Interaction of Two Novel 14-Epivitamin D₃ Analogs with Vitamin D₃ Receptor–Retinoid X Receptor Heterodimers on Vitamin D₃ Responsive Elements

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ABSTRACT

This study provides a detailed and exact evaluation of the interactions between vitamin D₃ receptor (VDR), retinoid X receptor (RXR), and vitamin D₃ responsive elements (VDREs) mediated by two novel 14-epianalogs of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], 19-nor-14-epi-23-yne-1,25(OH)₂D₃ (TX 522) and 19-nor-14,20-bisepi-23-yne-1,25(OH)₂D₃ (TX 527). Both analogs were more potent (14- and 75-fold, respectively) than 1,25(OH)₂D₃ in inhibiting cell proliferation and inducing cell differentiation. However, DNA-independent experiments indicated that both analogs had a lower affinity to VDR and that the stability of the induced VDR conformation, as measured by limited protease digestion assays, was similar (TX 527) or even weaker (TX 522) than that induced by the parent compound. However, DNA-dependent assays such as gel shift experiments revealed that those analogs were slightly more potent (3–7 times) than 1,25(OH)₂D₃ in enhancing binding of VDR-RXR heterodimers to a direct repeat spaced by three nucleotides (DR3) type VDRE. The functional consequences of the ligand-VDR-RXR-VDRE interactions observed in vitro were subsequently evaluated in transfection experiments. Both 14-epianalogs enhanced transcription of VDRE containing reporter constructs more efficiently than 1,25(OH)₂D₃ in COS-1 and MCF-7 cells regardless of the presence of ketoconazole. Transactivation activity is suggested to be a cell-specific process because maximal transcriptional induction and the half-maximal transactivation concentration for each reporter construct were different in both cell lines. The superagonistic transactivation activity closely resembled the biological potency of these analogs on the inhibition of MCF-7 cell proliferation. These data clearly indicate that superagonistic activity starts beyond the binding of the ligand-heterodimer (VDR-RXR) complex to VDRE and thus probably involves coactivator/corepressor molecules. (J Bone Miner Res 2001;16:625–638)

Key words: 1,25-dihydroxyvitamin D₃, analogs, 1,25-dihydroxyvitamin D₃ receptor, retinoid X receptor, 1,25-dihydroxyvitamin D₃ responsive element

INTRODUCTION

₁,₂₅-DIHYDROXYVITAMIN D₃ [1,25(OH)₂D₃], the active metabolite of vitamin D₃, is a major regulator of calcium and phosphate metabolism.¹² Moreover, 1,25(OH)₂D₃ is able to inhibit proliferation and induce differentiation of a wide variety of cells.³⁻⁶ However, the calcemic effects of 1,25(OH)₂D₃ hamper the application of pharmacologic doses of this compound. In the last decade, much effort has been made toward the development of analogs of...
1,25(OH)₂D₃ that are characterized by a clear dissociation of antiproliferative and calcemic activities. In the present study, two novel 14-epianalogs of 1,25(OH)₂D₃, TX 522 and TX 527, are discussed that have an enhanced antiproliferative and prodifferentiating capacity but have markedly reduced effects on calcium homeostasis. Therefore, these analogs have an appropriate profile for therapeutic applications and may be useful in the treatment of psoriasis, autoimmune diseases, and cancer. Currently, the compound TX 522 is under clinical investigation (phase II) for the treatment of psoriasis.

1,25(OH)₂D₃ and analogs exert their genomic actions through binding to the vitamin D₃ receptor (VDR). The main dimerization partner for VDR is retinoid X receptor (RXR), the receptor for 9-Ci cis-retinoic acid. This VDR-RXR heterodimer binds to specific DNA sequences (vitamin D₃ responsive elements [VDREs]). The VDR-RXR complex is thought to recruit specific coactivator proteins that mediate contacts with the basal transcription machinery. This whole complex is eventually responsible for the onset of gene transcription. The VDR-RXR heterodimer is shown to interact with different types of VDREs present in the promoter region of target genes. The classical DR3–type VDRE is composed of a direct hexanucleotide repeat separated by three interspacing nucleotides. The existence of other natural VDREs has been reported such as an inverted palindromic arrangement of two hexameric binding motifs referred to as IP9-type VDRE.

The aim of this study was to gain insight into the interaction of the 14-epianalogs with the VDR, the central protein mediating the genomic effects of 1,25(OH)₂D₃ and its analogs. Besides determination of the affinity to VDR, the stability of VDR conformation after interaction with either one of the analogs has been investigated. The effect of adding DNA (VDREs), to which the VDR-RXR complex can bind, on the conformational change of the VDR has been studied. In a second approach, the sensitivity of ligand enhanced interaction of VDR-RXR complexes with VDREs (DR3 and IP9 type) has been monitored by regular gel shift experiments. Finally, the data obtained from the previously described biochemical assays have been tested for reliability in cellular transfection experiments. The contribution of metabolites of 1,25(OH)₂D₃ or analogs to the transactivating potency and antiproliferative effects of these compounds was studied by performing experiments in the presence of ketoconazole. The ultimate goal of these assays was to find differences between 1,25(OH)₂D₃ and analogs in the ligand-VDR-RXR-VDRE interaction that may be relevant in explaining their different biological activity.

