Three Synthetic Vitamin D Analogues Induce Prostate-specific Acid Phosphatase and Prostate-specific Antigen while Inhibiting the Growth of Human Prostate Cancer Cells in a Vitamin D Receptor-dependent Fashion

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ABSTRACT
Numerous studies have indicated that the secosteroid hormone 1α,25-dihydroxyvitamin D₃ protects against the development of clinical prostate cancer (PC). Whether this hormone also has therapeutic potential for patients with advanced PC has not yet been evaluated. Several synthetic vitamin D analogues are now available that have reduced hypercalcemic effects and yet effectively induce differentiation in some cell types. For these reasons, these analogues may be safer and more effective for cancer therapy than the natural hormone. In the current study, 13 such analogues were screened for their abilities to inhibit the growth of PC cell lines. Three of the most consistently effective analogues (Ro 23-7553, Ro 24-5531, and Ro 25-6760) were then chosen for further analysis. Growth studies using clones of the JCA-1 cell line that were transfected with the vitamin D receptor cDNA indicate that the antiproliferative effects of these analogues require vitamin D receptor expression. Furthermore, these three analogues induce the secretion of prostate-specific acid phosphatase and prostate-specific antigen (two markers of the differentiated prostatic phenotype) in the cell line LNCaP. These in vitro studies suggest that Ro 23-7553, Ro 24-5531, and Ro 25-6760 should be further evaluated as therapeutic agents for the treatment of PC.

INTRODUCTION
Once androgens were discovered to regulate the growth, differentiation, and rate of apoptosis in the prostate (1–4), it seemed likely that PC could be effectively controlled or even cured, if total androgen ablation could be achieved in patients. This concept led to the development of numerous synthetic antiandrogens as well as gonadotropin-releasing hormone superagonists that are currently available and in use. Unfortunately, the results from major clinical trials using such compounds indicate that complete androgen ablation extends the survival time of patients with advanced PC by less than 3 months (5). Thus, the search for alternate therapeutic strategies continues. Recently, the efforts of several laboratories have shifted to exploring the promising role of the secosteroid hormone 1,25(OH)₂D₃ in reducing mortality from PC.

Sunlight exposure, even in cloudy cities, provides greater than 70% of the requirement for vitamin D in Caucasians (6) through the conversion of a light-sensitive cholesterol precursor in the skin to the prohormone vitamin D₃ (7). As early as 1937, well before vitamin D had been discovered, it was recognized that the mortality from many visceral cancers was significantly reduced in people who were exposed to the most sunlight (8, 9). More recent epidemiological studies have confirmed this observation in patients with colon cancer (10, 11), breast cancer (12, 13), and PC (14–16) and have attributed the protective effect of the sun specifically to elevated levels of vitamin D₃. More direct evidence was presented by Corder et al. (16), who found that the risk of developing clinical PC increases as serum levels of 1,25(OH)₂D₃ decrease.

It is now apparent that the mechanistic basis for the protective effects of 1,25(OH)₂D₃ on PC resides in the actions of specific nuclear VDRs in prostatic epithelial cells (17–23). When 1,25(OH)₂D₃ is added to the cell culture medium, it is capable of regulating the growth of several established PC cell lines (17–20), as well as primary cultures of benign hyperplastic and malignant PC cells (21). Those PC cell lines with the greatest constitutive VDR expression are also the most sensitive to the effects of 1,25(OH)₂D₃, suggesting the involvement of the VDR. Recent transfection analyses in our laboratory have confirmed that expression of the nuclear VDR is both necessary and sufficient to mediate these effects on growth (22, 23). In addition to the antiproliferative effects, 1,25(OH)₂D₃ also increases...

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3 The abbreviations used are: PC, prostate cancer; 1,25(OH)₂D₃, 1α,25-dihydroxyvitamin D₃; VDR, vitamin D receptor; PSA, prostate-specific antigen; PSAP, prostate-specific acid phosphatase; FBS, fetal bovine serum.
the secretion of prostate-specific antigen in the cell line LNCaP (18) and induces the expression of the catabolic enzyme 24-
hydroxylase in each of the seven PC cell lines tested (19).
Together, the biochemical and epidemiological data suggest that 1,25(OH)2D3 plays an important role in maintaining a state
of differentiation, while suppressing the malignant phenotype of localized PCs.

