Two Novel 14-Epi-Analogues of 1,25-Dihydroxyvitamin D₃ Inhibit the Growth of Human Breast Cancer Cells in Vitro and in Vivo

Lieve Verlinden,² Annemieke Verstuyf,² Mark Van Camp, Suzanne Marcelis, Katrien Sabbe, Xu-Yang Zhao, Pierre De Clercq, Maurits Vandewalle, and Roger Bouillon³

ABSTRACT

The biological activity of two novel 14-epi-analogues of 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), is described. Both analogues were at least 10 times more potent than 1,25(OH)₂D₃ in inhibiting in vitro cell proliferation and had much lower calcemic effects than 1,25(OH)₂D₃. Treatment with 1,25(OH)₂D₃, TX 522, or TX 527 in vitro was accompanied by an accumulation of cells in the G₁ phase of the cell cycle. Protein levels of cyclin C and cyclin D1 in in vitro cultures of MCF-7 cells were downregulated to 50 and 30%, respectively, of control levels at 72 and 120 h after stimulation. Protein levels of p21 and p27 at 72 h were significantly enhanced by 1,25(OH)₂D₃ and TX 522 but surprisingly not by TX 527. The inability of TX 527 to up-regulate p21 seemed to be cell type specific because p21 was induced in other cell types. Diminished phosphorylation of the retinoblastoma protein after treatment with 1,25(OH)₂D₃, TX 522, or TX 527 may ultimately contribute to the growth inhibition caused by these compounds. According to the data presented, the induction of apoptosis seemed not to be a major mechanism responsible for the growth-inhibitory effect of 1,25(OH)₂D₃ and analogues. Both 14-epi-analogues significantly retarded tumor progression (40% reduced compared with control mice) in an in vivo model of MCF-7 breast cancer cell lines established in nude mice. In conclusion, these novel analogues have the eligible profile to be tested as therapeutic agents for the treatment of hyperproliferative diseases such as breast cancer.

INTRODUCTION

1,25(OH)₂D₃,⁴ the active metabolite of vitamin D₃, is known to inhibit the proliferation of breast cancer cells in vitro (1–3) and to slow the progression of breast carcinoma in vivo (4). The major drawback related to the use of this compound is the calcemic effect, which prevents the application of pharmacological doses. A large number of analogues of 1,25(OH)₂D₃ displaying a clear dissociation of antiproliferative and calcemic effects have been reported (5). Structural modifications of the A-ring and side chain have been studied intensively, and most CD-ring modifications reported until now are substitutions on C11 (6) and modifications of C13/C18 (7, 8), C14 (9, 10), and C16 (11). In the present study, the in vitro and in vivo antiproliferative effects of two novel 14-epi-1,25(OH)₂D₃ analogues were examined.

The effects of 1,25(OH)₂D₃ are mainly mediated through binding to the VDR. Once bound, these nuclear receptors interact with specific DNA sequences as dimeric transcription factors that act as either activators or repressors of target genes (12). However, the knowledge of the precise molecular mechanism underlying the growth-inhibitory effect of 1,25(OH)₂D₃ and its analogues remains fragmentary. 1,25(OH)₂D₃ has a cell cycle-specific effect leading to accumulation of cells in G₁. Therefore, regulation of the activity of complexes between cyclins and cdk may be responsible for the antiproliferative effects (2, 3). Moreover, 1,25(OH)₂D₃ is able to up-regulate its own receptor expression, which may contribute to its resulting biological function (13).

This study presents two 14-epi-analogues of 1,25(OH)₂D₃ that are able to inhibit the proliferation of ER-positive MCF-7 breast cancer cells at 10-fold lower concentrations than 1,25(OH)₂D₃. In addition, both analogues are, unlike 1,25(OH)₂D₃, able to retard the progression of breast tumors in a nude mouse model with limited effects on calcemic parameters, pointing to the possible therapeutic application of these analogues in breast cancer. The contribution of programmed cell death to the growth-inhibitory effect of 1,25(OH)₂D₃, or analogues is only marginal, according to the data presented here. Therefore, gene regulation by these compounds has been studied in more detail.