### MATERIALS AND METHODS

1,25(OH)₂D₃ and analogs

1,25(OH)₂D₃ was a gift of M.R. Uskokovic (Hoffman-La Roche, Nutley, NY, USA) and J.P. van de Velde (Duphar, Weesp, The Netherlands). The 14-epianalogs, 19-nor-14-epi-23-yne-1,25(OH)₂D₃ (SDB 112/TX 522) and 19-nor-14,20-bisepi-23-yne-1,25(OH)₂D₃ (ZXY 1106/TX 527) originally were synthesized by M. Vandewalle and P. De Clercq from the University of Ghent (Belgium) (Table 1). The analogs TX 522 and TX 527 were obtained from Théramex S.A. (Monaco). [³H]1,25(OH)₂D₃ (specific activity, 180 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK) and [³H]TX 522 (specific activity, 68 Ci/mmol) were obtained from Théramex.

**Affinity for VDR and vitamin D binding protein**

The affinity of 1,25(OH)₂D₃ and its analogs to the VDR was evaluated by their ability to compete with [³H]1,25(OH)₂D₃ for binding to a high-speed supernatant from intestinal mucosa homogenates obtained from normal pigs as described previously. The relative affinity of the analogs was calculated from their concentration needed to displace 50% of [³H]1,25(OH)₂D₃ from its receptor compared with the activity of 1,25(OH)₂D₃ (assigned 100% value).

**Dissociation from the VDR**

Dissociation of [³H]1,25(OH)₂D₃ or [³H]TX 522 bound to cytosol from intestinal mucosa or from human HL-60 cells was measured at 25°C. Therefore, the cytosol was incubated at 25°C for 20 h in presence of 5 nCi [³H]1,25(OH)₂D₃ or 3 nCi [³H]TX 522. Thereafter, an excess (500 ng) of unlabeled ligand was added and the amount of bound radioactivity was measured at various time points. Bound and unbound steroids were separated after centrifugation of the samples that had been incubated for 30 minutes (4°C) in the presence of dextran-coated charcoal.

**Biological profile of compounds**

As a measure of cellular proliferation, [³H]thymidine incorporation of keratinocytes and MCF-7 cells (ATCC, Rockville, MD, USA), in the presence or absence of 10⁻⁸ M ketoconazole, was determined after a 72-h incubation period with various concentrations of 1,25(OH)₂D₃ or analogs. Ketoconazole was obtained from Janssen Research Foundation (Beerse, Belgium). Differentiation of HL-60 cells (ATCC) was measured by the 4-nitro blue tetrazolium (NBT) reduction assay after 72 h incubation. Osteocalcin production by osteosarcoma cells (ATCC) after a 72-h treatment with 1,25(OH)₂D₃ or analogs was measured as described previously.

Proliferation of human peripheral blood mononuclear cells (PBMCs) after stimulation with phytohemagglutinin and a 72-h incubation period with 1,25(OH)₂D₃ or analogs was assessed by measuring [³H]thymidine incorporation. The hypercalcemic effect of the analogs was tested in vitamin D-replete normal Naval Medical Research Institute (NMRI) mice by daily subcutaneous injections of 1,25(OH)₂D₃, analogs, or the solvent (arachis oil) during 7 consecutive days using serum and urinary calcium concentration as parameters.
### Table 1. Chemical Structures and Biological Activities of 14 Epianalogs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding studies</th>
<th>In vitro studies</th>
<th>In vivo</th>
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<tr>
<td></td>
<td>VDR $K_d$</td>
<td>DBP $K_d$</td>
<td>HL-60 EC$_{50}$ for NBT reduction</td>
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<tr>
<td>$1\alpha,25$(OH)$_2$D$_3$</td>
<td>$(1.1 \pm 0.5) \times 10^{-10}$ M</td>
<td>$(5.1 \pm 1.3) \times 10^{-8}$ M</td>
<td>$(3.5 \pm 0.2) \times 10^{-10}$ M</td>
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<tr>
<td>TX 522</td>
<td>$(6.2 \pm 1.9) \times 10^{-9}$ M</td>
<td>$(3.5 \pm 1.0) \times 10^{-8}$ M</td>
<td>$(1.8 \pm 0.3) \times 10^{-10}$ M</td>
</tr>
<tr>
<td>TX 527</td>
<td>$(1.6 \pm 1.1) \times 10^{-9}$ M</td>
<td>$(3.5 \pm 1.0) \times 10^{-8}$ M</td>
<td>$(1.1 \pm 1.4) \times 10^{-11}$ M</td>
</tr>
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</table>

