Effectiveness of Vitamin D Analogues in Treating Large Tumors and During Prolonged Use in Murine Retinoblastoma Models

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Objective: To investigate the effectiveness of the vitamin D analogues 1,25-(OH)2-16-ene-23-yne vitamin D3 (16,23-D3) and 1α-hydroxyvitamin D2 (1α-OH-D2) in inhibiting retinoblastoma growth in large tumors in a xenograft model and with prolonged use in a transgenic model.

Methods: For the large-tumor study, the xenograft athymic mouse/human retinoblastoma cell (Y-79) model was used. Subcutaneous tumors were allowed to grow to an average volume of 1600 mm3. Systemic treatment with one of the vitamin D analogues or with vehicle (control groups) was carried out for 5 weeks. For the long-term study, transgenic β-luteinizing hormone–large T antigen (LHβ-Tag) mice were systemically treated with one of the 2 compounds or vehicle (control groups) for up to 15 weeks. Tumor size and signs of toxicity were assessed.

Results: In the large-tumor study, tumor volume ratios for the 1α-OH-D2 and 16,23-D3 groups were significantly lower than those for controls (P<.002). No significant differences in tumor volume were seen between the 1α-OH-D2 and 16,23-D3 groups (P=.15). In the long-term study, the 1α-OH-D2 group showed significantly smaller tumor size compared with its control (P<.001). No significant difference was seen between the 16,23-D3 group and its control. Some toxic effects related to hypercalcemia were seen in both studies.

Conclusions: In athymic mice in the large-tumor study, both 1α-OH-D2 and 16,23-D3 were effective in inhibiting tumor growth compared with controls. In the long-term study, 1α-OH-D2 inhibited tumor growth but 16,23-D3 did not. Effective doses of both compounds caused hypercalcemia and a significant increase in mortality.

Clinical Relevance: Use of 1α-OH-D2 inhibited tumor growth in large tumors and with long-term treatment compared with controls. Because of hypercalcemia-related toxic effects seen in the present experiments, in clinical trials, serum calcium levels should be carefully monitored. This analogue may require use with drugs that lower serum calcium levels or use of relatively lower doses or skipped doses. The ideal alternative solution would be to identify vitamin D analogues that retain the antineoplastic action without the calcemic activity.


From the Departments of Ophthalmology and Visual Sciences (Drs Albert, Kumar, and Patel and Mss Darjatmoko and Lokken) and Biostatistics and Medical Informatics (Dr Lindstrom), University of Wisconsin Medical School, Madison; and Bone Care International, Middleton, Wis (Dr Strugnell).

VITAMIN D COMPOUNDS have been recognized for many years as having potential usefulness in the treatment of a variety of cancers, including retinoblastoma.1 A major obstacle to their use in human studies has been drug-induced hypercalcemia.1 Vitamin D analogues have previously been shown in preclinical studies of retinoblastoma using xenograft and transgenic models to cause apoptosis2 and to inhibit angiogenesis.2 Previous preclinical studies3 demonstrated that 2 commonly used forms of vitamin D, calcitriol (1,25-dihydroxyvitamin-D3) and ergocalciferol (vitamin D2), inhibited tumor growth but with marked hypercalcemia-related toxic effects in mouse xenograft and transgenic models. Also demonstrated was a similar inhibitory effect with the newer synthetic vitamin D analogues 1α-hydroxyvitamin D2 (1α-OH-D2),6 and 1,25-(OH)2-16-ene-23-yne vitamin D3 (16,23-D3),7 with reduced calcemic effects. All of these studies were carried out using small tumors treated for a relatively short time (5 weeks). The present studies were carried out to determine whether vitamin D analogues are effective in the treatment of retinoblastoma in larger tumors and remain effective with more prolonged administration.

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All research using mouse models of retinoblastoma conformed to the guidelines set by the Research Animal Resources Center of the University of Wisconsin and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. These guidelines permit large-tumor studies to be carried out in athymic mice but not in β-luteinizing hormone–large T antigen (LHβ-Tag) transgenic mice because of the relatively small size of the tumors occurring in the eyes and the morbidity associated with orbital extension. Likewise, long-term studies were suitable for the transgenic but not athymic mice.