MATERIALS AND METHODS

1,25(OH)₂D₃ and Analogues. 1,25(OH)₂D₃ was a gift of M. R. Uskokovic (Hoffman-La Roche, Nutley, NY) and J. P. van de Velde (Duphar, Weesp, The Netherlands). The analogues, KS 532, SDB 112/TX 522, and ZXY 1106/TX 527, were originally synthesized by M. Vandewalle and P. De Clercq from the University of Gent (Table 1). The 14-epi-analogues, TX 522 and TX 527, were obtained from Théramex S.A. (Monaco Cedex, Monaco). [³H]1,25(OH)₂D₃ (specific activity, 180 Ci/mmol) was purchased from Amersham (Buckinghamshire, United Kingdom).

Affinity to VDR and DBP. The affinity of 1,25(OH)₂D₃ and its analogues to the vitamin D receptor and binding to human DBP was determined as described previously (14).

Cell Proliferation Assays. As a measure of cellular proliferation, [³H]thymidine incorporation of MCF-7, T47D, and SK-BR-3 (ATCC, Rockville, MD) and keratinocytes was determined after a 72-h incubation period with various concentrations of 1,25(OH)₂D₃ or analogues (14). For cell cycle analysis, cells (10⁶) were trypsinized, washed, fixed, and stained with propidium iodide (Sigma Chemical Co., St. Louis, MO) as described previously (2). Samples were analyzed with a FACSORT flow cytometer (Becton Dickinson, Lincoln Park, NJ) using the CellFIT program.

Cell Differentiation Assays. Differentiation of HL-60 cells (ATCC) was measured by the nitro blue tetrazolium reduction assay after a 72-h incubation period in presence of 1,25(OH)₂D₃ or analogues or vehicle. Differentiation of MG-63 osteosarcoma cells (ATCC) was assessed by measuring osteocalcin production with a radioimmunoassay after a 72-h incubation period (14).

Detection of Apoptosis. Regulation of the expression of apoptosis regulators genes, bcl-2, bcl-x₁, bcl-xl, bax, and bcl-2, was measured using real-time quantitative RT-PCR. Total RNA was isolated by using the total RNA extraction kit from Roche (Palo Alto, CA). One µg of RNA was reverse transcribed, and PCR reactions on the resulting cDNA were performed in the ABI-prism 7700 sequence detector (Perkin-Elmer/Applied Biosystems, Foster City, CA). PCR primers and fluorogenic probes (6-carboxy-fluorescein as reporter and 6-carboxy-tetramethylrhodamine as quencher dye) for bcl-2, bcl-x₁, bax, and β-actin were purchased from Perkin-Elmer.

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⁴ The abbreviations used are: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; VDR, vitamin D receptor; cdk, cyclin-dependent kinase; KS 532, 19-nor-14-epi-1,25(OH)₂D₃; SDB 112/TX 522, 19-nor-14-epi-23-yne-1,25(OH)₂D₃; ZXY 1106/TX 527, 19-nor-14,20-bisepi-23-yne-1,25(OH)₂D₃; DBP, vitamin D binding protein; ATCC, American Type Culture Collection; ER, estrogen receptor; Rb, retinoblastoma; RT-PCR, reverse transcriptase-PCR.

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Summary of the *in vitro* and *in vivo* effects of 14-epi-analogues of 1,25(OH)2D3. The binding of 1,25(OH)2D3 and its 14-epi-analogues to VDR and human DBP is expressed by their dissociation constants. The antiproliferative effects of 1,25(OH)2D3 and analogues were measured on MCF-7 cells and keratinocytes and are expressed as the concentrations required for the half-maximal inhibition of [3H]thymidine incorporation. The prodifferentiating effects were determined using HL-60 cells (nitro blue tetrazolium 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)2D3 and its 14-epi-analogues was determined in mice by i.p. 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.

