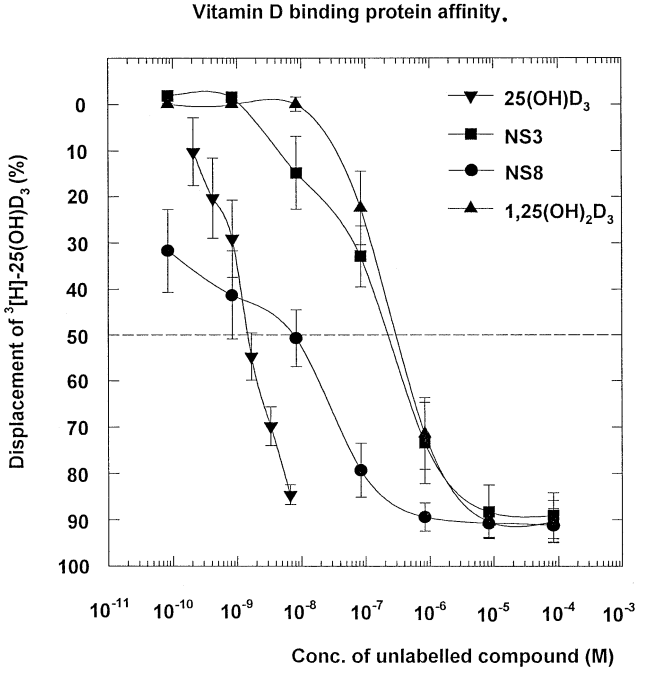


Figure 4 Displacement of [^{3}H]-25(OH)D$_3$ from sheep serum vitamin D binding protein by increasing concentrations of 1,25(OH)$_2$D$_3$, 25(OH)D$_3$, NS3 and NS8. A 25 (OH)D$_3$ assay system was used to determine the affinity of vitamin D$_3$ derivatives to serum DBP. DBP from sheep serum were incubated with 25(OH)[26,27-methyl-^{3}H] vitamin D$_3$ and increasing concentrations of vitamin D$_3$ derivatives for 2 h at 4°C. Bound and free [^{3}H]-25(OH)D$_3$ were separated by the addition of dextran coated charcoal suspension. Displacement of [^{3}H]-25(OH)D$_3$ was measured in a liquid scintillation counter. Experiments were carried out in duplicates.

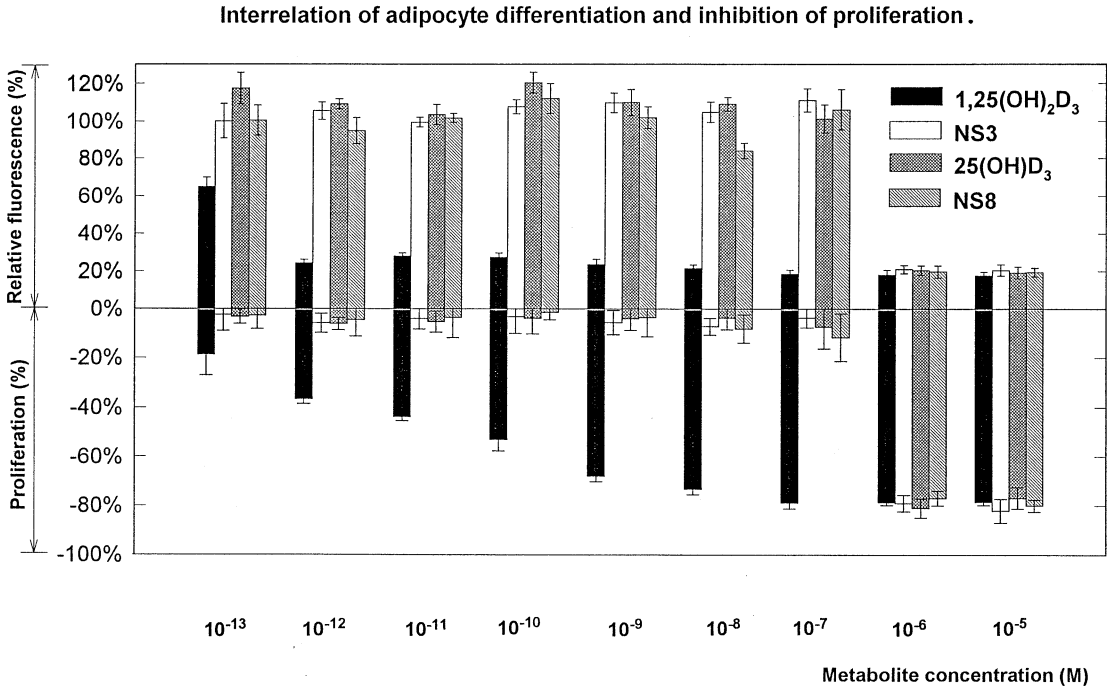


Figure 5 Interrelation of adipocyte differentiation and inhibition of cell proliferation of C3H10T1/2(BMP-4)-cells by $1,25(OH)_2D_3$, $25(OH)D_3$, NS3 and NS8. C3H10T1/2(BMP-4)-cells were cultured to confluence in 12-well plates. At confluence, the medium was replaced with DMEM supplemented with 10% FCS, 50 μg/mL ascorbate, and 10 mM β-glycerophosphate and treated with vitamin D_3 derivatives or solvent. Every 4–5 days, halve of the medium was replaced with fresh medium including the vitamin D_3 derivatives. After a culture period of 19 days, the cells were washed and after addition of nile red and washing, the neutral lipid content was determined in a Cytofluor 2350 fluorescence photometer (excitation 485 nm/emission 530 nm). Subsequently the cells were fixed in methanol treated with methylene blue solution for 20 min. After washing and lysis of the cells the absorbance was measured in a multiscan photometer at 650 nm and correlated with a standard curve. †, $1,25(OH)_2D_3$-induced cell lysis. Experiments were carried out in triplicates. The results shown are representative of two to three experiments.

A-ring is fixed in one of the two chair conformations. This seems to be unfavorable for at least one population of binding sites for which **NS8** has higher affinity than $25(OH)D_3$. Both chair conformations in **NS8** are, in contrast to **NS3**, energetically possible. However, it is unclear which specific A-ring conformation is favored for binding to the postulated population of high and low affinity binding sites and which other structural elements of the ligand are important for optimal binding.[25]

The in vitro biological effects of the examined 1β-vitamin D_3 derivatives are, as expected from the VDR binding data, weak compared to those of $1,25(OH)_2D_3$. However, a closer examination of the heterodimerization properties of the VDR in the presence of **NS8** and the unusually high affinity binding of **NS8** to new binding site(s) on the DBP, especially at low metabolite concentrations, could be interesting for the modulation of specific transcriptional activities and of pharmacokinetical characteristics of other highly biologically active vitamin D_3 derivatives.

Acknowledgments

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