Summary of the in vitro and in vivo effects of 14 epianalogs of $1,25$(OH)$_2$D$_3$. The binding of $1,25$(OH)$_2$D$_3$ and its 14-epianalogs to VDR and hDBP is expressed by their dissociation constants. The antiproliferative effects of $1,25$(OH)$_2$D$_3$ and analogs were measured on MCF-7 cells and keratinocytes and are expressed as the concentrations required for the half-maximal inhibition of $[^3]$Hthy midine incorporation. The prodifferentiating effects were determined using HL-60 cells (NBT-reduction) and MG-63 cells (osteocalcin secretion) and are expressed as the concentrations necessary for the half-maximal response in the differentiation assays. The in vivo activity of $1,25$(OH)$_2$D$_3$ and its 14-epianalogs was determined in mice by intraperitoneal injections during 7 consecutive days. This activity was expressed as the maximal dose that could be administered without exceeding a serum calcium concentration of 10 mg/dl, which is observed in untreated mice. All values represent mean and SD of at least three independent experiments.
Limited proteolytic digestion of VDR

Synthetic human VDR was prepared using an in vitro coupled transcription/translation reaction (Promega, Madison, WI, USA) following the instructions of the manufacturer. One microgram of VDR DNA inserted into the EcoRI site of pSG5 was in vitro transcribed using T7 RNA polymerase and translated in the presence of [35S]methionine in rabbit reticulocyte lysates. The limited protease digestion assay was carried out as described previously.5,24 Five microliters of in vitro synthesized VDR protein was incubated in presence of $10^{-7}$ M 1,25(OH)$_2$D$_3$, or analogs or with vehicle for 20 minutes at room temperature. Ligands were diluted in distilled water (0.01% ethanol). Thereafter, the VDR-ligand complex was digested with trypsin (1–300 µg/ml) for 15 minutes at room temperature. Trypsin was obtained from Roche (Palo Alto, CA, USA) and dissolved in distilled water. Digestion was stopped by adding 20 µl of denaturing sodium dodecyl sulfate (SDS)/sample buffer to 4 µl of the reaction mixture. Samples were heated for 5 minutes at 95°C and loaded on 12% Tris-glycine gels (Novex, San Diego, CA, USA). Gels were dried and labeled fragments were visualized after overnight exposure to Hyperfilm (Amersham, Buckinghamshire, UK) at $-80^\circ$C. Signals were quantified by means of a PhosphorImager (Molecular Dynamics, Piscataway, NJ, USA).

The persistence of VDR against proteolytic breakdown also was investigated in a time-course experiment in which the VDR, which was preincubated with ligand ($10^{-7}$ M), was partially digested with trypsin (final concentration of 10 µg/ml).

The ability of graded concentrations of the ligands to protect the VDR/RXR heterodimer from digestion by trypsin was assessed as follows: RXR and VDR were in vitro transcribed and translated and equal amounts of RXR and VDR were incubated for 15 minutes in the presence of ligand (or ethanol) in a final volume of 20 µl of binding buffer.25,26 The buffer was adjusted with 1 M KCl to a final salt concentration of 80 mM. The DR3- and IP9-type VDREs were labeled by a fill-in reaction using [³²P]deoxyctydosine triphosphate (dCTP) and the Klenow fragment of DNA polymerase I (Promega). Approximately 1 ng of the labeled probe (±50,000 cpm) was added to the receptor/ligand mixture and incubated further for 20 minutes at room temperature. The protein-DNA complex was resolved on an 8% nondenaturing polyacrylamide gel in Tris-borate-EDTA (TBE). Gels were dried and labeled fragments were visualized after overnight exposure to Hyperfilm (Amersham) at $-80^\circ$C.

Ligand-dependent gel shift assays

Equal amounts of in vitro translated VDR and RXR proteins were mixed and incubated in the presence of the indicated concentrations of 1,25(OH)$_2$D$_3$, analogs, or vehicle (ethanol) for 15 minutes at room temperature in a total volume of 20 µl of binding buffer. The buffer was adjusted with 1 M KCl to a final salt concentration of 80 mM. The DR3- and IP9-type VDREs were labeled by a fill-in reaction using [³²P]deoxyctydosine triphosphate (dCTP) and the Klenow fragment of DNA polymerase I (Promega). The reporter plasmid was a luciferase construct containing an androgen responsive element (AGCTTACAT-AGTACGTGATGTTCTCAAGG), to which the VDR-RXR heterodimer cannot bind, has been used in these experiments. After an additional 20 minutes of incubation, trypsin was added to the samples and incubation was continued for another 15 minutes. Digestion was stopped by adding 20 µl of denaturing SDS/sample buffer to 4 µl of the final reaction mixture. Samples were heated for 5 minutes at 95°C and loaded on a 12% Tris-glycine gel (Novex). Gels were dried and labeled fragments were visualized after overnight exposure to Hyperfilm (Amersham) at $-80^\circ$C.

