Toxicity and Dose-Response Studies of 1α-Hydroxyvitamin D2 in a Retinoblastoma Xenograft Model

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Background: Although calcitriol (1,25-dihydroxycholecalciferol) and vitamin D2 inhibit retinoblastoma growth in the athymic (nude) mouse xenograft (Y-79 cell line) model of retinoblastoma, they can cause severe toxicity.

Objective: To examine the toxicity of and dose-dependent response for the inhibition of tumor growth for 1α-hydroxyvitamin D2 (1α-OH-D2), an analogue with reduced systemic toxicity, in the athymic Y-79 mouse model.

Methods: Mice were randomized into treatment and control groups for 5-week toxicity and dose-response studies. Treatment was via oral gavage 5 times per week. Dose-response studies measured tumor inhibition and drug serum levels. Tumor size and body weight were measured weekly together with various criteria for toxicity. Animals were euthanized at the end of the treatment period. Tumors and kidneys were harvested, and serum was analyzed for calcium and drug levels.

Results: Doses of 0.1 to 1.2 µg/d were selected on the basis of toxicity studies for the dose-response trial. Tumor weight and volume in the 0.2-µg and 0.3-µg doses were significantly lower than in controls. Mortality rates and kidney calcification in mice treated with doses of 0.1 to 0.3 µg were lower than those observed in studies of calcitriol and vitamin D2.

Conclusion: A vitamin D analogue, 1α-OH-D2, inhibits tumor growth in this xenograft model of retinoblastoma with less toxicity than calcitriol and vitamin D2.

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ETINOBLASTOMA IS the most common intraocular malignancy of childhood, occurring once in 20000 live births worldwide.1 In 1966, Frederick Verhoeff, MD, who had studied this tumor type for more than 50 years, named it retinoblastoma,2 and initiated radiation treatment,3,4 stressed that patients with regressed retinoblastoma had tumors that were heavily calcified.5 He proposed using vitamin D as an inhibitor of proliferating retinoblastoma tumors.5,6

In 1974, our laboratory established the first human retinoblastoma cell line (Y-79 cell line) and subsequently confirmed the presence of high-affinity vitamin D receptors in this cell line.7,8 We have demonstrated that calcitriol and vitamin D2 inhibit the growth of Y-79 retinoblastoma cells in tissue culture, in a xenograft model of a human Y-79 retinoblastoma cell line grown in athymic (nude) mice, and in β-luteinizing hormone-Tag (LHB-Tag) mice, a transgenic model for hereditary retinoblastoma.6,8,10 In contrast to Verhoeff’s theory, this antineoplastic effect is unrelated to the serum calcium level. However, marked toxicity in the form of high mortality rates, hypercalcemia, and marked weight loss was seen in all animals treated with calcitriol and vitamin D2 at doses required to achieve a therapeutic effect.

Vitamin D analogues were developed in the 1990s with comparable antineoplastic activity but a reduced effect on calcium metabolism.11 The vitamin D analogue, 1,25-dihydroxy-16-ene-23-yne-vitamin D3 (16,23-D3) was tested in our laboratory and found to be effective against retinoblastoma tumors in LHB-Tag mice at doses that produced less toxicity than calcitriol and vitamin D2.12 This analogue has only recently been approved for experimental use in humans. We are unaware of any available results in adult patients with tumors, a prerequisite for human pediatric clinical trials.

In the current study, we report the toxicity and dose-dependent inhibition of tumor growth in the athymic Y-79 retinoblastoma model with 1α-hydroxyvitamin D2 (1α-OH-D2) (BoneCare International Inc, Madison, Wis).1 This vitamin D analogue was approved by the Food and
MATERIALS AND METHODS

All research using mouse models of retinoblastoma received institutional review board approval by the Research Animal Resources Center of the University of Wisconsin in Madison.

PRELIMINARY TOXICITY TRIAL

To assess the relative toxicity of 1α-OH-D3, a preliminary toxicity trial was conducted. Forty-eight athymic hybrid mice were divided into 6 treatment groups of 7 animals each, with a corresponding control group of 6 animals. The doses given were 0.1 µg, 0.2 µg, 0.3 µg, 0.6 µg, 1.2 µg, and 2.4 µg in 0.1 mL of solution. 1α-OH-D3 was prepared as described below, and animals’ baseline body weights were measured. The mice were treated via oral gavage 5 times per week for 5 weeks. Toxicity was assessed according to mortality rates, weight loss, serum calcium levels, and kidney calcification.