**COMPOUND PREPARATION**

Pure crystalline 1α-OH-D$_2$ was provided by Bone Care International and was prepared for administration with drug concentrations confirmed in the manner previously described elsewhere.$^{1,14}$ A solution of 1α-OH-D$_2$ was diluted in coconut oil to a concentration of 0.2 µg/0.1 mL for use in this study. Each treated mouse received 0.2 µg of 1α-OH-D$_2$ (approximately 10 µg/kg) per treatment. This dose has been previously demonstrated in toxicity and dose-response studies$^{1,14,15}$ to be the effective dose with the least toxicity. Pure crystalline 16,23-D$_3$ (provided by ILEX Oncology Inc, San Antonio, Tex) was prepared for injection as previously described elsewhere.$^{12,14,15}$ This drug was diluted in mineral oil to a concentration of 0.5 µg/0.1 mL. Each mouse in the treatment group received 0.5 µg of 16,23-D$_3$ (approximately 25 µg/kg) per treatment. This dose was found in previous toxicity and dose-response studies$^{1,14,15}$ to be the effective dose with the least toxicity.

**LARGE-TUMOR XENOGRAFT MODEL TREATMENT**

A total of 119 athymic “nude” mice (4-6 weeks old) were given dorsal subcutaneous injections of 2 × 10$^7$ Y-79 human retinoblastoma cells suspended in 0.5 mL of Iscove culture medium supplemented with 20% fetal bovine serum and basement membrane matrix suspensions. Details of culture methods have been previously described elsewhere.$^{1,14,15}$ After tumor cells were injected, all mice were maintained on a vitamin D– and calcium-restricted diet (PD; Purina Mills Inc, St Louis, Mo). This diet was used to minimize hypercalcemia and to control for any effect of vitamin D in the diet. Mice were randomized to 1 of 4 treatment groups: (1) 1α-OH-D$_2$ (0.2 µg per mouse; 40 animals), (2) gavage (GV) control (0.1 mL of coconut oil; 20 animals), (3) 16,23-D$_3$ (0.5 µg per mouse; 40 animals), or (4) intraperitoneal (IP) control (0.1 mL of mineral oil; 19 animals). The method of administration conformed with instructions provided by the respective pharmaceutical companies supplying the drugs to obtain maximum blood levels. Tumors were allowed to grow for 19 days to an average volume of 1600 mm$^3$ before initiation of treatment with either oral GV (1α-OH-D$_2$ and GV control groups) or IP (16,23-D$_3$ and IP control groups). Treatment was given 5 times per week for 5 weeks. Baseline tumor volume and animal body weight measurements were determined before the first treatment. Each animal was weighed, and its tumor was measured using calipers, twice per week during treatment and just before euthanization on the last treatment day as previously described elsewhere.$^{1,13,14}$ Investigators were masked to final tumor measurements and histopathologic examination findings but not to drug delivery method.

**LONG-TERM TREATMENT STUDY IN LHβ-Tag TRANSGENIC MICE**

The LHβ-Tag mice are a well-characterized transgenic model of retinoblastoma.$^{16}$ A total of 170 LHβ-Tag transgene-positive mice were randomized by sex and litter into 2 treatment groups and 2 control groups corresponding to the 4 treatment groups described for the large-tumor athymic study in the previous subsection. The presence of the transgene was confirmed by polymerase chain reaction.$^{16}$ These mice were maintained on the same vitamin D– and calcium-restricted diet as noted in the previous subsection. Each mouse in the 1α-OH-D$_2$ treatment group received 0.2 µg/d. Each mouse in the 16,23-D$_3$ treatment group received 0.3 µg/d. Treatment was given 5 times per week. One third of the mice in each group were treated for 5 weeks, one third for 10 weeks, and one third for 15 weeks. Baseline body weights were recorded before the first treatment, and animals were reweighed twice per week during treatment and before euthanization on the last treatment day. Details of the oral GV and IP methods of treatment with vitamin D analogues have been previously described elsewhere.$^{1,14,15}$ Doses were skipped for up to 2 consecutive days in mice that either developed weight loss (>20% of baseline weight) or severe lethargy, and treatment was resumed when the affected animals regained the lost weight or resumed normal activity. Investigators were masked to histopathologic examination findings and tumor measurement but not to drug delivery method.

**TUMOR MEASUREMENT AND HISTOPATHOLOGIC STUDY**

In athymic mice, tumor size was measured daily 5 times a week in 3 dimensions (length, width, and height) by means of calipers measuring to the nearest millimeter, and the volume was approximated by multiplying the 3 measurements. After euthanization, each tumor was excised, measured using calipers in 3 dimensions, and weighed. The tumors were fixed in 10% neutral buffered formalin for standard histopathologic processing. Five-micrometer sections were cut and stained with hematoxylin-eosin. In addition, von Kossa–stained preparations were made. These were examined microscopically, and the histologic features were recorded as previously described elsewhere.$^{1,13,14}$

