Toxicity and Dose-Response Studies of 1,25-$(OH)_2$-16-ene-23-yne Vitamin D_3 in Transgenic Mice[1]

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ABSTRACT

The vitamin D_3 analogue 1,25-$(OH)_2$-16-ene-23-yne vitamin D_3 (16,23-D_3) in doses with low systemic toxicity has been demonstrated to inhibit retinoblastoma growth in transgenic mice. This study examines the dose-dependent response for inhibition of tumor growth in transgenic mice with retinoblastoma and evaluates the *in vivo* toxicity of 16,23-D_3 in nontransgenic mice. Transgenic 8–10-week-old mice with retinoblastoma (n = 119) were randomly assigned to groups receiving 1.0, 0.75, 0.5, 0.35, 0.2, or 0.05 μg of 16,23-D_3 and a vehicle alone (control) group i.p. five times a week for 5 weeks. An additional control group received no injection. Eyes were enucleated one week after the end of treatment, and tumor areas were measured. To determine the toxic dose, transgene-negative littermates received 0.5, 1.0, 1.5, 2.5, 3.5, 4.5, or 5.0 μg of 16,23-D_3, and control groups received vehicle alone, 5 days a week for 5 weeks. Serum calcium levels were measured, and necropsies were performed on animals from each group. In the dose-response study, tumor growth inhibition was greatest in the group receiving 0.35 μg (55% inhibition; P = 0.0056) and was also significant in the group receiving 0.5 μg (42% inhibition; P = 0.036). The systemic toxic effects due to hypercalcemia occurred at doses of ≥1.0 μg. 16,23-D_3 inhibits tumor growth at doses ≥0.35 μg and shows toxic effects at doses ≥1.0 μg related to hypercalcemia in mice fed an unrestricted diet. No toxicity was observed with lower doses.

INTRODUCTION

Retinoblastoma is an ocular tumor that was described as a distinct entity by Wardrop in 1809 (1) and recognized to be a tumor derived from retinoblasts by Verhoeff in 1926 (2). Retinoblastoma occurs in both hereditary (bilateral) and nonhereditary (unilateral) forms. The genetic inheritance of this tumor has been extensively studied (3), and the retinoblastoma gene (*Rb*) was cloned in 1986 (4). Current methods of treatment include enucleation, external beam radiotherapy, scleral plaque brachytherapy, cryotherapy, photocoagulation, and chemotherapy (5). Unfortunately, irradiation, the most common nonsurgical treatment, carries an increased risk of secondary tumor formation in the irradiated field (6). The usefulness of the other therapies can be limited by the size and location of the tumor as well as the potential for secondary tumor formation (7, 8). Methods of chemoreduction in anticipation of conservative management are currently of extreme interest to ocular oncologists (5).

Verhoeff believed that calcification induced spontaneous regression in retinoblastoma and postulated that vitamin D might be an effective treatment (9). Vitamin D has been shown to inhibit the growth of retinoblastoma both *in vitro* (10) and *in vivo* in both the nude mouse (11) and transgenic models (12), but the effect is unrelated to either high serum calcium or calcium deposition in the tumor. Because human retinoblastoma cells have receptors for calcitriol, the cells should be sensitive to any antineoplastic drugs that exploit the presence of the receptor. The clinical usefulness of vitamin D has been limited by its toxic hypercalcemic effect (13).

16,23-D_3[3] is a promising vitamin D_3 analogue with decreased hypercalcemic properties (14). In leukemia (15, 16), breast cancer (17), prostate cancer (18), and colon cancer (19), 16,23-D_3 has the antineoplastic activity of the vitamin D analogues while having little calcium-mobilizing effect.