### Binding studies

<table>
<thead>
<tr>
<th>Compound</th>
<th>VDR K&lt;sub&gt;D&lt;/sub&gt;</th>
<th>DBP K&lt;sub&gt;D&lt;/sub&gt;</th>
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<tr>
<td>1α,25(OH)2D3</td>
<td>(1.1 ± 0.5) × 10&lt;sup&gt;−10&lt;/sup&gt; M</td>
<td>(5.1 ± 1.3) × 10&lt;sup&gt;−8&lt;/sup&gt; M</td>
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<tr>
<td>KS 532</td>
<td>(4.8 ± 1.0) × 10&lt;sup&gt;−10&lt;/sup&gt; M</td>
<td>(7.3 ± 1.2) × 10&lt;sup&gt;−8&lt;/sup&gt; M</td>
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<tr>
<td>TX 522</td>
<td>(5.3 ± 1.5) × 10&lt;sup&gt;−10&lt;/sup&gt; M</td>
<td>(1.0 ± 0.5) × 10&lt;sup&gt;−9&lt;/sup&gt; M</td>
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<tr>
<td>TX 527</td>
<td>(4.3 ± 0.6) × 10&lt;sup&gt;−10&lt;/sup&gt; M</td>
<td>(4.2 ± 1.7) × 10&lt;sup&gt;−9&lt;/sup&gt; M</td>
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### *In vitro* studies

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<tr>
<th>Compound</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; for NBT reduction</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; for osteocalcin</th>
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<tr>
<td>1α,25(OH)2D3</td>
<td>(3.5 ± 0.2) × 10&lt;sup&gt;−8&lt;/sup&gt; M</td>
<td>(3.5 ± 1.0) × 10&lt;sup&gt;−10&lt;/sup&gt; M</td>
</tr>
<tr>
<td>KS 532</td>
<td>(6.6 ± 1.9) × 10&lt;sup&gt;−8&lt;/sup&gt; M</td>
<td>(5.5 ± 1.9) × 10&lt;sup&gt;−8&lt;/sup&gt; M</td>
</tr>
<tr>
<td>TX 522</td>
<td>(6.2 ± 1.9) × 10&lt;sup&gt;−9&lt;/sup&gt; M</td>
<td>(1.5 ± 0.9) × 10&lt;sup&gt;−7&lt;/sup&gt; M</td>
</tr>
<tr>
<td>TX 527</td>
<td>(1.6 ± 1.1) × 10&lt;sup&gt;−9&lt;/sup&gt; M</td>
<td>(1.1 ± 1.4) × 10&lt;sup&gt;−10&lt;/sup&gt; M</td>
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### *In vivo*

| Compound     | 
|--------------|----------------|
| 1α,25(OH)2D3 | (1.8 ± 0.3) × 10<sup>−9</sup> M |
| KS 532       | (4.1 ± 1.4) × 10<sup>−9</sup> M |
| TX 522       | (3.0 ± 2.4) × 10<sup>−10</sup> M |
| TX 527       | (8.4 ± 1.8) × 10<sup>−10</sup> M |

### Calcium serum dose in μg/kg/day

- 0.1
- 30
- 40
- 10

### Expression of VDR and ER.

The effect of 1,25(OH)2D3 on the transcription of VDR and ER-α was quantified using real-time quantitative RT-PCR as described above. For VDR, primer sequences were TGGCGTTTCCCCTTGAATCATC and TGCGTTACTGATGAGGAAAG. For ER-α, primer sequences were GGACGCGACGATGCGTGC and TGCGTTACTGATGAGGAAAG. For the probe was TGGCGTTTCCCCTTGAATCATC and TGCGTTACTGATGAGGAAAG. Expression of ER-α was used to normalize expression of bcl-2, bcl-xl, bax, bcl-2, bcl-xl, bax, bcl-2, bcl-xl, bax, bcl-2, bcl-xl, bax.

### In Vivo Calcaemic Activity of 1,25(OH)2D3 and Analogues.

NMR1 mice were obtained from the Proefdierencentrum of Leuven (Belgium) and fed with a vitamin D-replete diet (0.2% calcium, 1% phosphorus, and 2000 units vitamin D/kg; Hope Farms, Woerden, the Netherlands). The hypercalcemic effect of the analogues was tested by daily s.c. injections of serial dilutions of 1,25(OH)2D3 or analogues for 7 consecutive days. Serum and urinary calcium were measured as calceimic parameters using a commercially available kit (Sigma Diagnostic).