In transgenic mice, after euthanization the eyes were enucleated and placed in 10% neutral buffered formalin for standard histopathologic sectioning. Four serially sectioned 3-µm-thick sections were cut from each of the superior, middle, and inferior areas of the globe and were stained with hematoxylin-eosin. The 4 sections from each globe area were examined under a microscope, and the section with the largest tumor from each of the 3 areas was used for measurement. The outline of the tumor in each section was traced from a microscopically digitized image, and the area was measured using image analysis software (Optimas version 6.5; Media Cybernetics, Silver Spring, Md). The 3 tumor areas from each representative portion of the globe were averaged together to obtain the mean tumor measurement of each eye (expressed in square micrometers). The measurements from both eyes were then averaged to provide the mean tumor area per mouse. Other histopathologic features were also evaluated.$^{1,13,14}$

**TOXIC EFFECT ASSESSMENT**

Serum samples from representative mice in each group were obtained just before euthanization from the axillary vessels and were analyzed for calcium levels by Marshfield Laboratories, Marshfield, Wis.

Kidneys, lungs, and liver were harvested from representative mice in each group in both arms of the study and were
The survival data for the study are presented in Tables 1 and 2. The 1α-OH-D$_2$ group showed significantly smaller tumor areas compared with the GV control group at each time point ($P<.001$). However, no significant difference was seen between the 16,23-D$_3$ group and the IP control group at any of the 3 times.

TOXIC EFFECTS IN THE LARGE-TUMOR AND LONG-TERM TREATMENT STUDIES

The survival data for the study are presented in Tables 1 and 2. There were significantly fewer survivors among athymic mice in the 1α-OH-D$_2$ group in the large-tumor study than in the GV control group (65% vs 90%; $P=.03$) (Table 1). There were also fewer survivors among the LHβ-Tag mice in the 1α-OH-D$_2$ group in the long-

### RESULTS

**TUMOR SIZE IN THE LARGE-TUMOR STUDY**

In the athymic mice with large tumors, mean change in tumor volume was analyzed as a proportional change during treatment (volume at treatment end divided by volume at treatment start) (Table 1). The tumor volume ratio for the 1α-OH-D$_2$ group was significantly lower than that for the GV control group, and the ratio for the 16,23-D$_3$ group was significantly lower than that for the IP control group ($P<.002$ for each). No statistically significant differences in tumor volume were seen between the 1α-OH-D$_2$ and 16,23-D$_3$ groups ($P=.15$).

**TUMOR SIZE IN THE LONG-TERM TREATMENT STUDY**

In the long-term study with LHβ-Tag mice, the tumor sizes after 5, 10, and 15 weeks of treatment are given in Table 2. The 1α-OH-D$_2$ group showed significantly smaller tumor areas compared with the GV control group at each time point ($P<.001$). However, no significant difference was seen between the 16,23-D$_3$ group and the IP control group at any of the 3 times.

**STATISTICAL ANALYSIS**

Tumor weight, tumor volume or area, animal weight, serum calcium level, and kidney calcification were analyzed using 1-way analysis of variance of tumor size to assess statistical differences among the groups. Pairwise comparisons were then performed to detect statistical differences between particular dose groups. The data from various measurements were transformed to the log scale before analysis to stabilize the variance. The change in animal weight (from first to last measurement) was restricted to animals that survived until the last measurement. The effect of dose of vitamin D analogues on mortality was assessed using a generalized linear model assuming binomial variability.

The effect of “layer” (ie, animal batch) on response was accounted for in all analyses by including a blocking term in the model. All significant global tests for effect of dose were followed by pairwise analyses to assess differences between specific dose groups.

In the long-term arm of study, comparisons of each drug’s efficacy over each time point (5, 10, or 15 weeks) were also performed.
term study than in the GV control group (83% vs 100%; \(P = .048\)) (Table 2).

Serum calcium levels for both study arms are given in Tables 1 and 2 and Table 3. All treatment groups had significantly higher serum calcium levels compared with their respective control groups (\(P \leq .02\)), except both of the 15-week treatment groups, which did not show any difference (\(P > .25\)). No difference in serum calcium levels was found between the 2 treatment modalities. The LHβ-Tag mice developed well-differentiated retinoblastoma, with rosettes, some calcification, and areas of necrosis. Treated animals had smaller tumors, but the degree of differentiation and the proportional amounts of calcification and necrosis were similar in treated and control animals. The athymic/Y-79 mice had poorly differentiated tumors, with an extensive and diffuse component of dead cells. The diffuse nature of the necrosis is similar to that seen in large intraocular retinoblastoma in enucleated human eyes. Because of the diffuse distribution of dead cells, the precise percentage of the tumor they represent cannot be accurately determined using the present techniques. It is our impression, however, that dead cells generally represented one third to two thirds of the cells present and were more numerous in treated eyes. The gradations of the kidney calcifications are given in Table 4. The treatment groups in both arms of the study showed a greater degree of calcification compared with controls, with the 1α-OH-D\(_2\) groups exhibiting greater severity of calcification compared with the 16,23-D\(_3\) groups. Gross and histopathologic examination of lung and liver tissues from randomly selected mice in each treatment group showed no evidence of metastatic lesions or other abnormalities.