The LHβ-Tag mouse is a well-characterized model of retinoblastoma (20) that has been used to evaluate both retinoblastoma pathogenesis and new therapeutic modalities, including the effectiveness of 16,23-D_3 in inhibiting tumor growth (21). Through some random integration event, the SV40-Tag has been placed under retinal or related regulatory control, resulting in the development of multiple retinal tumors shortly after birth. These are morphologically identical to human retinoblastoma. If untreated, the tumors invade the optic nerve and central nervous system and also give rise to blood-borne metastases, resulting in the death of the animal. Previous studies using 16,23-D_3 in other tumor models have shown that alternate-day administration or administration combined with a reduced calcium diet can minimize the amount of hypercalcemia and the related morbidity of the various vitamin D analogues, even at doses as high as 6.4 μg (18, 22, 23). The effectiveness of the drug and the toxicity levels have not been previously studied in mice on a "normal" (*i.e.*, non-calcium-restricted) diet, and the

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[3] The abbreviation used is: 16,23-D_3, 1,25-$(OH)_2$-16-ene-23-yne vitamin D_3.

Table 1 Summary of data obtained during the 5-week toxicity study

Group	No. of animals	Calcium (mg/dl)	Weight change (%)	Surviving animals at 5 weeks	Mortality (%)
Mineral oil	12	9.70	+27.74	11	8
16,23-D_3					
0.5 μg	6	9.00	+33.33	6	0
0.75 μg	6	8.15	+32.62	5	17
1.0 μg	6	10.30	+24.76	5	17[a]
1.5 μg	6	15.60	[b]	0	100
2.5 μg	6	19.37	[b]	0	100
3.5 μg	6	19.90		0	100
4.5 μg	6	20.50		0	100
5.0 μg	6	19.50		0	100

[a] One mouse died of traumatic injuries unrelated to the study.
[b] The groups receiving 1.5 and 2.5 μg were used only to assess the temporal characteristics of the toxic effects; thus, daily weights were not obtained.

present experiments were undertaken for these purposes. The dietary status is significant, because the hypercalcemic effects of vitamin D metabolites are attributed to an increased intestinal absorption of calcium rather than to the liberation of calcium from the bone (23–25).

MATERIALS AND METHODS

16,23-D_3 Compound Preparation and Administration. Pure crystalline 16,23-D_3, provided by Dr. Milan Uskokovic (Hoffmann-LaRoche, Nutley, NJ) and by Ilex (San Antonio, TX), was prepared and administered as described previously (21). Briefly, spectrophotometric analysis was used to determine the concentration. The compound has a UV-λ maximum absorption at 264 nm. The molecular extinction is ϵ14,120, and the molecular weight is 410.6. Stock solutions were prepared in 100% ethanol and diluted in mineral oil to make an injectable emulsion of the desired concentration in a final volume of 250 μl of mineral oil.

Toxicity Trial. Sixty LHβ-Tag-negative (non-tumor-bearing) littermates were randomized to treatment groups receiving 0.5–5 μg of 16,23-D_3 or to a mineral oil-receiving control group (Table 1). Injections were given five times/week for 5 weeks, followed by a 1-week washout period in which no injections were given. The drug was injected i.p. with a 25-gauge needle.

All animals were maintained on a nonrestricted diet (Harlan Teklad 7001). Toxicity was assessed by survival, daily weight, and clinical measurements of hypercalcemia, including poor grooming and lethargy. Animals were weighed before each injection and at the end of the study. Serum calcium levels were determined on day 5 from retrobulbar samples from three animals/group. Because the majority of animals died by the end of the first week, subsequent samples were not obtained.

Dose-Response Study. A total of 119 LHβ-Tag 8–10-week-old mice were randomized by sex and litter into six treatment groups and two control groups (Table 2). The presence of the transgene was confirmed by PCR (20). All mice were maintained on an unrestricted diet (Harlan Teklad 7001). Mice in the six treatment groups each received 0.05, 0.2, 0.35, 0.5, 0.75, or 1.0 μg of 16,23-D_3 in the mineral oil vehicle. One control group received only mineral oil injections, and another control group received no injections or other treatment. Injections were administered following the protocol described in the toxicity trial. Serum calcium was determined from samples taken from the subclavian artery at the time of death. All mice were weighed before receiving their daily injection and at the end of the study.

