Antineoplastic Effect and Toxicity of 1,25-Dihydroxy-16-ene-23-yne-vitamin D$_3$ in Athymic Mice With Y-79 Human Retinoblastoma Tumors

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**Objectives:** To evaluate the in vivo efficacy and toxicity of the 1,25-dihydroxy-16-ene-23-yne-vitamin D$_3$ (16,23-D$_3$) analogue in athymic nude mice injected with Y-79 human retinoblastoma cells and to compare the efficacy and toxicity of this compound with those of 1,25-dihydroxycholecalciferol (D$_3$, calcitriol).

**Methods:** Thirty athymic nude mice (4-6 weeks old) were injected subcutaneously with $1 \times 10^7$ Y-79 human retinoblastoma cells suspended in a 1:1 mixture of Iscove culture medium supplemented with 20% fetal bovine serum and basement membrane matrix suspension. Five days after tumor injection, the mice were randomized to 3 groups of 10 mice each. The first group served as a control group and received intraperitoneal injections of 0.25 mL of mineral oil (vehicle) 5 times a week. The second group received intraperitoneal injections of 0.05 µg of calcitriol in 0.25 mL of mineral oil (vehicle) 5 times a week. The third group received intraperitoneal injections of 0.5 µg of 16,23-D$_3$ in 0.25 mL of mineral oil 5 times a week. Injections were continued for 5 weeks, during which tumor size and mouse weight were individually measured. Toxicity was assessed by clinical measures such as lethargy, weight loss, and death. The mice were then killed and the size, volume, and weight of each tumor were determined. Also, in representative animals in each group, kidneys were evaluated for calcification and serum calcium concentration was measured.

**Results:** All experimental and control animals developed tumors subcutaneously. The 16,23-D$_3$-treated mice had significantly smaller average tumor size (1.55 cm$^3$) than the control mice (3.45 cm$^3$) ($P = .02$), less gain in average body weight from the beginning of treatment (2.4 g vs 3.5 g) ($P = .06$), and a 40% mortality. The calcitriol-treated mice did not have significantly smaller average tumor size (1.26 cm$^3$) than the 16,23-D$_3$-treated mice ($P = .35$), had significant body weight loss compared with the control animals (calcitriol-treated mice lost 4.03 g) ($P = .001$), and had a mortality of 90% by the completion of the experiment. Histologically, there was no difference in the degree of tumor necrosis and calcification between control and experimental mice. Serum calcium concentrations were equivalent between the control (2.15 mmol/L [8.6 mg/dL]) and experimental groups (calcitriol, 1.88 mmol/L [7.5 mg/dL] [$P = .97$]; 16,23-D$_3$, 2.15 mmol/L [8.6 mg/dL] [$P = .42$]). Mild bilateral renal tubular calcification occurred in 3 of 4 mice in the calcitriol-treated group and in 2 of 4 mice in the 16,23-D$_3$-treated group.

**Conclusions:** The growth of subcutaneous Y-79 human retinoblastoma cells in athymic nude mice is significantly reduced by treatment with intraperitoneal injections of 16,23-D$_3$. The antineoplastic effect of calcitriol is not statistically significantly different but is associated with significantly more toxicity. 1,25-Dihydroxy-16-ene-23-yne-vitamin D$_3$ may be a useful chemotherapeutic adjunct in the treatment of retinoblastoma.


**The Role of vitamin D as a regulator of calcium and phosphate metabolism has been known since early in this century.** However, recent interest has been generated by the finding of other biological functions of this class of compounds. Functional receptors for vitamin D have been found on numerous normal cell types that are not involved in mineral metabolism, including retina, brain, pituitary, pancreas, muscle, female reproductive organs, bone marrow, and thymus. Studies have also shown vitamin D receptors on numerous types of malignant cells, including retinoblastoma, breast, colon, renal, and lung carcinomas, as well as various malignant neoplasms of the reticular hematopoietic system. Studies on the effects of calcitriol (vitamin D$_3$) and its chemically modified analogues on these tumors have demonstrated a significant antineoplastic and differentiating effect.

Verhoef\(^{19}\) in 1966 proposed the use of vitamin D for the treatment of retino-
MATERIALS AND METHODS

EFFECT OF MATRIGEL ON IN VIVO RETINOBLASTOMA GROWTH

Six athymic nude mice were randomized to experimental and control groups, each containing 1 male and 2 females. The mice in the experimental group received $1 \times 10^7$ Y-79 retinoblastoma cells in 0.50 mL of a 1:1 mixture of Matrigel and Iscove culture medium supplemented with 20% fetal bovine serum (Mediatech Inc, Herndon, Va.) The control mice received $1 \times 10^7$ Y-79 retinoblastoma cells in 0.50 mL of Iscove culture medium with fetal bovine serum. The tumors were measured with calipers in 3 dimensions 5 times a week for 4 weeks, and the tumor volume was approximated in cubic centimeters by taking the product of the 3 measurements. At the completion of the experiment, the mice were killed in a carbon dioxide chamber and the tumors were excised and evaluated histologically.

