Superagonistic Action of 14-epi-Analogs of 1,25-Dihydroxyvitamin D Explained by Vitamin D Receptor-Coactivator Interaction

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ABSTRACT

Two 14-epi-analogs of 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃], 19-nor-14-epi-23-yne-1,25-(OH)₂D₃ (TX522) and 19-nor-14,20-bisepi-23-yne-1,25-(OH)₂D₃ (TX527), show enhanced antiproliferative (at least 10-fold) and markedly lower calcemic effects both in vitro and in vivo, compared with 1,25-(OH)₂D₃. This study aimed to evaluate their superagonistic effect at the level of interaction between the Vitamin D receptor (VDR) and coactivators. Mammalian two-hybrid assays with VP16-fused VDR and GAL4-DNA-binding-domain-fused steroid receptor coactivator 1 (SRC-1), transcriptional intermediary factor 2 (TIF2), or DRIP205 showed the 14-epi-analogs to be more potent inducers of VDR-coactivator interactions than 1,25-(OH)₂D₃ (up to 16- and 20-fold stronger induction of VDR-SRC-1 interaction for TX522 and TX527 at 10⁻¹⁰ M). Similar assays in which metabolism of 1,25-(OH)₂D₃ was blocked with VID400, a selective inhibitor of the 1,25-(OH)₂D₃-metabolizing enzyme CYP24, showed that the enhanced potency of these analogs in establishing VDR-coactivator interactions can only partially be accounted for by their increased resistance to metabolic degradation. Crystallization of TX522 complexed to the ligand binding domain of the human VDR demonstrated that the epi-configuration of C14 caused the CD ring of the ligand to shift by 0.5 Å, thereby bringing the C12 atom into closer contact with Val300. Moreover, C22 of TX522 made an additional contact with the CD1 atom of Ile268 because of the rigidity of the triple bond-containing side chain. The position and conformation of the activation helix H12 of VDR were strictly maintained. In conclusion, this study provides deeper insight into the docking of TX522 in the LBP and shows that stronger VDR-coactivator interactions underlie the superagonistic activity of the two 14-epi-analogs.

1,25-Dihydroxyvitamin D₃ [1,25-(OH)₂D₃], the biologically active form of vitamin D, plays a major role in bone metabolism and in calcium and phosphate homeostasis. In addition to this classic effect, 1,25-(OH)₂D₃ also has an antiproliferative and prodifferentiating effect on various normal as well as malignant cells (Bouillon et al., 1995). This makes 1,25-(OH)₂D₃ a candidate drug for cancer treatment. However, at the pharmacological doses needed for this application, 1,25-(OH)₂D₃ displays major calcemic side-effects (e.g., hypercal-

ABBREVIATIONS: 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃; VDR, vitamin D receptor; RXR, retinoid X receptor; LBD, ligand binding domain; TX522, 19-nor-14-epi-23-yne-1,25-(OH)₂D₃; TX527, 19-nor-14,20-bisepi-23-yne-1,25-(OH)₂D₃; pVPVDR, VP16-fused VDR; DBD, DNA binding domain; LBP, ligand binding pocket; DOTAP, N₁-[1-(2,3-dioleoyloxy)propyl]N,N,N-trimethylammonium methysulfate; CAT, chloramphenicol acetyltransferase; MES, 2-(N-morpholino)ethanesulfonic acid; TIF2, transcriptional intermediary factor 2; SRC-1, steroid receptor coactivator 1; DRIP, vitamin D receptor-interacting protein; h, human; LSD, least significant difference; MC903, calcipotriol; KH1060, analog 20-epi-22-oxa-24α,26α,27α-tri-homo-1,25(OH)₂D₃; vitamin D-3; Ro24-5531, 1α,25-dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol; ZK159222, 25-carboxylic ester analog of 1,25-(OH)₂D₃; BL314, 9,11-bisnor-16a-homo-20-epi-1,25(OH)₂D₃.
This study aims to investigate the influence of 1,25-(OH)2D3 on the agonistic profile of the two 14-epi-analogs TX522 and TX527, promoter (Rachez et al., 1998, 1999, 2000). Thus by recruiting the basal transcription machinery to enhance transcription by binding to RNA polymerase II and anchor other subunits of the complex to the receptor. When bound to the ligand binding domain (LBD) of the VDR and its preferred dimerization partner, the retinoid X receptor (RXR). Moreover, it was shown that the profile of the analogs could not be explained by the interaction between the VDR-RXR heterodimer and its target DNA sequences (vitamin D response elements) (Verlinden et al., 2001). The next step in the transcriptional process is the recruitment of coregulator molecules to the ligand-bound VDR. These findings point toward a possible key role for these coregulator molecules in the elucidation of the superagonistic action of the analogs.