Transactivation potency

COS-1 cells (150,000) or MCF-7 cells (200,000) were seeded in 6-well plates and grown overnight in phenol red free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were transfected using Fugene-6 (Roche) according to the instructions of the manufacturer. One microgram of the reporter plasmid and 0.5 µg each of pSG5-based receptor expression vectors for VDR and RXR were added to each well. Luciferase activity was measured using the luciferase assay system of Promega.
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RESULTS

Statistics

Data were first analyzed with the analysis of variance (ANOVA) for the one-way classification fixed effect model. The ANOVA determines whether the variation between and within groups is such that the groups can be compared, tests the equality of several population means, and indicates whether the factor tested was affected by the different treatments. The value of \( p < 0.05 \) was accepted as significant. This analysis was followed by a two-tailed Student’s \( t \)-test for unpaired samples, assuming equal variances (according to the results of ANOVA).

EC\(_{50}\) values for the various dose-response curves were calculated by using three different sigmoid regression equations (three- and four-parameter sigmoid regression equations and three-parameter Gompertz regression equation, Sigma plot). The EC\(_{50}\) values and figures presented in the text represent the mean and SE of the EC\(_{50}\) values obtained with those equations. The curves drawn in Figs. 3–6 represent the curve fitted by the four-parameter sigmoid regression equation.

The structure and biological profile of the analogs are summarized in Table 1. Both analogs are characterized by a deletion of C19, an epimerization of C14, and a 23-yn modification in the side chain. The only difference between both analogs is the orientation of C20, TX 527 being the 20-episomer of TX 522. These 14-epianalogs were more potent than the parent compound in inhibiting the proliferation of normal (keratinocytes) and malignant (MCF-7) cells. However, their capacity to suppress the proliferation of normal (keratinocytes) and malignant (MCF-7) cells. However, their capacity to suppress the proliferation of normal (keratinocytes) and malignant (MCF-7) cell lines was also measured in the presence of 10\(^{-7}\) M ketoconazole alone for 72 h did not affect MCF-7 cell proliferation.

The antiproliferative and prodifferentiating activity of 1,25(OH)\(_2\)D\(_3\) and both analogs to inhibit the proliferation of MCF-7 cells in 10% FCS, 10% charcoal-treated FCS, and 2% charcoal-treated FCS (data not shown). The EC\(_{50}\) value of 1,25(OH)\(_2\)D\(_3\) decreased 10-fold when its antiproliferative potency was determined in medium containing charcoal-treated FCS. However, the EC\(_{50}\) values of both analogs decreased to a similar extent and their relative potencies compared with 1,25(OH)\(_2\)D\(_3\) remained approximately identical.

These 14-epianalogs were selected based on their potent antiproliferative capacity and their dissociation of antiproliferative and calcemic activities for evaluation of their interaction with VDR, RXR, and VDREs to investigate the pretranscriptional process initiated by these analogs.

DNA-independent interaction of 1,25(OH)\(_2\)D\(_3\) and analogs with VDR

Affinity of analogs to VDR and dissociation from VDR:
The affinity of 1,25(OH)\(_2\)D\(_3\) for the pig duodenal mucosa VDR was 1.9 ± 0.5 \times 10^{16} \text{ M}^{-1} \text{ (mean ± SD; } n = 7; \text{ Tables 1 and 2). The 14-epianalogs displayed a binding affinity of approximately 30–50% when compared with the affinity of 1,25(OH)\(_2\)D\(_3\), which was set to 100%. Using \([\text{H}]1,25(\text{OH})_2\text{D}_3\) and \([\text{H}]\text{TX 522}, the dissociation rate constant of receptor-bound ligand was investigated. In pig mucosa cytosol, the dissociation rate constant was 0.0132 minutes\(^{-1}\) for \([\text{H}]1,25(\text{OH})_2\text{D}_3\) and 0.0819 minutes\(^{-1}\) for \([\text{H}]\text{TX 522 (Fig. 1A). Also, in the cytosol from human HL-60 cells, the dissociation rate constant of \([\text{H}]\text{TX 522 was markedly higher than that of } [\text{H}]1,25(\text{OH})_2\text{D}_3 \text{ (0.1902 minutes}^{-1} \text{ and 0.0358 minutes}^{-1}, \text{ respectively; Fig. 1B).}

Limited proteolytic digestion of VDR in the absence of DNA: The conformation of VDR changes after interaction with 1,25(OH)\(_2\)D\(_3\) or its analogs and this can be shown using a partial enzymatic digestion of in vitro synthesized human VDR. A modification of the VDR conformation will lead to a different accessibility of cleavage sites within the VDR molecule and hence to an altered digestion profile. VDR was synthesized in vitro with \(^{35}\text{S}\)methionine and was visualized after separation on a Tris-glycine gel and autoradiography as three bands of 51, 48, and 45 kDa, which probably represent different forms of the VDR.