### In Vivo Antiproliferative Activity of 1,25(OH)2D3 and Analogues.

An *in vivo* breast cancer model was set up by injecting s.c. a mixture of 5 × 10<sup>6</sup> MCF-7 cells and Matrigel (Becton Dickinson) in the flanks of female nude mice, which were given estrone (10 mg/l; Sigma) in their drinking water. After 3 months, tumors were cut in small pieces (2 mm<sup>3</sup>) and could be used for transplantation. Part of the tumor pieces was used to maintain a continuous *in vivo* breast cancer model. For experiments, tumor pieces were transplanted in 5-week-old female mice, which were given estrone in their drinking water and kept on a calcium-poor diet (<0.2% calcium). Treatment with 1,25(OH)2D3 or analogues, diluted in arachis oil, was started 4 days after tumor transplantation and was given every other day by i.p. injections. Tumor volume was measured twice weekly using a caliper and calculated using the formula (π<sup>a</sup>b<sup>2</sup>)<sup>2</sup>/(4a), where a is the length of tumor; b, width of tumor. The total number of tumors evaluated per treatment group was at least 44 (divided over three independent experiments).

The number of mitotic figures present in tumors from control and treated mice was also determined. Therefore, tumors were cut into 4-μm slices, which were stained with H&E. Slices were examined at a 400-fold magnification and overlaid with a raster. For each slice, the number of mitotic figures was counted in 10 different fields, and the average count was used for statistical analysis.

![Diagram of binding studies](https://example.com/diagram)
evaluation. At least 10 tumors of each treatment group were analyzed in this way. The calcemic effects of the compounds were evaluated by measuring serum and bone calcium content, and body weight was monitored during the whole treatment period.

**Statistics.** Data were first analyzed with the ANOVA for 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. \( P < 0.05 \) 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).

**RESULTS**

The analogues of 1,25(OH)\(_2\)D\(_3\) described here are all epimers at C14 (Table 1). In the natural CD-ring configuration, the C18 methyl and C14 hydrogen are trans oriented. Reorientation of the hydrogen on C14 into the cis configuration [14-epi-1,25(OH)\(_2\)D\(_3\)] decreased the affinity to VDR and DBP but also markedly diminished its calcemic effect. This effect may be explained by the tendency of 14-epi-1,25(OH)\(_2\)D\(_3\) to isomerize into the previtamin configuration (9, 10). This isomerization is prohibited by deleting the 19-methylene group on the A-ring. Yet, the calcemic activity of these compounds remained very low. The biological activity of the 19-nor-14-epi-1,25(OH)\(_2\)D\(_3\) analogue (KS 532) was more or less comparable with the activity of the parent compound, depending on the cell type investigated. The 23-yne modification, known to be successful in the parent hormone for its biological activity, was introduced in the side chain of KS 532, resulting in the analogue 19-nor-14-epi-23-yne-1,25(OH)\(_2\)D\(_3\) (TX 522). Introduction of this triple bond increased the affinity to VDR, whereas the binding affinity to DBP was decreased. However, the antiproliferative and prodifferentiating capacity was strongly enhanced [2–17 times more potent in comparison with 1,25(OH)\(_2\)D\(_3\)]. The 20-epimer of TX 522, 19-nor-14,20-bisepi-23-yne-1,25(OH)\(_2\)D\(_3\) (TX 527), was even more potent than TX 522 in inhibiting cellular proliferation or inducing differentiation, but its calcemic activity was also increased (still 50–100 times lower than the parent hormone). Because the analogues TX 522 and TX 527 are potent inhibitors of cell proliferation and inducers of cell differentiation with minimal calcemic effects, their effects on breast cancer cell growth has been studied in further detail, and their mode of action was compared with that of 1,25(OH)\(_2\)D\(_3\).

**Growth Inhibition of Breast Cancer Cells Associated with G\(_1\) Arrest.** Proliferation of the ER-positive MCF-7 breast cancer cells was dose-dependently inhibited by 1,25(OH)\(_2\)D\(_3\) and was characterized by an EC\(_{50}\) of approximately 5 \( \times \) 10\(^{-8}\) M (Fig. 1A). The EC\(_{50}\) of both 14-epi analogues was, however, 10 times lower than that of the parent molecule. Cell cycle analysis revealed that a 72-h treatment with 10\(^{-8}\) M 1,25(OH)\(_2\)D\(_3\) caused a significant increase in the percentage of cells in the G\(_1\) phase (64% versus 55% in control cultures; \( P < 0.01 \)), whereas the proportion of S-phase cells decreased (14% versus 24% in control cultures; \( P < 0.01 \); Fig. 1B). This shift in cell cycle distribution was more pronounced when MCF-7 cells were treated with 10\(^{-8}\) M TX 522 or TX 527; 75% of cells were found in the G\(_1\) phase of the cell cycle (\( P < 0.01 \)), and the percentage of actively proliferating cells was decreased to 6% of the total population (\( P < 0.01 \); Fig. 1B). A 10-fold higher analogue concentration (10\(^{-7}\) M) did not induce a further shift in cell cycle distribution. The cell cycle distribution of MCF-7 cells that were treated with 10\(^{-7}\) M 1,25(OH)\(_2\)D\(_3\) was similar to that of cultures that were incubated with 10\(^{-8}\) M TX 522 or TX 527 (data not shown).