DOSE-DEPENDENT RESPONSE FOR INHIBITION OF TUMOR GROWTH

A total of 292 athymic (nude) mice at ages ranging from 8 to 10 weeks were given dorsal subcutaneous injections of 1 × 10⁴ human retinoblastoma cells from a cultured Y-79 cell line. Details of culture methods have been described previously. Because of limitations in available housing for the large number of animals needed, this study was done in several layers of treated and control animals until group sizes of at least 30 animals per group were reached. The tumors were allowed to grow for 5 days prior to the start of treatment. Pretreatment body weight was recorded. For 2 weeks prior to treatment with 1α-OH-D3, and throughout the treatment period, the animals were placed on a vitamin D- and calcium-deficient diet (Vitamin D/Calcium Deficient PD; Purina Mills Inc, St Louis, Mo) to reduce hypercalcemia.

The mice were randomized into 5 1α-OH-D3 treatment groups and a corresponding control group (37 animals in the 0.1 µg dose group, 60 animals in the 0.2 µg group, 55 animals in the 0.3 µg group, 45 animals in the 0.6 µg group, and 40 animals in the 1.2 µg group; 35 animals were in the control group). 1α-OH-D3 was provided by BoneCare International Inc in a crystalline form, which was then dissolved with 100% ethanol for a stock solution of 2.98 mg/mL. This solution was diluted in coconut oil to concentrations corresponding to drug doses of 0.1 µg, 0.2 µg, 0.3 µg, 0.6 µg, and 1.2 µg per 0.1 mL. Spectrophotometric analysis was used to confirm the drug concentrations. The mice in the control group were given 0.1 mL of coconut oil. Stock solutions of the drug were prepared weekly and stored in amber glass bottles at −40°C to protect the compound from degradation due to temperature or UV light.

Animals were treated 3 times per week for 5 weeks by oral gavage using a 1-in, 1.25-mm-ball diameter steel gavage needle (22GX; Becton Dickinson, Franklin Lakes, NJ) attached to a 1-mL syringe. Each animal was weighed twice a week during treatment. Approximately 2 hours after the last dose at the fifth week, animals were euthanized by cervical dislocation using isofluorane anesthesia. Immediately prior to euthanasia, serum was collected for the determination of calcium and vitamin D metabolite levels. The subcutaneous tumors were harvested, measured, and weighed. Tumor volume was also measured using water displacement. The kidneys were removed from representative animals in each treatment group and histologically examined for calcification. Toxicity was assessed according to mortality rate, decreased body weight, increased lethargy, and kidney calcification.

Animal care followed standards set by the Research Animal Resources Center at the University of Wisconsin and previously published guidelines for mice treated with

PRELIMINARY TOXICITY STUDY

Mortality data for the preliminary toxicity study are shown in Figure 1. From these results, we selected doses of 0.1 µg, 0.2 µg, 0.3 µg, 0.6 µg, and 1.2 µg per day for the dose-response efficacy study.

DOSE-RESPONSE EFFICACY STUDY

All animals in the 5 treatment groups and control group had visible tumors at the start of treatment that remained observable until the end of the study. There was no significant difference in tumor size between the groups on day 1, when treatment was initiated. Mortality rates for all dose groups appear in Figure 2. As expected from the preliminary toxicity study, high mortality rates were observed in the 0.6 µg and 1.2 µg dose groups. The 1.2 µg dose group had only 3 surviving animals at the end of the treatment period. Because of the small number of surviving animals (3 of 12), the 1.2 µg dose group is not included in the data analyses.

At the end of the study, all tumors were harvested and confirmed to be retinoblastomas using light microscopic examination. Tumor size showed a strong inverse relationship to dose groups of 1α-OH-D3, with statistically significant differences in tumor size between controls and each drug group (P <.03) (Table). Tumor weight and volume showed similar strong relationships with dose. Statistically significant differences in weight and volume existed between controls and 0.2 µg and 0.3 µg dose groups (P <.004 and P <.003, respectively) (Table and Figure 3). A decrease in tumor weight and volume was also seen in the 0.6 µg dose group, but statistical significance was not reached, possibly

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vitamin D compounds. Animals were monitored daily for signs of toxicity, such as lethargy and weight loss, by lab personnel and by veterinarians responsible for the Animal Care Unit at the Clinical Science Center at the University of Wisconsin. Treatment was withheld for animals that were severely lethargic for a maximum of 2 consecutive days, allowing them to regain weight and recover normal physical activity. Animals that experienced marked weight loss or were extremely ill were euthanized prior to treatment completion on the advice of the attending veterinarians.