When VDR was incubated in the presence of a limited amount of trypsin, the receptor broke down to fragments of different sizes (approximately 27 kDa and 26 kDa). A final concentration of 10 \(\mu\)g/ml trypsin was sufficient to completely digest the unliganded receptor (data not shown). Incubation of VDR with 1,25(OH)\(_2\)D\(_3\) (10\(^{-7}\) M) before digestion with trypsin led to a digestion profile consisting of three fragments of different sizes (approximately 27, 26, and 23 kDa; Fig. 2A). The three fragments are thought to represent parts of the VDR ligand binding domain and possess 9 or 10 methionine molecules. After quantification of all fragments, it was possible to estimate the amount of receptor stabilized due to binding of the ligand.

Incubation for 15 minutes in the presence of a final concentration of 150 \(\mu\)g/ml of trypsin was required to digest...
completely the 1,25(OH)₂D₃-bound VDR. A very similar digestion profile was observed when VDR was incubated with TX 527 before digestion with trypsin (Fig. 2A). Interestingly, the conformation induced by the analog TX 522 was more prone to degradation with trypsin because only a 15-minute treatment in the presence of 50 μg/ml of trypsin was required to digest completely the receptor (Fig. 2A). Moreover, a 1-hour incubation period with 10 μg/ml of trypsin was sufficient to digest completely the VDR pre-treated with 10⁻⁷ M TX 522 while approximately 60% of the total receptor input persisted in the case of pretreatment with the parent molecule or TX 527 (Fig. 2A). Similar results were obtained when the receptor, pretreated with 1,25(OH)₂D₃ or 14-epianalogs, was digested with other proteases such as chymotrypsin and proteinase K (data not shown).

### Table 2. Summary of Biochemical and Biological Data

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<th>TX 522</th>
<th>TX 527</th>
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<td>Affinity measurements</td>
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<tr>
<td>VDR-RXR</td>
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<td><strong>DNA-dependent assays</strong></td>
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<td>DNA-dependent conformational change of VDR* (limited proteolytic digestion)</td>
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<td>VDR-RXR-VDRE</td>
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<tr>
<td>DR3 VDRE</td>
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<tr>
<td>IP9 VDRE</td>
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<td>VDR-RXR binding to VDRE*</td>
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<td>In COS-1 cells</td>
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<tr>
<td>DR3 VDRE</td>
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<td>2000</td>
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<td>Antiproliferative effects on MCF-7 cells</td>
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<tr>
<td>DR3 VDRE</td>
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<td>6200</td>
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<tr>
<td>IP9 VDRE</td>
<td>180</td>
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Summary of the biochemical and biological effects of 1,25(OH)₂D₃ and 14-epianalogs. The effects are expressed as percentage activity (at 50% dose response) in comparison with 1,25(OH)₂D₃ (=100% activity). All experiments were at least performed three times with similar results.

*Affinity of 1,25(OH)₂D₃ to VDR at 4°C and pH 7.4: 1.9 × 10⁻¹⁰ M⁻¹.

Limited proteolytic digestion assays: the concentration of 1,25(OH)₂D₃ necessary for half-maximal stabilization of human VDR was 7.5 × 10⁻⁹ M (human VDR alone), 4.0 × 10⁻⁸ M (human VDR and human RXR), 3.2 × 10⁻⁹ M (hVDR, human RXR, and DR3 VDRE), 6.0 × 10⁻⁹ M (human VDR, human RXR, and IP9 VDRE).

Limited proteolytic digestion assays: the concentration of 1,25(OH)₂D₃ required for half-maximal ligand-enhanced hVDR-hRXR binding to a DR3- and IP9-type VDRE was 1.2 × 10⁻¹⁰ M and 1.0 × 10⁻¹⁰ M, respectively.

Transfection experiments in COS-1 cells: the concentration of 1,25(OH)₂D₃ for the half-maximal transactivation of a DR3 reporter construct was 6.0 × 10⁻⁹ M and 5.1 × 10⁻⁹ M for an IP9 reporter construct.

Transfection experiments in MCF-7 cells: the concentration of 1,25(OH)₂D₃ for the half-maximal transactivation of a DR3 reporter construct was 4.9 × 10⁻⁹ M and 2.9 × 10⁻⁹ M or an IP9 reporter construct.

Transfection experiments in MCF-7 cells: the percentages transactivation activity (at 50% dose response) of 1,25(OH)₂D₃, TX 522, and TX 527 in presence of ketoconazole were expressed relative to the transactivation activity of 1,25(OH)₂D₃ in absence of ketoconazole.

Antiproliferative effects: the concentration of 1,25(OH)₂D₃ necessary for half-maximal inhibition of MCF-7 cell proliferation was 4.5 × 10⁻⁸ M.