**In Vivo Activity of 1,25(OH)\(_2\)D\(_3\) and Analogues.** The antiproliferative capacity of 1,25(OH)\(_2\)D\(_3\) and its analogues was also studied in an in vivo breast cancer model established in female nude mice. 1,25(OH)\(_2\)D\(_3\) was given at the highest tolerable dose in these mice (5 \( \mu \)g/kg/2 days), but this dose was unable to affect tumor growth (Fig. 2A). A 36 and 45% decrease in tumor volume at the end of the experiments (26–28 days after transplantation) was observed when mice were treated with TX 522 (80 \( \mu \)g/kg/2 days) and TX 527 (25 \( \mu \)g/kg/2 days), respectively. Tumors of control and treated mice were sliced and stained, and the number of mitotic figures was determined. The average number of mitotic figures present in a counting field of tumor slices from control mice was 76 \( \pm \) 7 (Fig. 2B). Treatment with 1,25(OH)\(_2\)D\(_3\) did not affect the number of mitotic figures. The number of mitotic figures was substantially decreased in tumors of mice treated with TX 522 (24 \( \pm \) 5; \( P < 0.01 \)) or TX 527 (22 \( \pm \) 8; \( P < 0.01 \)).

None of the applied analogues had a significant effect on body weight during the experiment (data not shown). However, a significant increase in serum calcium (10.6 mg/dl compared with 9.3 mg/dl in control mice; \( P < 0.01 \)) and decrease in tibia calcium (4.1 mg compared with 7.6 mg in control animals; \( P < 0.01 \)) was noticed when mice were treated with 5 \( \mu \)g/kg 1,25(OH)\(_2\)D\(_3\) every other day.
reach significance for treatment with $10^{-7}$ M 1,25(OH)$_2$D$_3$, whereas it did for treatment with $10^{-7}$ M TX 522 and TX 527 (from 3% in control cultures to 7% in cultures treated with TX 522 or TX 527). The same tendency was observed after a 120-h incubation period.

(d) Neither 1,25(OH)$_2$D$_3$ nor these 14-epi-analogues were able to induce DNA strand breaks as investigated by the terminal deoxynucleotidyl transferase-mediated nick end labeling method (data not shown).

(e) Finally, no significant differences could be found in the number of cells displaying morphological characteristics of programmed cell death. Moreover, no major differences in the degree of programmed cell death could be demonstrated in the tumors from nude mice treated with vehicle, 1,25(OH)$_2$D$_3$, TX 522, or TX 527 (data not shown).

As an internal control for the in vitro apoptosis assays, MCF-7 cells were treated with a combination of 1,25(OH)$_2$D$_3$ ($10^{-7}$ M) and TNF-α (10 ng/ml). Caspase activity in lysates of these cells was doubled after 24 h of treatment. Moreover, 20% of MCF-7 cells were Annexin

(Fig. 3). The analogue TX 522 had no effect on serum calcium, whereas it decreased the calcium content of the tibia, albeit not significantly (6.1 mg). The analogue TX 527, applied at a 5-fold higher dose than 1,25(OH)$_2$D$_3$ (25 μg/kg/2 days), significantly increased the serum calcium content (10.4 mg/dl; P = 0.014) and induced a slight decrease in tibia calcium content.

**Induction of Apoptosis.** Because not every cell type displays all classical features of apoptotic cell death, different protocols for analyzing the possible induction of programmed cell death were used:

(a) In a first approach, no significant alterations in the expression of bcl-2- and bax-related genes were found in MCF-7 cells that were incubated with 1,25(OH)$_2$D$_3$ or analogues ($10^{-7}$ M) at any time point investigated (24, 48, 72, and 120 h; data not shown).