From these data, it would seem that raising 1,25(OH)2D3
levels in men with PC may have a therapeutic effect. How-
ever, the well-recognized hypercalcemic effect of 1,25(OH)2D3
precludes its widespread use as a chemopreventive or chemotherapeutic agent. For these reasons, intensive efforts
have been directed at the development of synthetic vitamin D analogues that are more potent at inducing cellular differ-
tiation than 1,25(OH)2D3 but have similar or reduced effects
on calcium homeostasis. A number of these synthetic ana-
tergues have been shown to have biological effects on a wide
variety of cells, including those of the hematopoetic system
(24, 25), colon (26, 27), bone (28, 29), and breast (30–32).
Recently, a few of these analogues have been tested on
human PC cells with promising results (33–35). However,
much of this data were acquired using PC cell lines that
were maintained in RPMI 1640 medium supplemented
with 10% FBS from Hyclone Laboratories (Logan, UT), without
the use of antibiotics. Transfected cell lines were cultured in the
presence of 0.25 mg/ml geneticin (Life Technologies, Inc.,
Grand Island, NY). Cells were passaged weekly and were incu-
bated at 37°C in 95% air and 5% CO2. All cultures have
remained free from mycoplasma infection.

**Growth Assays: Nontransfected Cell Lines.** Cell
monolayers were trypsinized, and 24-well tissue culture dishes
were seeded with 5 × 104 cells per well in RPMI 1640 supple-
mented with 10% FBS. After a 24 h incubation to allow cell
attachment, the medium was replaced with fresh RPMI + 10%
FBS containing a 1:1000 (v/v) dilution of ethanol vehicle or the
appropriate concentration of vitamin D3 analogue. Cell cultures
were incubated for 4 days and were then harvested for DNA
quantitation by Hoechst 33258 fluorescence (36, 37). Each
condition was carried out in triplicate or quadruplicate. The
effects of the various analogues on growth are expressed as a
percent of that obtained with the ethanol vehicle control.

**Growth Assays: Transfected Cell Lines.** These assays
were performed similarly to those described above with the
following modifications: geneticin was added to the growth
medium at a final concentration of 0.25 mg/ml (active); cultures
were fed every 48 h with fresh RPMI + 10% FBS containing a 1:1000 (v/v) dilution of ethanol vehicle or the
appropriate concentration of vitamin D3 analogue; and the monolayers were harvested for DNA
quantitation after a total of 6 days of hormone treatment.

**Quantitation of Secreted PSA and PSAP.** LNCaP cul-
tures that were used for the growth assays above were simulta-
naneously assayed for the secretion of PSA and PSAP into the
culture medium. After 4 days of hormone treatment, the medium
from each well was removed and centrifuged at 400 × g to
pellet any floating cells and debris. Half of the supernatant was

### Table 1 Synthetic vitamin D analogues tested

<table>
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<tr>
<th>Analogue no.</th>
<th>Chemical name</th>
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<tr>
<td>Ro 23-4194</td>
<td>1,25-Dihydroxy-26,27-hexafluorocholecalciferol</td>
</tr>
<tr>
<td>Ro 23-6536</td>
<td>1,25-Dihydroxy-22E-ene-26,27-hexafluorocholecalciferol</td>
</tr>
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<td>Ro 23-7498</td>
<td>1,25-Dihydroxy-23-yn-cholecalciferol</td>
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<td>Ro 23-7553</td>
<td>1,25-Dihydroxy-16-ene-23-yn-cholecalciferol</td>
</tr>
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<td>Ro 24-0291</td>
<td>1,25S-Dihydroxy-26-trifluoro-22E-ene-cholecalciferol</td>
</tr>
<tr>
<td>Ro 24-2201</td>
<td>1,25-Dihydroxy-16,23E-diene-cholecalciferol</td>
</tr>
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<td>Ro 24-2637</td>
<td>1,25-Dihydroxy-16-ene-cholecalciferol</td>
</tr>
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<tr>
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<td>1,25-Dihydroxy-16,23E-diene-26,27-hexafluorocholecalcifer</td>
</tr>
<tr>
<td>Ro 25-9022</td>
<td>1,25-Dihydroxy-16,23E-diene-26,27-hexafluoro-19-nor-cholecalcifer</td>
</tr>
<tr>
<td>Ro 26-7670</td>
<td>1,25-Dihydroxy-16-ene-26,27-hexafluoro-14-nor-cholecalcifer</td>
</tr>
<tr>
<td>Ro 26-2198</td>
<td>1,25-Dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor-cholecalcifer</td>
</tr>
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**MATERIALS AND METHODS**

**Vitamin D3 Analogues.** 1,25(OH)2D3 was a generous gift from B. Borsje (Duphar B.V., Weesp, The Netherlands).
The synthetic vitamin D3 analogues listed in Table 1 were synthesized by Hoffmann-La Roche (Nutley, NJ).