### Table 3. Serum Calcium Levels in the Large-Tumor and Long-term Treatment Studies

<table>
<thead>
<tr>
<th>Dose Group</th>
<th>Large-Tumor Study</th>
<th>Long-term Treatment Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum Calcium, mg/dL</td>
<td>5wk</td>
</tr>
<tr>
<td>1α-OH-D(_2) (0.2 µg), 10 µg/kg</td>
<td>10.2</td>
<td>11.7</td>
</tr>
<tr>
<td>GV control</td>
<td>9.3</td>
<td>9.1</td>
</tr>
<tr>
<td>16,23-D(_3) (0.2 µg), 25 µg/kg</td>
<td>10.2</td>
<td>10.6</td>
</tr>
<tr>
<td>IP control</td>
<td>8.7</td>
<td>8.43</td>
</tr>
</tbody>
</table>

**Abbreviations:** See Table 1.

**SI conversion factor:** To convert serum calcium to millimoles per liter, multiply by 0.25.

### Table 4. Severity of Kidney Calcifications in Animals at the End of the Study

<table>
<thead>
<tr>
<th>Dose Group</th>
<th>Large-Tumor Study</th>
<th>Long-term Treatment Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mice, No.†</td>
<td>0</td>
</tr>
<tr>
<td>1α-OH-D(_2) (0.2 µg), 10 µg/kg</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>GV control</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>16,23-D(_3) (0.5 µg), 25 µg/kg</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>IP control</td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>

**Abbreviations:** See Table 1.

*Kidney calcifications: 0, grade 0 (none); I, grade I (mild); 1 to 7; II, grade II (moderate); 8 to 15; III, grade III (severe), greater than 15.

†Number of animals whose kidneys, liver, and lungs were examined.

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**COMMENT**

Retinoblastoma is the most common intraocular malignancy of childhood, occurring once in every 20,000 live births worldwide. Current methods of treatment include enucleation, external beam radiotherapy, scleral plaque brachytherapy, cryotherapy, photocoagulation, and chemotherapy. Although current treatment methods have achieved survival of 90%, there remains a need for improved treatment alternatives to provide better visual results and to decrease the risk of secondary nonocular cancers in hereditary retinoblastoma.

In recent years, there has been strong interest in systemic chemotherapy and, particularly, “chemoreduction” in the treatment of retinoblastoma. There are 4 general circumstances in the treatment of retinoblastoma in which chemotherapy is used: (1) to shrink tumors in eyes with visual potential that are too large to treat with local methods to a size at which photocoagulation, cryotherapy, thermotherapy, or radioactive plaques can be administered; (2) in patients younger than 1 year who have advanced bilateral tumors that require external beam radiotherapy for cure; (3) as a single modality (which rarely obtains a permanent response); and (4) for the treatment of extraocular spread. Numerous serious short-term sequelae and less severe complications can occur. These drugs are mutagenic, and the development of secondary nonocular cancers is a well-documented risk. New retinoblastomas have been reported to develop in the eyes of patients undergoing treatment with systemic chemotherapy.

Verhoeff, in 1966, hypothesized that calcification induced spontaneous regression in retinoblastoma and suggested that treatment with vitamin D might prove effective. Vitamin D has since been shown to inhibit the growth of retinoblastoma in vitro and in vivo in the athymic mouse model and in the transgenic mouse model.
The effect, however, is unrelated to either high serum calcium levels or calcium deposition in the tumor, and, in fact, the clinical usefulness of vitamin D is limited by the toxic effects associated with hypercalcemia. The antineoplastic mechanism of action of vitamin D compounds used in models of human retinoblastoma has been demonstrated to be apoptosis due to increased expression of p53 and p21 and also inhibition of angiogenesis. Vitamin D receptor messenger RNAs, which are necessary for this antineoplastic effect, have been demonstrated in Y-79 retinoblastoma cells, WERI-1 cells, LHB-Tag tumors, and 23 consecutive freshly removed human retinoblastoma specimens using reverse transcriptase polymerase chain reactions.