(b) In a second approach, no induction of the effector caspase-3 could be detected in lysates of MCF-7 cells that were treated with vehicle, 1,25(OH)$_2$D$_3$, or its analogues during different time periods (24, 48, 72, and 120 h; data not shown).

(c) The proportion of Annexin V-positive cells was slightly increased in MCF-7 cells after a 72-h incubation period with either $10^{-7}$ M 1,25(OH)$_2$D$_3$ or analogues (Fig. 4). This increase did not
Next, changes in the expression of cell cycle regulators were investigated. As representatives of the INK4 family, protein levels of p15 and p19 have been measured. However, no induction of those proteins could be observed after a 72-h incubation period with 10^{-7} M 1,25(OH)_{2}D_{3} or TX 522, respectively (P < 0.01). Again, the analogue TX 527 was not able to induce an enhanced production of p27. Therefore, p27 protein levels were also investigated in the other breast cancer cell lines. However, no induction of p27 by 1,25(OH)_{2}D_{3} or any other of the 14-epi-analogues, the amount of highly phosphorylated Rb (ppRb) was decreased 5-fold (Fig. 5A). Additionally, the evolution of p27 was investigated after treatment with 1,25(OH)_{2}D_{3} or TX 527 during 72 h (Fig. 5B). A significant 4- and 6-fold increase in p21 protein production was observed when MCF-7 cells were treated with 1,25(OH)_{2}D_{3} and TX 522, respectively (P < 0.01). Surprisingly, the level of p21 protein after a 72-h treatment with the analogue TX 527 was not significantly different from that in control cultures (1.5-fold induction compared with control).

Therefore, a time course experiment was set up, and p21 protein levels were measured at different time points during a 120-h incubation period. The p21 protein level was approximately constant and never significantly different from that in control cultures (data not shown). Thereafter, p21 protein levels, after treatment with 1,25(OH)_{2}D_{3} or analogues, were also measured in other cell types to investigate whether the same discrepancy in induction of p21 was found. A 3-fold up-regulation was found in normal keratinocytes that had been incubated for 48 h with 10^{-7} M 1,25(OH)_{2}D_{3} and a 4-fold up-regulation in the ER-negative SK-BR-3 cell line. Both TX 522 and TX 527 enhanced p21 production to the same extent as 1,25(OH)_{2}D_{3} (data not shown). Additionally, the evolution of p27 was investigated after treatment with 1,25(OH)_{2}D_{3} (Fig. 5B). p27 protein levels were also significantly enhanced by a 72-h incubation period with 10^{-7} M 1,25(OH)_{2}D_{3} or with TX 527; however, the degree of induction was lower than that of p21 (P < 0.01). Again, the analogue TX 527 was not able to induce an enhanced production of p27. Therefore, p27 protein levels were also investigated in the other breast cancer cell lines. However, no induction of p27 by 1,25(OH)_{2}D_{3} or any other of the analogues was found, either in the SK-BR-3 or in the T47D cell line (data not shown).

Because active complexes between cyclins and cdks phosphorylate the Rb protein so that transcription factors are released (15, 16), protein levels of Rb after incubation with 1,25(OH)_{2}D_{3} were measured (Fig. 5C). After a 72-h incubation period in the presence of 10^{-7} M 1,25(OH)_{2}D_{3} or these 14-epi-analogues, the amount of highly phosphorylated Rb (ppRb) was decreased 5-fold (P < 0.01).

Expression of VDR and ER. The transcription levels of VDR and ER were investigated using RT-PCR (Fig. 6). Transcription of VDR was doubled after a 72-h incubation period with 10^{-7} M 1,25(OH)_{2}D_{3} (P < 0.01). The analogues TX 522 and TX 527, applied at 10^{-7} M, caused a 3-fold up-regulation of VDR expression levels in MCF-7 cells [significantly different from control cultures but not from those treated with 1,25(OH)_{2}D_{3}]. Protein levels of VDR were found to be up-regulated to the same extent as the mRNA after treatment with 1,25(OH)_{2}D_{3} or analogues (data not shown). Transcription levels of ER were, however, not altered after a 72-h incubation period in the presence of 1,25(OH)_{2}D_{3} or analogues (data not shown).
DISCUSSION