ANALYSIS OF TUMOR SIZE

Tumors were measured externally 3 times per week using calipers calibrated in 1-mm increments, according to methods previously described in our laboratory. A single investigator performed all tumor measurements. Tumor volume on day 1 of treatment was used as a baseline measurement. After the animals were euthanized, the tumors were dissected from the subcutaneous location and measured using calipers in a similar manner. These measurements were identified as end-of-study tumor volume values and are referred to as tumor size. Tumor weight was recorded to the nearest 0.01 mg. Tumor volume was measured by suspending the tumors in water and measuring displacement. All tumors were fixed in 10% buffered formalin, processed histologically, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. The slides were examined using light microscopy to confirm the presence of retinoblastoma.

ANALYSIS OF TOXICITY TO THE KIDNEYS

Kidneys were harvested immediately following euthanasia from 6 mice in the 0.1 µg and 0.2 µg groups, 12 mice in the 0.3 µg group, 9 mice in the 0.6 µg group, and 3 mice in the 1.2 µg group. Each kidney was assigned a unique identifying number, fixed in 10% buffered formalin, and processed for light microscopy examination. Three hematoxylin-eosin–stained sections of each kidney were examined. Renal calcification was assessed by a single masked reviewer and rated as none, mild, moderate, or severe.

ANALYSIS OF BLOOD FOR HYPERCALCEMIA

Prior to euthanasia, serum was collected from the axillary veins of all study mice and sent for calcium measurement by an independent commercial laboratory (Marshfield Laboratories Inc, Marshfield, Wis).

SEWER VITAMIN D METABOLITE ANALYSIS

Prior to euthanasia, serum was collected from the axillary veins of all study mice and sent for quantitation of serum concentrations of 1,25(OH)2D3, the active metabolite of 1α-25(OH)D3. This analysis was performed at BoneCare International using solid-phase extraction followed by high-performance liquid chromatography purification and radioimmunoassay. The detection limit for 1,25(OH)2D3 in this assay was 17 pg/mL (44.2 pmol/L). A minimum of 0.5 mL of serum was required to perform serum vitamin D metabolite analysis, which necessitated the pooling of samples within dose groups.

STATISTICAL ANALYSIS

Tumor size, weight, and volume as well as animal weight were analyzed using a 1-way analysis of variance (ANOVA) test to detect statistical differences in tumor size among the groups. Pairwise comparisons were then performed to detect statistical differences between particular dose groups. Tumor size, weight, and volume were transformed to the log scale before analysis to obtain approximately normal distributed residuals. Differences were considered significant at P<.05.

Kidneys were harvested and histologically processed from 15 mice in the control group, 6 mice each in the 0.1 µg and 0.2 µg groups, 12 mice in the 0.3 µg group, 9 mice in the 0.6 µg group, and 3 mice in the 1.2 µg group. One masked reviewer (R.J.G.) graded calcification as none, mild, moderate, or severe. The calcification of all kidneys was rated as none to mild with the exception of 1 kidney in the 0.3 µg dose group (moderate), 2 kidneys in the 0.6 µg dose group (moderate), and 1 kidney in the 1.2 µg dose group (moderate). No kidneys were rated as having severe calcification.

SEWER VITAMIN D METABOLITE ANALYSIS

Serum obtained from the mice prior to euthanasia was pooled and analyzed at BoneCare International for levels of 1,25(OH)2D3. There was a strong relationship between dose and serum level of the drug (Figure 4). However, the available volume of serum for analysis was limited, so statistical analysis could not be performed (N=8).