The function and the importance of coregulators, comprising both coactivators and corepressors, in nuclear receptor-mediated gene transcription have been described at length. Coactivators interacting with the VDR include the p160 family members, such as steroid receptor coactivator 1 (SRC-1), GRIP1/transcriptional intermediary factor 2 (TIF2), and ACTR (reviewed by Rachez and Freedman, 2000). These coactivators recruit histone acetyltransferase activity and create a permissive chromatin surrounding for gene transcription. Corepressors recruit histone deacetylase activity and inhibit gene transcription, both coactivators and corepressors, in nuclear receptor-mediated gene transcription have been described at length.}

Materials and Reagents

The GAL4 DNA-binding domain cloning vector pM, the activation-domain cloning vector pVP16, and the reporter construct pG5CAT are part of the Mammalian Matchmaker Two-Hybrid Assay kit (BD Biosciences Clontech, Erembodegem, Belgium). The pVPVDR construct was made by cloning the 1284-base pair fragment of the pAS2VDR construct, which was obtained from D. Feldman (Stanford University School of Medicine, Stanford, CA), into the pVP16 vector using the BamHI and SalI restriction sites. The fragment was subcloned into the BamHI and XbaI restriction sites of the pVPVDR construct. The GAL4-DNA-binding domain containing constructs pGALTIF2.4 (AA 624-1010) and pSG424SRC1NIR (AA 570–782) were obtained from H. Grone

Verlinden et al., 2000). The pMDRIP205 construct, a fragment of DRIP205 (AA 510–787) was generated by PCR with the forward primer 5'-CGCGGATCCACTGTCCCTCAT-3' and the reverse primer 5'-TGTCCTAGAGGCTGGGCAATCATCACTA-3'. The fragment was subcloned into the BamHI and XbaI restriction sites of the pM vector. The GAL4-DNA-binding domain containing constructs pGALTIF2.4 (AA 624-1010) and pSG424SRC1NIR (AA 570–782) were obtained from H. Grone.
Transient Transfection Assays

COS-1 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Biochrom KG, Berlin, Germany) supplemented with GlutaMAX I, 100 U/ml penicillin, and 100 μg/ml streptomycin (In Vitrogen, Merelbeke, Belgium). Approximately 1.5 × 10⁶ cells were seeded 24 h before transfection in 6-well plates. Cells were transfected with DOTAP liposomal transfection reagent (Roche Diagnostics, Mannheim, Germany) as specified by the manufacturer. The amounts of plasmid DNA used for transfection were 300 ng for the GAL4-fusion, 300 ng for the VP16-fusion, and 1.5 μg for the CAT-reporter plasmid. Cells were treated with 1,25-(OH)₂D₃, TX522, TX527, VID400, or vehicle (ethanol) 24 h after transfection. CAT-amounts were assayed 24 h thereafter with a CAT-enzyme-linked immunosorbent assay (Roche Diagnostics) according to the manufacturer's instructions and were corrected for total protein contents.

Cell Proliferation Assays

As a measure of cell proliferation, [³H]thymidine incorporation of breast cancer MCF-7 cells (American Type Culture Collection) was determined after a 72-h incubation with various concentrations of 1,25-(OH)₂D₃ analogs, or vehicle as described previously (Verstuyf et al., 1998).

Crystallography

Expression, Purification, and Crystallization. The LBD of the human VDR (residues 118–427) was cloned in pET28b expression vector to obtain an N-terminal hexahistidine-tagged fusion protein, and overproduced in Escherichia coli BL21 (DE3) strain. Cells were grown in Luria-Bertani medium and subsequently induced for 6 h at 20°C with 1 mM isopropyl thio-

Expression, Purification, and Crystallization

Crystallography

Cell Proliferation Assays

Crystallography

Effects of 1,25-(OH)₂D₃ and 14-epi-Analogs on the Interaction between VDR and Coactivator Molecules.