The current study presents novel analogues of 1,25(OH)₂D₃ that are characterized by the epi configuration at C14. Previously published analogues with a 14-epi configuration have only minor calcemic effects, which might be partially explained by their tendency to isomerize to the previtamin configuration (9, 10). More stable compounds arise by deleting the C19 methyl group because transition to the previtamin configuration is then impossible. The antiproliferative and prodifferentiative capacity of these 14-epi-19-nor analogues was greatly enhanced by introducing modifications in the side chain. Interestingly, the calcemic activity of these compounds was not (TX 522) or only marginally (TX 527) affected by these side chain modifications. As a result, the compounds TX 522 and TX 527 are examples of 1,25(OH)₂D₃ analogues that show a clear dissociation of antiproliferative and calcemic effects. The antiproliferative activities of TX 522 and TX 527 were more extensively investigated in MCF-7 breast cancer cells. Both 14-epi-analogues were able to inhibit the proliferation of these breast cancer cells at 10-fold lower concentrations than the parent hormone in vitro. Moreover, treatment with either one of these 14-epi-analogues was able to significantly retard tumor progression in an in vivo breast cancer model without major effects on calcemic parameters for TX 522. Histological examination of tumors failed to demonstrate any effect on cell differentiation or programmed cell death. Thus, it is hypothesized that tumor reduction in treated animals is attributable to either direct growth inhibition of the cancer cells or to indirect effects on tumor angiogenesis (17). The possible mechanisms for the antiproliferative effects of 1,25(OH)₂D₃ were further investigated in in vitro experiments. 1,25(OH)₂D₃ and both 14-epi-analogues enhanced the transcription of the VDR coding gene and increased its protein levels. This finding is in agreement with previous reports (13, 18) and suggests that this up-regulation of VDR may enhance the actions of the compound and enhance its subsequent biological effects. Cell cycle analysis demonstrated an accumulation of cells in the G₁ phase after treatment with 1,25(OH)₂D₃ or analogues. A family of cdks, the activity of which is regulated through binding of cyclin regulatory molecules, drives progression through the cell cycle (15, 16). Therefore, regulation of cyclin abundance constitutes one way of affecting the activity of the cyclin/cdk complexes (19, 20). Cyclin D1 is frequently amplified in breast cancer tumors, and its expression is both necessary and sufficient for the G₁ phase progression in breast cancer cells (19). Protein levels of cyclin D1 were 3-fold reduced after a 120-h treatment period with 1,25(OH)₂D₃, TX 522, or TX 527. Because of the late time point of modulation of cyclin D1, it is suggested that reduced cyclin D1 levels are not responsible for the onset of growth inhibition by these compounds. However, they may contribute to the diminished growth of MCF-7 cells as cyclin D1 is known to act as a cdk-independent activator of the ER, and it is able to up-regulate ER-mediated transcription (21). Cyclin C, a putative G₁ cyclin, was originally isolated through its ability to rescue a Saccharomyces cerevisiae strain deficient in the production of G₁ cyclins CLN1–3 (22). Unlike for cyclins D1 and E, which were identified using the same approach, there is still little evidence that this cyclin is indeed critical for the G₁-S transition. It has been demonstrated that the cyclin C/cdk8 complex is capable of phosphorylating the COOH-terminal domain of RNA-polymerase II (23). Moreover, cyclin C has been reported to be able to stimulate E₂F and Sp1-mediated transcription, suggesting that it affects a general pathway of transcriptional activation (24). A 2-fold down-regulation of cyclin C protein levels was observed in MCF-7 cells after treatment with these 14-epi-analogues, suggesting that decreased cyclin C levels may contribute to their growth-inhibitory effect.