**Cell Cultures.** We have used a panel of seven human PC cell lines that have been described previously (19). The
development and characterization of the JCA-1 stable transfectants
(JC-VEC#7 and JC-VDR#25) have also been described (22). All
cell lines were maintained in RPMI 1640 medium supplemented
with 10% FBS from Hyclone Laboratories (Logan, UT), without
the use of antibiotics. Transfected cell lines were cultured in the
presence of 0.25 mg/ml geneticin (Life Technologies, Inc.,
Grand Island, NY). Cells were passaged weekly and were incu-
bated at 37°C in 95% air and 5% CO2. All cultures have
remained free from mycoplasma infection.
Effects of the three least potent synthetic vitamin D analogues on growth of the ALVA-31 human PC cell line. Cells were cultured for 4 days in the presence of increasing concentrations of 1,25(OH)2D3 (●) or the designated analogues (○, Ro 24-0291; ▲, Ro 24-2637; ◦, Ro 24-2201). Growth was measured by quantitation of the DNA content of the cell monolayer. Each condition was conducted in triplicate. Growth with the ethanol vehicle was designated as 100%. In contrast, the data in Fig. 2 show three of the most sensitive analogues, each inhibiting ALVA-31 growth in a dose-dependent fashion, with maximal effect at 10-8 and 10-7 M. At these concentrations, growth was approximately 37% of the vehicle control. At the same time that growth was inhibited, PSA and PSAP secretion were stimulated. Interestingly, maximal effects on PSAP secretion also required 10-8 to 10-7 M 1,25(OH)2D3, although PSA secretion plateaued at a 10-fold lower dose. Little data are available on the mechanism by which 1,25(OH)2D3 acts to stimulate the expression of these proteins; however, the different doses required for maximal induction suggests that the mechanisms may be distinct. As presented in Fig. 4, B–D, the synthetic vitamin D analogues behaved similarly to the natural metabolite in their ability to inhibit LNCaP growth and simultaneously induce PSA and PSAP secretion. Once again, all three analogues appeared to be more potent than 1,25(OH)2D3. This is especially apparent at consistently effective in repeated trials. It is noteworthy that three additional analogues showed significant inhibitory effects in the preliminary screening. However, the results varied with repeated trials using different lots of the compounds, making the data difficult to interpret. These analogues included Ro 23-4194, Ro 23-6536, and Ro 25-9022. Portrayed in Fig. 3 are the chemical structures of the three least potent and the three most potent analogues.

The LNCaP human PC cell line is often viewed as one of the better models for studying PC because it has retained several features of the in vivo disease. First, it has remained androgen sensitive but not androgen dependent throughout serial culturing. Second, it has retained the ability to secrete two androgen-regulated markers of the prostatic phenotype. These include PSA (38, 39) and PSAP (40). As we have demonstrated previously (18, 19), the LNCaP cell line also contains comparatively high numbers of functional VDRs (approximately 3000/cell). In addition to regulating the growth of this cell line (18–20), 1,25(OH)2D3 induces PSA secretion (18). Therefore, we next examined the effects of the more potent vitamin D analogues in this system. The results of these studies are presented in Fig. 4, in which values for growth, PSA, and PSAP, obtained with an ethanol control, were designated as 100%. As shown in Fig. 4A, 1,25(OH)2D3 reduced the growth of LNCaP cells in a dose-dependent fashion, with maximal effect at 10-8 and 10-7 M. At these concentrations, growth was approximately 37% of the vehicle control. At the same time that growth was inhibited, PSA and PSAP secretion were stimulated. Interestingly, maximal effects on PSAP secretion also required 10-8 to 10-7 M 1,25(OH)2D3, although PSA secretion plateaued at a 10-fold lower dose. Little data are available on the mechanism by which 1,25(OH)2D3 acts to stimulate the expression of these proteins; however, the different doses required for maximal induction suggests that the mechanisms may be distinct. As presented in Fig. 4, B–D, the synthetic vitamin D analogues behaved similarly to the natural metabolite in their ability to inhibit LNCaP growth and simultaneously induce PSA and PSAP secretion. Once again, all three analogues appeared to be more potent than 1,25(OH)2D3. This is especially apparent at consistently effective in repeated trials. It is noteworthy that three additional analogues showed significant inhibitory effects in the preliminary screening. However, the results varied with repeated trials using different lots of the compounds, making the data difficult to interpret. These analogues included Ro 23-4194, Ro 23-6536, and Ro 25-9022. Portrayed in Fig. 3 are the chemical structures of the three least potent and the three most potent analogues.