To determine the effect of 1,25-(OH)₂D₃ and the two 14-epi-
alogs TX522 and TX527 on the interaction between VDR and the coactivator TIF2, a mammalian two-hybrid system with pVPVDR, a GAL4-DNA binding domain-fused TIF2.4 (pGALTIF2.4), and a chloramphenicol-acetyltransferase-reporter construct (pG5CAT) were used. To exclude any possible bias caused by the two activation domains of TIF2 (AD1 and AD2), the coactivator fragment TIF2.4 (AA 624-1010) was used. This fragment contains the nuclear receptor interacting domain but lacks AD1 and AD2. Transient transfection of these plasmids into COS-1 cells and subsequent treatment of these cells with 1,25-(OH)₂D₃, TX522, or TX527 yielded clear differences in CAT-reporter gene expression (Fig. 2). Cells treated with 10⁻¹⁰ M and 10⁻⁹ M TX522 or TX527 had clearly higher VDR-TIF2.4 interaction (1.6- and 2.3-fold, respectively, for TX522 and 2.7- and 2.7-fold, respectively, for TX527) than cells treated with 1,25-(OH)₂D₃ at the same doses. At 10⁻⁸ M, only TX527 induced a significantly higher VDR-TIF2.4 interaction; at 10⁻⁷ M, there was no more difference between 1,25-(OH)₂D₃ and either of the two analogs. TX522 required approximately 10-fold lower concentrations than 1,25-(OH)₂D₃ for the induction of half-maximal VDR-TIF2.4 interaction, whereas TX527 required approximately 20- to 50-fold lower concentrations for the same induction. Cotransfection of either pVPVDR or pGALTIF2.4 alone together with pG5CAT and subsequent treatment with 1,25-(OH)₂D₃, TX522, or TX527 yielded no significant or ligand-dependent reporter gene expression (data not shown).

Similar experiments were performed with the coactivator SRC-1 and again a fragment of the coactivator (AA 570–782;
pSG424SRC1NIR), which contains the nuclear receptor interacting domain but lacks AD1 and AD2, was used. Even at $10^{-11}$ M, the 14,20-bisepi-analog TX527 caused a 6-fold higher VDR-SRC-1 interaction than 1,25-(OH)$_2$D$_3$; at $10^{-10}$ M, a 16- and 20-fold stronger interaction was observed in TX522- and TX527-treated samples (Fig. 3). In cells treated with $10^{-9}$ M TX522 and TX527, VDR-SRC-1 interaction was clearly, although not statistically significantly, higher than in 1,25-(OH)$_2$D$_3$-treated samples. At $10^{-8}$ M there was no longer a difference between 1,25-(OH)$_2$D$_3$ and either of the analogs. Based on the half-maximal induction of VDR-SRC-1 interaction, TX522 and TX527 are both at least 10 times more potent than 1,25-(OH)$_2$D$_3$. Cotransfection of either pVPVDR or pSG424SRC1NIR alone together with pG5CAT and subsequent treatment with 1,25-(OH)$_2$D$_3$, TX522, or TX527 yielded no significant or ligand-dependent reporter gene expression (data not shown).

Next, we used DRIP205, which is devoid of histone acetyltransferase activity but instead is part of a larger DRIP complex that recruits RNA polymerase II. A fragment (AA 510–787) containing the two nuclear receptor interaction motifs NR1 and NR2 was fused to the GAL4-DBD (pMDRIP205) and cotransfected with pVPVDR and pG5CAT in COS-1 cells, which were then treated with the different ligands (Fig. 4). At $10^{-10}$ M, TX522 and TX527 induced a 4- and 10-fold higher VDR-DRIP205 interaction than 1,25-(OH)$_2$D$_3$. These differences increased to 6-fold for TX522 and stayed at 10-fold for TX527 at $10^{-9}$ M and dropped to 2- and 2.6-fold for TX522 and TX527, respectively, at $10^{-8}$ M. TX522 and TX527 require 30- and 40-fold lower doses to obtain the VDR-DRIP205 interaction induced by 1,25-(OH)$_2$D$_3$ at $10^{-8}$ M. Cotransfection of either pVPVDR or pMDRIP205 alone together with pG5CAT and subsequent treatment with 1,25-(OH)$_2$D$_3$, TX522, or TX527 did not yield significant or ligand-dependent reporter gene expression (data not shown).