Cdk-inhibitory proteins can also influence progression through the cell cycle (15, 16). As representatives of the Ink4 family, p15 and p19 have been studied, and their protein levels were found to be unchanged by 1,25(OH)₂D₃ or one of the 14-epi-analogues during a 72-h incubation period. It has been reported earlier that accumulation of cells in the G₁ phase of the cell cycle after stimulation with 1,25(OH)₂D₃ occurs, chronologically consistent with the up-regulation of p21 and p27, which belong to the Cip/Kip family of inhibitors (2, 3). Therefore, it was investigated if p21 and p27 protein levels were equally enhanced by the 14-epi-analogues. The analogue TX 522 up-regulated p21 and p27 to the same extent as the parent hormone. Most surprisingly, the analogue TX 527, which only differs from TX 522 by the stereochemistry of C20, was not able to enhance the protein levels of p21 or p27 at any investigated time point (0–120 h after stimulation). However, this inability to up-regulate these cdk inhibitors seemed to be cell specific because the analogue TX 527 did enhance p21 protein levels in the T47D and SK-BR-3 breast cancer cells and in normal keratinocytes. This observation illustrates the analogue and cell type specificity of the regulation of cell cycle-related genes. Analogue specificity may be partially explained at the biochemical level. Because VDR conformation alters upon binding of the ligand, it is conceivable that interaction with its heterodimerization partner retinoid X receptor and subsequently with coactivators and corepressors will change (12, 25). This can then finally lead to differences in gene transcription. In support of this hypothesis, it is demonstrated via limited protease digestion (data not shown) that the analogue TX 522 induced a less stable VDR conformation compared with 1,25(OH)₂D₃ and TX 527. The cell specificity might essentially be explained in the same way. It may be hypothesized that different coactivator or corepressor molecules prevail in different cell types. The mutual proportion of these different molecules may then influence the composition of the preinitiation complex and affect the regulation of gene transcription. The inability of the analogue TX 527 to enhance protein levels of p21 also questions the critical role of p21 in the growth inhibition caused by 1,25(OH)₂D₃ and its analogues. In a previous report, it is already described that a simultaneous treatment of MCF-7 cells with 1,25(OH)₂D₃ and transforming growth factor-β
neutralizing antibodies completely abolishes the induction of p21, whereas proliferation of these cells is still inhibited (2). Moreover, growth inhibition of squamous cell carcinoma by 1,25(OH)2D3 is reported to be accompanied by a decrease of p21 protein levels both in vitro and in vivo (26). The importance of the p21 protein may also be questioned, because this inhibitor is not enhanced in T47D or in SK-BR-3 cells. It is generally accepted that the ultimate goal of activated cdks is the phosphorylation of the Rb protein, which upon phosphorylation releases transcription factors required for progression to the S phase of the cell cycle (15, 16). The amount of hyperphosphorylated Rb was markedly decreased by 1,25(OH)2D3 and both 14-epi-analougues, suggesting the existence of redundant pathways for phosphorylation of Rb and subsequent growth control.

Programmed cell death may also be involved in growth inhibition by 1,25(OH)2D3 and has been reported to occur in MCF-7 breast cancer (4). In this study, a number of approaches were used to investigate the induction of apoptosis at different chronological stages of the process. No significant changes were found in the transcription of the pro-apoptotic gene bax and the antiapoptotic genes bcl-2 and bcl-xL. Proteins of these apoptosis-related genes can interfere with the release of cytochrome c, which is believed to precede the activation of the caspases (27, 28). Activity of caspase-3, generally involved in the process of apoptotic cell death, was not altered upon treatment with 1,25(OH)2D3 at any of the time points investigated (24, 48, 72, and 120 h after stimulation). There is also no proof for the fragmentation of DNA in MCF-7 cells after stimulation with 1,25(OH)2D3 or any of the 14-epi-analougues. A marginal increase in the number of cells expressing Annexin V is observed in MCF-7 cells that are incubated in the presence of TX 522 or TX 527. Therefore, it is suggested that the contribution of programmed cell death to the growth-inhibitory effects of 1,25(OH)2D3 and the investigated 14-epi-analougues is only marginal.

In conclusion, the 14-epi-analougues TX 522 and TX 527 are potent inhibitors of breast cancer proliferation both in vitro and in vivo. Their selectivity profile based on data obtained in vitro on MCF-7 cells and in vivo calcemic effect (mouse serum calcium levels) exceeds severalfold that of the best analogues of 1,25(OH)2D3 yet published when measured with the same methods in the same laboratory. Therefore, these compounds have the ideal profile to be tested as therapeutic agents for benign (e.g., psoriasis) and malignant (e.g., breast cancer) hyperproliferative diseases.

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