ANIMALS

Thirty female athymic nude mice were kept in a laminar air-flow room under sterile conditions. The details of animal care concerning athymic mice treated with vitamin D and its analogues have been previously reported. The mice were 4 to 6 weeks old at the start of the experiment. There were no more than 4 mice per cage. They were fed a vitamin D–and calcium-deficient, irradiated diet (Purina Mills Inc, St Louis, Mo). Five days after tumor injection, the mice were randomized into 3 groups of 10 mice each, corresponding to the control, calcitriol-treated, and 16,23-D$_3$–treated mice.

TUMOR INJECTION AND ANALYSIS

The Y-79 human retinoblastoma cell line was cultured and suspended in Iscove culture medium with fetal bovine serum. Cell counts were done with a hemocytometer with the use of the trypan blue dye exclusion test to verify cell viability. This suspension was then mixed in a 1:1 ratio with Matrigel basement membrane matrix suspension to a concentration of $1.0 \times 10^7$ cells per 0.50 mL. The mice were then injected with 0.50 mL of this suspension subcutaneously in the dorsal midscapular region. The tumor size was measured daily 5 times a week in 3 dimensions by means of calipers and the volume was approximated by multiplying the 3 measurements. At the completion of the experiment, tumors were excised and histological study was performed on the tumors with hematoxylin-eosin stain. In addition, von Kossa–stained preparations were examined for the presence of calcium.

DRUG TREATMENT

Intraperitoneal injections with mineral oil, calcitriol, and 16,23-D$_3$ were started 5 days after tumor injection, to allow the tumor time to grow and become established. Pure crystalline 16,23-D$_3$ (provided by Milan Uskokovic, PhD, Hoffmann-LaRoche Inc, Nutley, NJ, and by Ilex Inc, San Antonio, Tex) was prepared for injection as previously described. The experimental mice received 0.05 µg of calcitriol or 0.5 µg of 16,23-D$_3$ in 0.25 mL of mineral oil vehicle intraperitoneally 5 times a week for 5 weeks (Monday through Friday). Injections were withheld if any signs of toxic effects developed, such as lethargy or weight loss greater than 25% during a 1-week period. The control mice received 0.25 mL of mineral oil intraperitoneally 5 times a week for 5 weeks.

KIDNEY EVALUATION

Both kidneys of 4 mice randomly selected from each group were evaluated histologically with hematoxylin-eosin and von Kossa stains for evidence of calcification and metastases.

STATISTICAL ANALYSIS

Serum calcium level, change in body weight, and final tumor volume were compared by $t$ tests where the variances in the treatment groups were estimated separately. No data transformation was found to stabilize the variance without introducing nonnormal data distributions. The need for estimating separate variances ruled out analysis of variance as an approach for these data. Because of the insufficient number of mice for statistical analysis in the D$_3$ group, the final tumor volumes were compared 3 days before the animals were killed.

blasta. He based his suggestion on the frequent observation of calcification in retinoblastoma, especially when it undergoes spontaneous or induced regression. He reasoned from this that calcifying agents such as vitamin D may therefore cause regression of these tumors. Studies have now demonstrated the presence of vitamin D receptors in human retinoblastoma cells in vitro as well as a significant antineoplastic effect of calcitriol on retinoblastoma cells in vitro, retinoblastoma xenografts in athymic mice, and heritable, spontaneously developing retinoblastoma in transgenic mice. These studies also documented marked hypercalcemia and high morbidity and mortality occurring with doses sufficient for tumor inhibition.

The occurrence of this toxic effect has led to the search for chemically modified analogues of calcitriol that retain its antineoplastic properties but not its calcemic effects. One of the most promising of these synthetic analogues is 1,25-dihydroxy-16-ene-23-yne-vitamin D$_3$ (16,23-D$_3$). It causes 10 to 25 times less hypercalcemia and retains the antineoplastic properties of the parent compound. This vitamin D analogue has been shown to have significant antineoplastic effect against retinoblastoma tumors in transgenic mice and an improved toxicity profile. However, its effect on human retinoblastoma cells in vivo has not been evaluated. In this study, we investigated the antineoplastic effect of 16,23-D$_3$ on Y-79 human retinoblastoma cells injected subcutaneously into athymic nude mice.