Antiproliferative effects: the percentages antiproliferative capacity (at 50% dose response) of 1,25(OH)₂D₃, TX 522, and TX 527 in presence of ketoconazole were expressed relative to the antiproliferative capacity of 1,25(OH)₂D₃ in absence of ketoconazole.
Subsequently, dose-response curves for 1,25(OH)₂D₃ and both analogs were performed to calculate an EC₅₀ value for each compound. This is the concentration required to stabilize half of the receptor amount protected after a limited protease digestion. These dose-response curves have been performed both in the absence (data not shown) and presence of RXR with essentially the same results (Table 2). The results of the limited proteolytic digestion in the presence of RXR are discussed because they are used as reference for further experiments.

**DNA-dependent interaction of analogs with VDR-RXR complex**

Limited proteolytic digestion of VDR in the presence of DNA: Subsequently, DNA-dependent conformational alterations and stability of labeled VDR were studied by performing dose-response experiments for each compound in the presence of DR3- or IP9-type VDREs. 1,25(OH)₂D₃, TX 522, or TX 527 did generally slightly enhance the maximal amount of VDR recovered after trypsin treatment (data not shown). However, for the ligands, no significant difference was found between the EC₅₀ values obtained in the absence of DNA and those in the presence of the VDREs (DR3 or IP9).

As a control to this experiment, an androgen responsive element, to which the VDR-RXR heterodimer cannot bind, has been added to the VDR-RXR heterodimer before the addition of trypsin. As expected, this control DNA element did not affect the maximal amount of receptor stabilized or the calculated EC₅₀ values (data not shown).

**Ligand-dependent gel shift:** The influence of ligand on the actual binding of the VDR-RXR complex to DNA was investigated by ligand-dependent gel shift experiments with a labeled DR3- and IP9-type VDRE (Fig. 4). Increasing concentrations of 1,25(OH)₂D₃, TX 522, or TX 527 led to increased amounts of VDR-RXR bound to DNA. The maximal amount of ligand-VDR-RXR complexes that could be bound to DNA was comparable for 1,25(OH)₂D₃ and the two analogs when they were run in a side-by-side assay (data not shown).

Dose-response curves were set up for each compound to calculate the concentration required for a half-maximal ligand-enhanced VDR-RXR binding to DNA. For 1,25(OH)₂D₃, this concentration was almost identical for both response elements; the EC₅₀ value was (2.1 ± 1.0) × 10⁻¹⁰ M on the DR3 response element and (1.1 ± 0.1) × 10⁻¹⁰ M on the IP9-type VDRE (Table 2). For TX 522, there was a clear difference in these EC₅₀ values. Surpris-
ingly, lower concentrations of TX 522 in comparison with 1,25(OH)$_2$D$_3$ were needed to obtain half-maximal VDR-RXR binding to the DR3 responsive element [EC$_{50}$ : (3.0 ± 1.2) × 10$^{-11}$ M]. The EC$_{50}$ value was (9.7 ± 0.5) × 10$^{-10}$ M for the IP9-type VDRE, which was substantially higher than that of 1,25(OH)$_2$D$_3$. This selectivity to bind preferentially to the DR3-type response element was less pronounced for the 20-epianalog TX 527. The EC$_{50}$ value for the DR3-type VDRE was (6.0 ± 2.3) × 10$^{-11}$ M, which was lower than that of 1,25(OH)$_2$D$_3$ and (1.3 ± 0.7) × 10$^{-10}$ M for the IP9-type VDRE, which was comparable with 1,25(OH)$_2$D$_3$.