**Fig. 3.** Effect of 1,25-(OH)$_2$D$_3$ and 14-epi-analogs on the interaction VDR-SRC-1. COS-1 cells were transfected with pVPVDR, pSG424SRC1NIR, and the pG5CAT reporter and treated with 1,25-(OH)$_2$D$_3$, with TX522 and TX527 at the indicated doses, or with vehicle. CAT accumulation was normalized to total protein content and expressed as relative CAT amounts (RCA). Results shown are the mean ± S.E.M. of at least three independent experiments performed in triplicate. *, RCA significantly different from RCA for 1,25-(OH)$_2$D$_3$-treated samples; $p < 0.05$ according to Fisher's LSD multiple-comparison test.

**Fig. 4.** Effect of 1,25-(OH)$_2$D$_3$ and 14-epi-analogs on the interaction VDR-DRIP205. COS-1 cells were transfected with pVPVDR, pMDRIP205, and the pG5CAT reporter and treated with 1,25-(OH)$_2$D$_3$, with TX522 and TX527 at the indicated doses, or with vehicle. CAT accumulation was normalized to total protein content and expressed as relative CAT amounts (RCA). A representative experiment of three independent experiments is shown. Data shown are the mean ± S.E.M. of triplicate samples. *, RCA significantly different from RCA for 1,25-(OH)$_2$D$_3$-treated samples; $p < 0.05$ according to Fisher's LSD multiple-comparison test.
shifts in EC50 values for TX522 and TX527 after addition of VID400 were only 2.5- and 1.5-fold, respectively.

**Crystal Structure of VDR-TX522.** To obtain crystals of the hVDR LBD complexes, we used a hVDR LBD mutant lacking 50 residues in the loop connecting helices H2 and H3. The same construct was previously used to solve the structure of the hVDR LBD bound to 1,25-(OH)2D3 and to several synthetic ligands (Rochel et al., 2000; Tocchini-Valentini et al., 2001, 2004). This mutant has the same biological properties (binding, transactivation in several cell lines, heterodimerization) as the hVDR LBD wild-type (Rochel et al., 2001). The crystals were obtained in similar conditions and were isomorphous. The structure of VDR-TX522 has been refined at a resolution of 1.9 Å. The experimental data and refinement statistics are summarized in Table 1. After refinement of the protein alone, the map shows an unambiguous electron density where the ligand fits.

The hVDR LBD complexes adopted the canonical conformation of all previously reported agonist-bound nuclear receptor LBDs with 12 to 13 α-helices organized in a three-layered sandwich. In all the structures of hVDR bound to agonist ligands, a single conformation of the complex was observed. The position and conformation of the activation helix H12 was strictly maintained. The ligands adopted the same orientation in the pocket (Fig. 7). An adaptation of their conformation was observed to maintain the hydrogen bonds forming the anchoring points. Compared with the structure of hVDR-1,25-(OH)2D3 complex, the atomic models of hVDR bound to TX522 show root-mean-square deviations of 0.39 Å on all atoms. The ligand is buried in the predominantly hydrophobic pocket that is conserved in all complexes. The sizes of the ligands are 381 Å3 and 374 Å3 for 1,25-(OH)2D3 and TX522, respectively. The volume of the ligand binding cavity is 660 Å3, and the two ligands occupy 57% of the pocket.