We have used the athymic nude mouse model because it has long been recognized to provide useful information regarding the in vivo evaluation of hetero-
transplanted human tumors.24,25 Athymic mice are homozygous for a recessive mutation that leads to agenesis of the thymus gland, and are therefore immunodeficient.26 This allows heterotransplanted tumors to grow uninhibited by the immune response. We have used these mice previously to study the effect of calcitriol and ergocalciferol on retinoblastoma.20,21 In the present experiment we used them to study the effect of 16,23-D3 on human retinoblastoma in vivo.

A preliminary experiment was performed to evaluate the effect of Matrigel basement membrane matrix (Becton Dickinson Labware, Bedford, Mass) on retinoblastoma tumor cell growth. This is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins. Its major component is laminin, followed by collagen type IV, heparan sulfate proteoglycans, entactin, and nidogen.27 It also contains transforming growth factor β, fibroblast growth factor, tissue plasminogen activator,28 and other growth factors that occur naturally in the Engelbreth-Holm-Swarm tumor. This matrix has proved effective for the attachment and differentiation of both normal and transformed anchorage-dependent cells.29,30 We evaluated its effect on human retinoblastoma cell growth in vivo.

#### RESULTS

**PRELIMINARY MATRIGEL STUDY**

All mice in the Matrigel group developed tumors. One of the control mice showed no evidence of tumor either grossly or on histological examination of the subcutaneous tissue at the injection site. The tumors in the Matrigel group grew significantly larger than those in the control group (Figure 1). Final average tumor size was 2.8 cm³ in the experimental group vs 0.52 cm³ in the control group. Histologically, no qualitative difference in morphological features, calcification, or necrosis was present between the control and experimental groups.

**TUMOR SIZE**

All of the experimental and control mice developed single or multiple tumors at the site of injection. Data regarding tumor size are summarized in the Table. The rate of growth and final volume of tumor were greater in the control mice than in the treated mice (Figure 2 and Figure 3). Mice treated with 16,23-D3 showed significantly smaller final tumor size (1.55 cm³) than the control mice (3.45 cm³) (P = .02). The tumor volume of the calcitriol-treated mice (1.26 cm³) analyzed on the 32nd day was not significantly different from that of the 16,23-D3–treated mice (P = .35). (On the 35th day when the experiment was terminated, there were insufficient calcitriol-treated mice surviving to permit analysis.) The final
average weights of the tumors (Figure 4) parallel the volume measurements closely.

TOXIC EFFECTS

All of the mice in the calcitriol-treated group appeared lethargic as indicated by inactivity. They lost an average of 4.03 g during the treatment period vs an average weight gain of 5.50 g in the control mice ($P = .001$) (Figure 5) and suffered a 90% mortality rate (Figure 6). One mouse in this group died on the fifth day of treatment secondarily to injection trauma. These mice received only an average of 2.4 injections per mouse per week as opposed to the scheduled 5 injections per week because of clinical evidence of toxicity as defined above. Seven of the 9 mice died in the 2-day period in the week when injections were not done. All of the control mice survived to the end of the experiment. There was no significant difference in the measured serum calcium concentration levels between the control (2.15 mmol/L [8.6 mg/dL]) and experimental groups (calcitriol, 1.88 mmol/L [7.5 mg/dL] [$P = .42$]; 16,23-D3, 2.15 mmol/L [8.6 mg/dL] [$P = .97$]).

HISTOLOGICAL EVALUATION

Histological evaluation of the tumors in both the control and experimental mice disclosed sheets of small cells with scanty cytoplasm and prominent, dark-staining nuclei consistent with retinoblastoma. No qualitative difference in tumor morphological characteristics as regards calcification, mitotic figures, differentiation, and necrosis was present between tumors in the control and experimental groups.

RENAI CALCIFICATION

Three (75%) of 4 mice examined in the calcitriol-treated group showed evidence of moderate bilateral renal calcification. In the 16,23-D3–treated group, 2 (50%) of 4 mice had trace renal calcification. None of the control mice showed renal calcification. The calcification was confined to the renal tubules.

The results of this study demonstrate the antineoplastic efficacy of 16,23-D3 in the treatment of human retinoblastoma cells in vivo, and show less toxicity than was observed with similar treatment with calcitriol. In our preliminary experiment, we demonstrated the positive effect of Matrigel basement membrane matrix on the growth of retinoblastoma cells in vivo, with tumors growing more consistently, earlier, and more rapidly than in the control group.