Our initial studies were with calcitriol (the active form of vitamin D3) and ergocalciferol (vitamin D2). These compounds consistently inhibited tumor growth by more than 50% compared with controls. In the case of transgenic mice, animals were observed in the treatment groups with total regression of tumors. This was proven by serially sectioning the globes. All the negative animals were rechecked to make sure that they carried the transgene. In this study, 6 of 10 animals in the high-dose calcitriol group and 4 of 12 animals in the low-dose calcitriol group showed no tumors, whereas 16 control animals had tumors. The difference was statistically significant as assessed by a chi² test for independence. However, only 25% to 50% of animals survived 5 weeks of treatment in the calcitriol and ergocalciferol treatment groups given doses adequate to cause tumor inhibition or regression.

In the athymic/Y-79 xenograft treated with calcitriol, 81% inhibition of tumor growth was seen compared with controls, and vitamin D inhibition ranged from 58% to 66%, but no regression was seen in these tumors. The real-time polymerase chain reaction expression of vitamin D receptors is barely perceptible in the Y-79 retinoblastoma line. It is far lower than the 23 retinoblastoma samples taken from enucleated eyes from different patients. In contrast, the LHB-Tag tumors have a greater degree of vitamin D receptor expression. This difference seems to be related to the dose of drug response. We concluded that calcitriol and ergocalciferol showed impressive antineoplastic activity but caused hypercalcemic activity that was too excessive for them to be used in human clinical trials.

We then turned our attention to 2 synthetic vitamin D analogues, 16,23-D3 and 1α-OH-D3, which have antineoplastic effects similar to calcitriol and ergocalciferol but with reduced hypercalcemic activity. In short-term experiments (5 weeks) in small tumors, 16,23-D3 therapy caused 55% inhibition in LHB-Tag xenografts compared with controls. These experiments described in the present article were intended to determine the effectiveness and the toxic effects of treatment with 16,23-D3 and 1α-OH-D3 in large tumors and in tumors receiving long-term therapy. The size of tumors that could be treated and the duration of treatment were limited by the guidelines of the University of Wisconsin Medical School Animal Care and Use Committee regarding suffering and stress caused to animals. Specifically, we wanted to determine whether drug-resistant cell lines develop with prolonged use of these drugs or with large tumors, as well as the drugs' comparative effectiveness in these situations. These experiments simulate the circumstances of treatment for extrapolocular spread. In the large-tumor studies, 16,23-D3 therapy achieved 62% growth inhibition compared with controls, and 1α-OH-D3 therapy achieved 51% inhibition of tumor growth compared with controls. These determinations are based on tumor size alone. However, as determined by histopathologic analysis, there was a diffuse distribution of dead cell in the tumors that constituted one third to two thirds of the cells present, and we observed that the dead cells were more numerous in treated animals. We are adapting techniques to accurately quantify numbers of viable, common, necrotic, and apoptotic cells.

In the long-term treatment studies using transgenic mice only, 1α-OH-D3 therapy seemed to be effective. In these experiments, there was 92% regression at 10 weeks and 70% regression at 15 weeks compared with controls. Some treated eyes seemed to have totally regressed tumors on the basis of the slides examined, but because serial sections were not examined, this cannot be stated with certainty. All of the groups that were effectively treated showed statistically significant hypercalcemia and associated increased mortality compared with controls (Tables 1, 2, and 3). Although these toxic effects and mortality rates are considerably less than those seen with calcitriol and vitamin D3 treatment, it remains a potential deterrent to proceeding to clinical trials.

An Investigational New Drug application for 1α-OH-D3 as a cancer treatment was submitted to the Food and Drug Administration in 1996, and an application for 16,23-D3 was submitted in 1999. In phase 1 trials for treatment of prostate cancer, 1α-OH-D3 exhibited tumor growth suppression for stabilization with low but reversible toxic effects. This drug is currently being used in phase 2 human trials of prostate cancer (George Wilding, MD, oral communication, October 7, 2003).

Because of their mechanism of action, which differs from that of existing retinoblastoma chemotherapeutic agents, and the fact that they are nonmutagenic agents, we believe that these compounds have potential use in eye-preserving and, in cases of extrapolocular spread, life-preserving treatment as a component of multidrug therapy or possibly as a potential single-modality treatment. In clinical trials of 1α-OH-D3 and 16,23-D3, hypercalcemia-related toxic effects would need to be carefully monitored, controlling the dose given and possibly

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skipping doses in response to elevated serum calcium levels. Drugs that lower serum calcium levels may need to be used. The ideal alternative solution would be to identify vitamin D analogues that retain the antineoplastic action but have no calcemic activity.

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REFERENCES