The need for improved treatment alternatives for retinoblastoma is generally acknowledged. The most widely
used current treatment, external beam radiation, is associated with a 35% or higher risk of secondary cancers in patients with bilateral retinoblastoma studied during a 30-year period.\textsuperscript{18-20} Radiation therapy also increases the risks of adverse visual consequences as well as cosmetic and functional abnormalities. Large retinoblastoma tumors and those with vitreous seeding respond poorly to radiation therapy and often require enucleation. Chemotherapy, particularly triple therapy using carboplatin with vincristine sulfate and teniposide, is increasingly being used for the chemoreduction of tumors but can be mutagenic and is linked to an increased risk of secondary cancers in treated patients.\textsuperscript{1,20,21}

In our study, we tested a vitamin D analogue, 1\textalpha/-OH-D\textsubscript{2}, that was recently approved for human use against a human cell line of retinoblastoma (Y-79) and that was subcutaneously injected into athymic mice. Previous studies with this compound have focused primarily on the characterization and metabolic action of the drug,\textsuperscript{13,14,22} phase 1 clinical trials for prostate cancer (George Wilding, MD, written communication, June 2001), and its efficacy in the treatment of secondary hyperparathyroidism.\textsuperscript{13} This is the first study that describes the antineoplastic properties of 1\textalpha/-OH-D\textsubscript{2} in an animal model of retinoblastoma. Our results indicate that 1\textalpha/-OH-D\textsubscript{2}, a compound not known to be a mutagen, can inhibit tumor growth in a nontoxic dose range.

Using the data generated from the preliminary toxicity experiment, we designed a study to examine combined dose-response and toxicity characteristics in this mouse model. Animals receiving doses of 0.1 to 0.3 µg of 1\textalpha/-OH-D\textsubscript{2} had similar survival percentages (68%, 65%, and 62%, respectively, in each dose group) (Figure 2). With 1\textalpha/-OH-D\textsubscript{2}, the mortality rate in the control group was 15% and ranged from 32% to 38% in the groups receiving lower effective treatment doses. Comparing this with treatment of similar mice with vitamin D\textsubscript{2}, the latter compound resulted in a 0% mortality rate in the controls and a 46% mortality rate in the low-dose (2.8 mg/kg) group.\textsuperscript{6} This dose of vitamin D\textsubscript{2} had a comparable tumor effect with 0.1 to 0.3 µg of 1\textalpha/-OH-D\textsubscript{2}. Looking at previous results with calcitriol in athymic mice, we found a control mortality rate of 10% in the control group and 60% for the therapeutic dose that was comparable with 0.1 to 0.3 µg of 1\textalpha/-OH-D\textsubscript{2}.\textsuperscript{10} On the basis of mortality rate, 1\textalpha/-OH-D\textsubscript{2} appears less toxic than vitamin D\textsubscript{2} or calcitriol.

We suggest that the mortality rates in this study can be attributed to 3 factors in addition to the calcemic effect of the drug: (1) the learning curve of the personnel treating the animals (for 1\textalpha/-OH-D\textsubscript{2}, gastric tubes were used to deliver the drug as opposed to intraperitoneal injection for vitamin D\textsubscript{2} and calcitriol); (2) the sensitivity of the immunocompromised athymic mouse model; and (3) the stress of the 0.1% calcium diet (a full-calcium diet was previously used with vitamin D\textsubscript{2} and calcitriol). Current studies in our laboratory using similar doses of 1\textalpha/-OH-D\textsubscript{2} in mice with intact immune systems have resulted in lower mortality rates (D. M. A., unpublished data, August 2001).

Tumor size, weight, and volume were observed to be reduced the most significantly in the 0.3 µg dose group when compared with controls. However, reduction in animal weight was also highly significant in the 0.3 µg dose

**Table 1.** Mortality rate of all animals in the preliminary toxicity study with 1\textalpha/-hydroxyvitamin D\textsubscript{2} (1\textalpha/-OH-D\textsubscript{2}). Control animals received coconut oil.