Transactivating potency of 14-epianalogs

The capacity of 1,25(OH)$_2$D$_3$ and the 14-epianalogs to enhance the transcription of reporter genes carrying the DR3 and IP9 VDREs used in previous experiments was investigated in transient transfection experiments (Fig. 5). Treatment of COS-1 cells, transfected with the DR3 or IP9 reporter construct with 1,25(OH)$_2$D$_3$ or analogs (10$^{-7}$ M) led to a 7- or 8-fold increase of transcription levels when compared with cells that had been incubated with vehicle. 1,25(OH)$_2$D$_3$ did not show a preference in the activation of the DR3- or IP9-type VDRE regulated transcription and EC$_{50}$ values for both reporter constructs were nearly identical, respectively, (4.9 ± 1.5) × 10$^{-9}$ M and (4.7 ± 0.5) × 10$^{-9}$ M (Table 2). The 26-fold lower concentrations of TX 522 compared with 1,25(OH)$_2$D$_3$ were sufficient to obtain half-maximal enhanced transcription levels of the DR3 reporter construct [EC$_{50}$ : (1.9 ± 0.1) × 10$^{-10}$ M], whereas 4-fold lower concentrations were required for half-maximal
transactivation of the IP9 reporter construct \( [\text{EC}_{50}] = (1.1 \pm 0.1) \times 10^{-9} \) M. This DR3 preference also was observed for TX 527 but to a lesser extent. The \( \text{EC}_{50} \) value of TX 527 for the DR3 reporter construct was \( (7.2 \pm 4.2) \times 10^{-10} \) M while that for the IP9 reporter construct was \( (1.6 \pm 1.0) \times 10^{-9} \) M. Thus, the analog TX 527 also proved to be more potent than 1,25(OH)$_2$D$_3$ in enhancing the transcription levels of these reporter constructs. To investigate the cell-type specificity of the activity of these compounds, breast cancer MCF-7 cells have been transfected with the same constructs using identical experimental conditions (Fig. 6). The maximal induction by the addition of ligand that could be obtained in this cell line was much higher than that in COS-1 cells for both the DR3 and the IP9 reporter construct. The maximal induction of transcriptional activation by 1,25(OH)$_2$D$_3$ was 160-fold for the DR3 reporter construct and 35-fold for the IP9 reporter construct. The \( \text{EC}_{50} \) values obtained for 1,25(OH)$_2$D$_3$ were almost identical for both response element reporter constructs and were similar to the values obtained in COS-1 cells \( [(6.3 \pm 1.9) \times 10^{-9} \) M for the DR3 and \( (1.9 \pm 0.2) \times 10^{-9} \) M for the IP9 reporter construct; Table 2]. The analogs TX 522 and TX 527 again were more potent in enhancing the transcription of these reporter constructs. However, the DR3 selectivity of TX 522 and TX 527 could not be confirmed in this cell line. For TX 522 the \( \text{EC}_{50} \) value was \( (3.2 \pm 1.1) \times 10^{-10} \) M for the DR3 and \( (8.0 \pm 1.3) \times 10^{-11} \) M for the IP9 reporter construct. The \( \text{EC}_{50} \) values for TX 527 were \( (7.2 \pm 1.9) \times 10^{-11} \) M for the DR3 reporter construct and \( (4.4 \pm 1.1) \times 10^{-11} \) M for the IP9 construct, indicating that the analog TX 527 was more potent than TX 522 in activating transcription in MCF-7 cells whereas the opposite was seen in COS-1 cells.

Experiments in the presence of ketoconazole \( (10^{-7} \) M) were performed to assess the importance of metabolites of 1,25(OH)$_2$D$_3$ or analogs in the enhancement of transcription activation (Fig. 6). The maximal fold induction by 1,25(OH)$_2$D$_3$ was increased from 160- to 188-fold in the presence of ketoconazole in MCF-7 cells transfected with the DR3 reporter construct and from 36- to 42-fold in MCF-7 cells transfected with the IP9 construct. However, no significant changes in the maximal fold induction or in the calculated \( \text{EC}_{50} \) values were found either for 1,25(OH)$_2$D$_3$ or for analogs TX 522 and TX 527 (Table 2).
Moreover, the dissociation rate of the analog TX 522 from pig and human VDR was approximately 6-fold higher than that of 1,25(OH)2D3, indicating that this compound is very quickly released from the receptor.

Subsequently, ligand-induced changes in VDR conformation were investigated because subtle deformations of the ligand binding pocket may modulate the ability of VDR to dimerize, be phosphorylated, bind to VDREs, or interact with coactivators and repressors involved in target gene activation.17,18,24,31,32 Especially, interaction with and positioning of the VDR (trans)activation function 2 (AF-2) domain seems to be a major factor in determining the stability and transactivation potency of the receptor.33–35

**FIG. 4.** Ligand-dependent gel shift experiment. Equal amounts of in vitro translated human VDR and human RXR were incubated with graded ligand concentrations before the addition of [32P]-labeled DR3- or IP9-type VDREs. (A) A representative experiment for 1,25(OH)2D3 on a DR3-type VDRE (left panel) and on an IP9-type VDRE (right panel) is shown. The amount of protein-complexed VDREs was quantified on a Bioimager in relation to nonliganded VDR-RXR heterodimers. (B) Dose-response curves for 1,25(OH)2D3 (●), TX 522 (▼), or TX 527 (□) on a DR3-type VDRE (left panel) and on an IP9-type VDRE (right panel) have been performed. Each data point represents the mean of triplicates and bars indicate SDs. EC50 values were calculated from dose-response curves.

VDR conformation was studied with the well-established limited protease digestion assay.24,25,30 The ligand-induced modification of VDR conformation was first investigated in a DNA-independent assay whether or not in the presence of the RXR dimerization partner. The VDR conformation induced by 1,25(OH)2D3 and TX 527 was similarly protected against proteolytic breakdown whereas that induced by TX 522 appeared to be more prone to proteolytic degradation, as illustrated by time course and dose-response experiments using different proteases. This finding confirms once more that an altered stereochemistry of C20 can dramatically affect the stability of ligand-induced VDR conformation.30,32,36,37 The fact that the analog TX 522 dissociates very quickly from its receptor may explain the
relative instability of the VDR conformation induced by TX 522. Dose-response curves determining the effect of ligand on VDR conformation were performed both in the absence (data not shown) and in the presence of RXR with essentially the same results, suggesting that the addition of RXR does not alter the stability of VDR conformation.