The interactions between the ligands and the receptor involve hydrophobic contacts and electrostatic interactions. The A and secoB rings present conformations similar to those of the natural ligand (Fig. 7). Because of the epi-configuration of C14, the CD rings are shifted by 0.5 Å. The distance between the C12 atom of TX522 and that of 1,25-(OH)2D3 is 0.5 Å. Because of the triple bond of the side chain and the reversed configuration of C14, the C21 atom is shifted by 0.4 Å. The distance between the 1-hydroxy and the 25-hydroxy groups varies from 13.3 Å for TX522 to 13.0 Å for 1,25-(OH)2D3 complex. All the residues forming the binding pocket adopt the same conformation as in the VDR-1,25-(OH)2D3 structure. All contacts between the ligand and the

### TABLE 1

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**RMSD**, root mean square deviation.
proteins observed in the VDR-1,25-(OH)₂D₃ complex. The C12 shift induces a closer contact of this atom to Val300 (H6) in the VDR-TX522. Because of its rigidity, the side chain of TX522 takes another pathway in the pocket and makes an additional contact with the CD1 atom of Ile268 (H5) at 3.7 Å of C22 atom instead of 4.3 Å for 1,25-(OH)₂D₃ (Fig. 8).

**Discussion**

Ever since the discovery of the antiproliferative and prodifferentiation action of 1,25-(OH)₂D₃ in the 1980s, efforts have been made to develop superagonistic analogs of 1,25-(OH)₂D₃ with a dissociation between the antiproliferative effect and the calcemic side effects (Bouillon et al., 2003). The two 14-epi-analogs TX522 and TX527 display markedly enhanced antiproliferative potencies coupled to reduced calcemic effects. Our previous attempts to unravel the superagonistic profile of these two analogs at the level of their molecular mode of action have demonstrated that the superagonism is not caused by enhanced binding to the VDR, by differences at the level of heterodimerization between ligand-bound VDR and RXR, or by enhanced binding of the VDR-RXR heterodimer to vitamin D response elements (Verlinden et al., 2001). These findings led to the hypothesis that the specific profile of these analogs might be explained at the next level of transcriptional regulation by 1,25-(OH)₂D₃: the recruitment of coactivator molecules. Recent studies describe the effect of different analogs of 1,25-(OH)₂D₃ on the interaction of VDR with coactivators (Takeyama et al., 1999; Issa et al., 2002). The present study investigates the 14-epi-ana-logs TX522 and TX527 and their influence on VDR-mediated recruitment of coactivator molecules. Recent studies describe the effect of different analogs of 1,25-(OH)₂D₃ on the interaction of VDR with coactivators (Takeyama et al., 1999; Issa et al., 2002). The present study investigates the 14-epi-analogs TX522 and TX527 and their influence on VDR-mediated recruitment of the coactivators TIF2, SRC-1, and DRIP205. A stronger induction of the interaction between VDR and each of the three coactivators was observed in cells treated with TX522 or TX527 compared with those treated with 1,25-(OH)₂D₃. For TIF2, the higher induction was most obvious at 10⁻⁹ and 10⁻¹⁰ M concentrations of the different ligands, for SRC-1 at 10⁻¹⁰ and 10⁻¹¹ M concentrations, and for DRIP205 at 10⁻⁸, 10⁻⁹, and 10⁻¹⁰ M concentrations. On average, at least 10-fold lower doses than for 1,25-(OH)₂D₃ are sufficient for the two analogs to induce equivalent VDR-coactivator interactions. Likewise, a recent study with the superagonistic analog 2-methylene-19-nor-(20S)-1,25-(OH)₂D₃ demonstrated this analog to be significantly more potent in inducing VDR interaction with SRC-1 and DRIP205 in a mammalian two-hybrid assay (Yamamoto et al., 2003). ZK159222, a 25-carboxylic ester analog of 1,25-(OH)₂D₃ with antagonistic action, on the other hand, was found to be un-

![Fig. 7. Comparison of the ligand conformations of 1,25-(OH)₂D₃ (blue) and TX522 (green) in their VDR ligand binding pockets.](image)