<table>
<thead>
<tr>
<th>Dose Group</th>
<th>Body Weight, g</th>
<th>Tumor Weight, g</th>
<th>Tumor Size, mm\textsuperscript{2}</th>
<th>Tumor Volume, mm\textsuperscript{3}</th>
<th>Serum Calcium, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.43 ± 0.64</td>
<td>1.76 ± 0.51</td>
<td>2914.88 ± 713.03</td>
<td>2914.83 ± 83.33</td>
<td>8.33</td>
</tr>
<tr>
<td>0.1 µg</td>
<td>18.58 ± 0.86</td>
<td>1.33 ± 0.56</td>
<td>1688.09 ± 594.23</td>
<td>1688 ± 68.8</td>
<td>9.55</td>
</tr>
<tr>
<td>0.2 µg</td>
<td>17.01 ± 0.95</td>
<td>0.63 ± 0.30</td>
<td>1087.23 ± 436.18</td>
<td>1087 ± 66.8</td>
<td>11.66</td>
</tr>
<tr>
<td>0.3 µg</td>
<td>15.46 ± 0.69</td>
<td>0.73 ± 0.23</td>
<td>1102.09 ± 291.18</td>
<td>1102 ± 66.8</td>
<td>11.25</td>
</tr>
<tr>
<td>0.6 µg</td>
<td>15.81 ± 0.71</td>
<td>0.96 ± 0.31</td>
<td>1251.49 ± 344.38</td>
<td>1251 ± 66.8</td>
<td>11.42</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD unless otherwise indicated. To convert serum calcium values to SI units (mmol/L), multiply by 0.25.
group vs the control group. Tumor size, tumor volume, and tumor weight were not significantly different between the 0.2 µg and 0.3 µg groups, whereas animal weight was significantly different from controls in those groups (Table). This may indicate that between the doses of 0.2 µg and 0.3 µg in this model, there is a relatively large difference in the level of toxicity but a small difference in tumor inhibition.

Inhibition of tumor growth with corresponding low toxicity is an ideal outcome when searching for alternatives for cancer treatments. As noted previously, other vitamin D compounds and analogues have been studied in our laboratory and are inhibitors of retinoblastoma in studies of Y-79 cell lines and mouse models.6,9,18 We demonstrated that, although vitamin D3 and calcitriol (1,25-dihydroxycholecalciferol) were effective in inhibiting retinoblastoma tumors in athymic and transgenic mice, they were highly toxic.6,10 All groups treated with these agents experienced hypercalcemia, weight loss, and death. A synthetic analogue of calcitriol, 16,23-D3 (1,25-dihydroxy-16-ene-23-yne-vitamin D3), has shown an impressive tumor-inhibiting effect in previous experiments.12,15,24 In our studies with 16,23-D3, in the athymic Y-79 retinoblastoma model and a transgenic mouse model, toxicity was a limiting factor in dose selection.12,24 In comparing the present study with a similar study of 16,23-D3 in the same mouse model,15 1α-OH-D2 appears to be similar in tumor reduction capability at 0.3 µg/d in comparison with 0.5 µg/d of 16,23-D3 (ie, an approximately 44%-45% reduction in mean tumor size when compared with controls) while having a similar survival rate to animals treated with 16,23-D3.

The mechanism of action of 1α-OH-D2 and other synthetic analogues and vitamin D compounds in limiting tumor growth is not fully understood. Calcitriol and its analogues inhibit cellular proliferation in several malignant cell types including retinoblastoma and breast, colon, renal, and lung carcinomas.25-28 It is hypothesized that the antiproliferative effects of these compounds are mediated by a vitamin D receptor–linked mechanism, although exceptions have been reported.29-32 Considerable evidence indicates that the antineoplastic and differentiating effects of vitamin D compounds result from alterations in the fundamental cellular processes of proliferation, differentiation, and apoptosis. The resulting key biochemical events are related to activation of cyclin-dependent kinase inhibitors such as p21.33-36

Calcitriol and vitamin D analogues can induce apoptosis in leukemic (HL60) cells as well as human breast cancer and colon cancer cell lines.37-39 Recent studies have shown that human retinoblastoma and retinoblastoma cell lines are extremely susceptible to p53-mediated apoptosis,40 and preliminary studies in our laboratory have shown that Y-79 xenografts in athymic mice treated with 16,23-D3 result in tumor growth attenuation through increased apoptotic cell death (Robert W. Nickells, PhD, and Daniel M. Albert, MD, unpublished data, November 1999). We hypothesize that 1α-OH-D2 has a similar mechanism of action against retinoblastoma tumors in the athymic mouse, and studies are planned to determine if this is correct. We believe that 1α-OH-D2 has potential value in the treatment of human retinoblastoma; a phase 1 clinical trial is being developed.

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