Complex formation between VDR-RXR heterodimers and specific VDREs is considered as a major molecular step in the specific activation of vitamin D responsive genes and is shown to induce DNA bending possibly facilitating the transcription assembly process.\(^{11,38}\) Therefore, DNA-dependent modifications in VDR conformation and the formation of ligand-VDR-RXR complexes on DNA were investigated. To evaluate DNA-dependent VDR conformation, VDREs (DR3 and IP9 type) were added to the ligand-bound VDR-RXR complex before the addition of trypsin. Although the maximal amount of VDR molecules that could be protected against proteolytic breakdown generally was enhanced by the addition of DNA, no significant increase in the stabilization of the VDR conformation was observed.

These observations illustrate that the limited proteolytic digestion assay, whether or not in the presence of RXR or VDREs, is not able to estimate the biological activity of 1,25(OH)\(_2\)D\(_3\) analogs.

Gel shift experiments were performed to investigate ligand-enhanced binding of VDR-RXR heterodimers to different types of radioactively labeled response elements (DR3 and IP9). Although 1,25(OH)\(_2\)D\(_3\) did not show a preference for one of the response elements, such selectivity was clearly present for the analog TX 522 and to a lesser extent for the analog TX 527. Surprisingly, a slightly lower concentration of TX 522, when compared with 1,25(OH)\(_2\)D\(_3\), was sufficient to obtain half-maximal ligand-enhanced binding of the VDR-RXR complex to a DR3-type response element. Higher concentrations of TX 522 were required to reach half-maximal binding to the IP9-type VDRE, stressing the importance of the VDRE sequence.

These gel shift experiments indicate that the compound TX 522, despite the induction of a less stable VDR conformation both in DNA-independent and -dependent assays, is able to induce high affinity binding of the VDR-RXR complex to DNA. It may therefore be hypothesized that ligand-induced alterations in VDR conformation and in VDR-RXR interactions may enhance the binding of VDR-RXR to DNA. However, the main reason for the superagonistic activity of the analogs probably is not entirely situated at the level of DNA binding because more or less comparable concentrations of 1,25(OH)\(_2\)D\(_3\) and analogs were required to obtain half-maximal ligand-enhanced binding.

The functionality of the ligand-VDR-RXR-VDRE interactions observed in biochemical assays was subsequently evaluated in transfection experiments using reporter constructs carrying the same VDREs. The superagonistic profile of these 14-epianalogs was revealed in those experiments. Indeed, these compounds proved to be more potent than 1,25(OH)\(_2\)D\(_3\), both in COS-1 cells and in MCF-7 cells, in inducing transcription from reporter constructs carrying a DR3- or IP9-type VDRE. These data stress the importance of coactivator/corepressor molecules in determining the eventual transcriptional regulation of target genes.
COS-1 cells, lower concentrations of TX 522 and TX 527 were required for half-maximal transactivation of the DR3 reporter construct than for the IP9 reporter construct, confirming the DR3 selectivity of these analogs. However, such selectivity to transactivate preferentially a DR3-type VDRE could not be shown for these 14-epianalogs when these transfections were performed in MCF-7 cells. The maximum levels of transcriptional induction obtained in MCF-7 cells were markedly higher than the maximum levels in COS-1 cells, and the maximum levels of transcriptional induction in MCF-7 cells were higher for both DR3- and I9-type reporter constructs compared with the maximum levels in COS-2 cells (Figs. 5 and 6). This difference could be related to protein levels of VDR and RXR in each cell type. However, both cell lines were cotransfected with the same amount of VDR and RXR expression vectors. Reducing the amount of cotransfected VDR and RXR in MCF-7 cells led to a reduced transcriptional induction of the reporter constructs (data not shown). These diverging transactivating results illustrate the cell-type specificity of the regulation of gene transcription by vitamin D analogs. It might be hypothesized that different coactivator and corepressor proteins prevail in different cell types. The mutual proportion of these different regulator proteins together with the ligands’ preference for any of these proteins may influence the composition of the basal transcription machinery and affect regulation of gene transcription.17,18

Transfection experiments in the presence of ketoconazole indicated that the transactivation results obtained for TX 522 and TX 527 were not caused by their metabolites.
In conclusion, two 14-epianalogs of 1,25(OH)2D3 are discussed, which, despite the induction of a similar or even weaker stability of VDR conformation, are more potent than 1,25(OH)2D3 in enhancing VDR-RXR complex formation on a DR3-type VDRE, suggesting that modifications in VDR conformation and VDR-RXR interaction enhance the binding of VDR-RXR heterodimers to DNA. However, the coactivator proteins and basal transcription factors are the main determining factors for revealing the superagonistic transactivating and biological potency of these compounds. Thus, the whole transcriptional machinery and the cell type are to be taken into account to define the selective action of vitamin D3 analogs.

ACKNOWLEDGMENTS

We thank N. Adje and J.C. Pascal at Theramex for the synthesis of TX 522 and TX 527. This work was supported by the Flemish Fund for Scientific Research (G.0233.97) and by Theramex S.A.

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Received in original form February 25, 2000; in revised form November 7, 2000; accepted December 5, 2000.