![Fig. 8. Superposition of the VDR-1,25-(OH)₂D₃ (yellow) and VDR-TX522 (blue) complexes. The view is restricted to the ligand binding pocket. Only residues closer than 4.0 Å are shown. The two residues Val300 and Ile268 making different contacts with TX522 are highlighted in red. The ligands 1,25-(OH)₂D₃ and TX522 are shown in stick representation, with carbon and oxygen atoms in gray and red, respectively. The hydrogen bonds are shown as red dashed lines.](image)
assay with pVPVDR and pMDRIP205. In samples treated with 1,25-(OH)_2D_3 but not in samples treated with TX522 or TX527, VDR-DRI2P205 interaction increased 3-fold after addition of VID400. Despite this increase, the difference in potency to induce VDR-DRI2P205 interaction between 1,25-(OH)_2D_3 and the two analogs remained significant. Growth inhibition assays on MCF-7 breast cancer cells treated with a combination of 1,25-(OH)_2D_3, TX522, TX527, and VID400 yielded comparable results. Addition of VID400 caused a 10-fold decrease in the EC_{50} value for 1,25-(OH)_2D_3, whereas the decreases in the EC_{50} value for TX522 and TX527 were only 2.5- and 1.5-fold. The resulting EC_{50} value for 1,25-(OH)_2D_3, however, remained clearly higher than the EC_{50} values for TX522 and TX527. These findings demonstrate that differences in VDR-coactivator interaction could not be completely accounted for by different rates of metabolism through the 24-hydroxylase-pathway between 1,25-(OH)_2D_3 and the two analogs TX522 and TX527.

Another level at which the superagonistic profile of TX522 and TX527 can be explained would be the docking of the analogs in the LBD of the VDR and the possible subsequent conformational changes at the carboxyl terminal helix 12 (H12) of the VDR, which might influence coactivator binding. Upon ligand binding in the LBP of the LBD, H12, much like a mouse-trap, closes off the pocket and provides a surface upon which coactivator molecules can bind through the LXXLL-motif in their nuclear receptor interacting domains. Superagonistic action of analogs, such as that of the 20-epi-analogs, for instance, would then originate from conformational changes in the VDR-LBD that occur after docking of the analog in the LBP and that result in selective VDR-coactivator interaction and in an increased resistance of the VDR to proteolytic digestion. However, recent crystallographic studies of the VDR complexed to the natural ligand as well as to the superagonistic 20-epi-analogs MC1288 and KH1060 have clearly demonstrated that there is almost no difference in protein conformation between the LBD with 1,25-(OH)_2D_3 and the LBD with MC1288 or KH1060. (Rochel et al., 2000; Tocchini-Valentini et al., 2001). These findings support the idea of one single agonistic conformation of the VDR-LBP to which the different ligands adapt. A most recent study, however, demonstrates that, when complexed to a Gemini-analog with two side chains at C20 and an increase in volume of approximately 25% compared with 1,25-(OH)_2D_3, the conformation of the zebrafish VDR-LBD (the LBP to which the different ligands adapt) is 3-fold after addition of VID400. Despite this increase, the difference in protein conformation between the LBD with 1,25-(OH)_2D_3 and the LBD with VID400 yielded comparable results. Addition of VID400 caused a 10-fold decrease in the EC_{50} value for 1,25-(OH)_2D_3, whereas the decreases in the EC_{50} value for TX522 and TX527 were only 2.5- and 1.5-fold. The resulting EC_{50} value for 1,25-(OH)_2D_3, however, remained clearly higher than the EC_{50} values for TX522 and TX527. These findings demonstrate that differences in VDR-coactivator interaction could not be completely accounted for by different rates of metabolism through the 24-hydroxylase-pathway between 1,25-(OH)_2D_3 and the two analogs TX522 and TX527. Growth inhibition assays on MCF-7 breast cancer cells treated with a combination of 1,25-(OH)_2D_3, TX522, TX527, and VID400 yielded comparable results. Addition of VID400 caused a 10-fold decrease in the EC_{50} value for 1,25-(OH)_2D_3, whereas the decreases in the EC_{50} value for TX522 and TX527 were only 2.5- and 1.5-fold. The resulting EC_{50} value for 1,25-(OH)_2D_3, however, remained clearly higher than the EC_{50} values for TX522 and TX527. These findings demonstrate that differences in VDR-coactivator interaction could not be completely accounted for by different rates of metabolism through the 24-hydroxylase-pathway between 1,25-(OH)_2D_3 and the two analogs TX522 and TX527.

In conclusion, this study shows that the enhanced potency to induce VDR-coactivator interactions is the basis for the superagonistic profile of TX522 and TX527.


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