We chose to evaluate the tumors in a subcutaneous location as opposed to the eye. One reason for this was that the tumors are more easily and reliably measured in this location. Additionally, tumor growth can quickly become affected by mechanical pressure from the sclera. In a subcutaneous location, these tumors can grow with-
out mechanical restriction as a confounding factor. Because vitamin D and its analogues are fat soluble, they freely cross the blood-ocular barrier\(^{16}\); therefore, any effect on tumor growth in a subcutaneous location can be inferred to occur in an intraocular location as well.

Our initial experiment with Matrigel basement membrane matrix showed a significant positive effect on tumor growth. We recognize that this matrix may introduce numerous potentially confounding biochemical and mechanical factors to the tumor growth, but since it was used in both the control and experimental mice, any such factors will cancel each other out, and treatment effect can be inferred to occur from the 16,23-D\(_3\) alone.

Mortality and toxicity, as evidenced by lethargy and weight loss, were highest in the calcitriol-treated group. This occurred despite the fact that injections were withheld in this group when the mice exhibited extreme lethargy and weight loss. Most of the deaths occurred during the 2 days (ie, Saturday and Sunday) when they were not injected. Previous studies in our laboratory\(^{22}\) and in other laboratories\(^{9}\) have demonstrated that naturally occurring calcitriol is extremely toxic at doses with antineoplastic efficacy. The calcitriol-treated mice were included as a positive control. The mortality in the 16,23-D\(_3\)-treated mice was significantly less. However, the 16,23-D\(_3\) group did not show the preceding weight loss and lethargy that we saw in the premorbid calcitriol-treated mice.

We were not able to demonstrate a significant difference in serum calcium levels between the experimental and control groups from blood obtained on the final day of the experiment. It should be noted, however, that the marked reduced calcemic effects of 16,23-D\(_3\) compared with calcitriol have been well documented in other studies.\(^{9,22,23}\) This could be because the blood was obtained after the 2-day weekly hiatus in treatment, and the calcemic effect may have already peaked and resolved when the samples were obtained. We found more severe calcification in the kidneys of the calcitriol-treated mice than the 16,23-D\(_3\)-treated mice. In a previous study carried out on transgenic mice with ocular tumors,\(^{23}\) no mortality was seen in mice receiving 0.5 mg of 16,23-D\(_3\). The 40% mortality occurring in the present experiment may be related to the greater size and different location of the tumors as well as the immunodeficient nature of the mice.

The mechanism of the antineoplastic action of vitamin D\(_3\) and its analogues is not fully understood. Verhoeff\(^{18}\) based his hypothesis of the antineoplastic action of these compounds on the frequent observation of spontaneous calcification in treated and regressed tumors. However, this hypothesis of its mechanism of action has not found support in recent experiments.\(^{32}\) The response to vitamin D occurs through the binding of an intracellular receptor, which subsequently effects transcriptional activity within the nucleus. However, direct effects also can occur through the cell membrane, mediated by G proteins, a calcium gradient, and protein kinase C.\(^{10}\) The antineoplastic effects appear to occur through inhibition of growth, induction of cellular differentiation, and antiangiogenic effects.\(^{9,11-13}\)

In a more recent experiment, we noted that smaller or more unhealthy mice tend to have smaller tumors (unpublished data, 1987). In that experiment, control mice that were injected with a more toxic vehicle weighed less and had smaller tumors. However, the tumors in both of these groups were larger than those in the analogue-treated groups. Therefore, tumor size does appear to correlate with the general health, nutritional status, and body weight of the mice. It is of interest, however, that although the 16,23-D\(_3\)-treated mice in this study weighed significantly more and appeared much healthier than the calcitriol-treated group, the size of their tumors was similar.

In summary, we have demonstrated that the vitamin D analogue 16,23-D\(_3\) has antineoplastic properties against Y-79 human tumor cells in athymic mice, and that this effect was not statistically different from that of calcitriol to a significant degree. Furthermore, the toxicity and mortality with this analogue is significantly less than with calcitriol.

The current practice of using radiation therapy for retinoblastoma is associated with a significant increase in the incidence of secondary neoplasms in the field of radiation.\(^{33,34}\) This has led to the study of chemotherapeutic agents for this tumor.\(^{35}\) However, many of these compounds are known mutagens and therefore may also carry a risk of secondary neoplasms. Vitamin D analogues, if proved effective in human trials, may help obviate these risks. The findings in this study, along with previous studies of this analogue,\(^{22}\) establish a rationale for future human clinical trials with the use of this compound as adjuvative therapy. The study of other new synthetic vitamin D analogues that may have enhanced antineoplastic efficacy and reduced toxicity\(^{14-17}\) is also of interest.

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REFERENCES

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