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Effective Vitamin D Analogues on Prostate Cancer Cells

10^{-10} \text{M}, where 1,25(OH)_{2}D_{3} had very little effect on growth or differentiation, although the analogues were capable of inducing greater than 50% of their maximal effect. These results suggest that these nonhypercalcemic vitamin D analogues are capable of regulating growth and differentiation of human PC cells in a similar fashion to the natural metabolite and that they are consistently more potent in doing so.

We also tested the ability of the same three synthetic vitamin D analogues to inhibit the growth of PC cell lines that express fewer VDRs (data not shown). Although some of the analogues inhibited the growth of individual cell lines, these data were difficult to interpret because the majority of these cells do not respond to 1,25(OH)_{2}D_{3}. This could be due to the fact that the synthetic analogues are more potent and require fewer functional VDRs to mediate the effects on growth. Alternatively, it may be that these analogues are functioning in a VDR-independent fashion. To distinguish between these two possible mechanisms of action, we used stably transfected clones of the JCA-1 cell line that were previously developed in our laboratory (22), as described below.

The cell line JCA-1 inherently expresses very low numbers of VDRs and is not growth-inhibited by 1,25(OH)_{2}D_{3} (19). The transfection of this cell line with a sense-oriented VDR cDNA produced the clone JC-VDR#25. This clone stably expresses approximately 2700 VDRs per cell and is sensitive to the antiproliferative effects of 1,25(OH)_{2}D_{3} (22). Vector-transfected clones did not exhibit these characteristics. Therefore, we chose to use these transfectants to determine whether VDR expression was also requisite for the growth-regulatory properties of the three synthetic vitamin D analogues used in the current study. The growth of the vector-transfected control clone JC-VEC#7 (Fig. 5A) is not inhibited in a dose-dependent fashion by any of the three synthetic analogues, nor are these cells affected by the natural metabolite 1,25(OH)_{2}D_{3}. In contrast, JC-VDR#25 is inhibited in a dose-dependent fashion (Fig. 5B), similar to that observed with the ALVA-31 cell line (Fig. 2). Again, analogue Ro 25-6760 appears to be the most potent with significant inhibitory effects, even at the lowest concentration (10^{-11} \text{M}). The other two analogues are also more potent than 1,25(OH)_{2}D_{3}, although the maximal efficacies of all four compounds appear essentially the same. These data indicate that the inhibitory effects of these synthetic analogues are also mediated through the VDR. This finding is in agreement with that proposed by other investigators using quite different methods of analysis (26, 30).

DISCUSSION

The chemotherapeutic potential of 1,25(OH)_{2}D_{3} in the treatment of various human solid tumors cannot be fully exploited due to the hypercalcemic side effects of this hormone. As a solution to this problem, less hypercalcemic analogues have been synthesized that demonstrate antineoplastic activity both in vivo and in vitro. For example, Ro 25-6760 has been shown to be 14-fold more potent than 1,25(OH)_{2}D_{3} at inhibiting the growth of HL-60 leukemia cells (24). Likewise, the analogues Ro-23-7553, Ro-24-5531, and Ro 25-5317 were statistically more effective than 1,25(OH)_{2}D_{3} at inhibiting [3H]thymidine incorporation into MCF-7 breast carcinoma cells (30). Furthermore, Ro 24-5531 and Ro 23-7553 were found to have the greatest antimitogenic activity of 8 analogues tested on Caco-2 colon carcinoma cells (27). In general, these studies
Fig. 4  LNCaP cell growth and differentiation: dose-dependent effects of three vitamin D analogues. LNCaP cells were grown for 4 days in the presence of increasing concentrations of: A, 1,25(OH)2D3; B, Ro 23-7553; C, Ro 24-5531; or D, Ro 25-6760. The culture media were assayed for secreted PSA (●) and secreted PSAP (▲). The DNA content (○) of the cell monolayers was quantitated to measure cell growth. Data points, means of triplicate samples expressed as a percent of the ethanol vehicle control; bars, SE.

have revealed that multiple bonds at C-16 and C-23, as well as hexafluorination of C-26 and C-27, seem to increase the antiproliferative potential of this hormone, while reducing its hypercalcemic properties. Additionally, these structural modifications have been shown to prevent side chain cleavage (41) and alter the catabolism of the molecule (42), characteristics that are likely to further enhance the biological potency of the analogues.

Comparatively few studies have examined the activity of the synthetic vitamin D analogues on human PC cells. The first of such studies was published by Schwartz et al. (33), who used the analogues Ro 24-2287, Ro 24-2201, and Ro 24-2637. Each of these was found to inhibit LNCaP cell growth to a similar extent as does the natural hormone 1,25(OH)2D3. Our results with the latter two compounds are in agreement with this; however, in comparison to the other analogues we tested, these were some of the least effective at inhibiting PC cell growth.

Instead, Ro 25-6760, Ro 24-5531, and Ro 23-7553, in descending order, were consistently the most potent at reducing growth and promoting differentiation. In 1995, Skowronski et al. (35) examined five synthetic analogues, including Ro 24-2637, and concluded that the introduction of a double bond at C-22, a hydroxyl group at C-24, and cyclopropyl modification of the side chain produced the greatest antiproliferative and differentiating activity in LNCaP cells. Additional studies using animal models further support our findings with these analogues. For example, Ro 24-5531 was shown to effectively reduce and even prevent the development of breast cancer induced by N-nitroso-N-methylurea in the rat (31). Also of importance is that when fed continuously to the rats, this analogue did not elevate serum calcium through the course of the study. Lastly, Schwartz et al. (34) recently reported that the growth of the PC cell line PC-3 was reduced in nude mice by only 15% using the analogue Ro 23-7553. This modest reduction in growth may be due to the fact

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that PC-3 cells have relatively low numbers of endogenous VDRs and are less sensitive to the antiproliferative effects of 1,25(OH)2D3 as compared to the cell lines ALVA-31 and LNCaP (19). This observation is also in agreement with our finding that a sufficient number of VDRs must be present for 1,25(OH)2D3 or the related analogues to effect growth. Together, these data suggest that the VDR status of a patient’s tumor would have to be determined before therapy because only those patients whose tumors express sufficient numbers of VDRs would be expected to respond favorably. Another concern with vitamin D treatment for PC is that the 35-65% reduction in growth that we observed may be too modest to alter the course of the disease in a patient. It should be considered, however, that prostatic cancers have an unusually slow growth rate with doubling times greater than 2 years in 79% of the patients (43). For this reason, even modest decreases in proliferation may significantly extend their survival time, especially if treatment is initiated in the earlier clinical stages. It should also be kept in mind that during such clinical testing a paradoxical rise in serum PSA and PSAP might occur in patients as tumor growth is inhibited, due to the differentiating properties of these compounds.

Numerous laboratories have explored the possible mechanisms by which the synthetic vitamin D analogues are working. Interestingly, it has been concluded that the relative affinities of the analogues for the VDR does not generally explain their potency (26, 30, 44). Instead, the differences in potency are often correlated with altered dimerization and increased transcriptional activity on certain vitamin D response elements (29, 44–46). A variety of vitamin D response elements have now been reported, some preferring VDR homodimers for activation of gene transcription, whereas others prefer heterodimers with different retinoid X receptor isoforms. Perhaps it is this great diversity in functional VDR complexes that allows for many of the synthetic analogues to induce differentiation of some cell types without inducing the osteoclastic resorption of bone. With continued pharmacological advances, it may eventually be possible to synthesize designer vitamin D analogues that can target individual cell types, thus reducing unwanted side effects when used therapeutically. For example, certain analogues would have maximal activity for inhibiting the growth of PC cells without also suppressing the immune system or inducing hypercalcemia. Likewise, other analogues may target the immune system for treatment of leukemia. To achieve such tailored drug specificity, it will be necessary to identify the differences in hormone response elements and transcription factors that mediate the effects of 1,25(OH)2D3 in the prostate versus other cell types. Certainly, continued research and development of synthetic vitamin D analogues should prove to be valuable in the advancement of this field.

In summary, we have tested a variety of synthetic vitamin D analogues on the growth and differentiation of PC cells. Our results indicate that the dual modification 16-ene, 23-yne produced an analogue with greater potency and similar efficacy to the parent hormone. Additional modification by hexafluorination at C-26 and C-27 and the deletion of C-19 produced even greater increases in potency. These analogues (Ro 23-7553, Ro 24-5531, and Ro 25-6760) inhibited growth and stimulated PSA and PSAP secretion with greater than 50% maximal activity at 10−10 M. Experiments using cells that were transfected with the VDR cDNA indicate that the antiproliferative effects are mediated through the VDR. Our data, in conjunction with the results from other laboratories, indicate that analogues Ro 23-7553, Ro 24-5531, and Ro 25-6760 are promising therapeutic compounds for the treatment of PC that warrant further evaluation in a clinical setting.

REFERENCES


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