The eyes were enucleated, fixed in 10% neutral buffered formalin, and submitted for routine histological processing. Five-μm-thick H&E-stained sections through the anatomical center of the globe in the pupil-optic nerve plane were examined. The outline of tumors in each eye was traced from a digitized image, the area of the trace was determined by the Optimas software (Bioscan, Edmonds, WA), and the average cross-sectional area for each eye was calculated (see below). The histopathological appearance of the tumors was characterized by scoring the degree of involvement of the retina and other ocular structures, the severity of necrosis, the number of mitotic figures, and the degree of calcification and differentiation. The extent and character of the inflammation was also graded.

Statistical Methods. Toxicity, measured by the percentage of mortality, weight loss, and calcium levels, was compared among groups using Fischer's exact test, ANOVA, and the Kruskal-Wallis test, respectively.

The measure of tumor size used is the average of the square root of the cross-sectional tumor area for the two eyes. The square root transformation was used to equalize the variance in the treatment groups. A one-way ANOVA was used to test for the effect of dose, followed by paired *t* tests when a significant effect was found. The Splus statistical package was used for all calculations (26).

RESULTS

Toxicity Trial. The results of the toxicity study are summarized in Table 1. All mice in the groups receiving 1.5, 2.5, 3.5, or 4.5 μg died on days 6 and 7. One mouse in the group receiving 5.0 μg survived for 2 weeks, and another survived for 4 weeks. 16,23-D_3 showed systemic toxic effects due to hypercalcemia in all mice receiving doses of ≥1.5 μg. The serum calcium level in these groups averaged 18.73 ± 0.91 mg/dl (normal, 3.2–8.5 mg/dl), and animals in these groups weighed less than controls. Microscopic examination demonstrated metastatic calcification of the peritoneum, viscera, and, particularly, the renal tubules.

Dose-Response Study. A summary of the results of the dose-response data is given in Table 2. Five of the seven (71%) mice in the group receiving 1.0 μg died by week 4 and exhibited signs of toxicity similar to those of the mice in the toxicity trial receiving that dose. Serum calcium measurements were not obtained from these mice. Necropsy examination of these animals revealed widespread calcification that was most marked in the kidneys. This dose was felt to be toxic, and this group was excluded from the analysis. A total of 102 of the remaining 112 mice (91.1%) completed the study. No signs of toxicity were observed in any of the mice completing the study. Ten mice (8.9%) died. Necropsy results on the nonsurvivors treated with doses of <1.0 μg revealed mild sterile peritonitis but no calcification of any organs or tissues. These animals were eliminated

Table 2 Summary of data obtained during the dose response study

Group	No. of mice	Survival (%)	Calcium (mg/dl)	Weight change (%)	Tumor size[a]	% Tumor growth inhibition[b]
Untreated	4	100	10.30		400.54 ± 136.64	12.36
Mineral oil	18	95	9.20	+34.83	457.02 ± 59.17	0
16,23-D_3						
0.05 μg	18	100	9.40	+30.08	478.67 ± 55.78	−4.74
0.2 μg	18	89	6.65	+37.68	451.43 ± 59.17	1.22
0.35 μg	18	89	8.88	+27.40	205.68 ± 57.40	55.00
0.5 μg	18	89	8.45	+28.56	263.48 ± 59.17	42.35
0.75 μg	18	89	8.53	+22.91	317.94 ± 61.11	30.43
1.0 μg[c]	7	29	8.00	+25.49	Not measured	Not measured

[a] Average of the square root of the right and left eye tumor area ± SE.
[b] Percentage of tumor growth inhibition = 1 − percentage of mineral oil control.
[c] This group was excluded from tumor measurements.

Table 3 Summary of variations in dosing and diet in studies of 16,23-D_3

Ref. no.	Tumor	Model	Dose	Diet	Period of time administered	Toxicity
22	None	Mouse	6.4 μg q.o.d.[a]	Calcium free		None
28	Leukemia	Mouse	2.0 μg q.o.d.	Normal		None
18	Prostate	Nude mouse	1.6 μg q.o.d.	Normal		None
21	Retinoblastoma	Transgenic mouse	0.05 μg q.d.	Calcium free	5 wk	None

[a] q.o.d., every other day.

from the study and were not included in the tumor size determination. There was no statistically significant difference in the serum calcium measurements among the groups. Tumor growth inhibition in animals receiving 16,23-D_3 ranged from no inhibition (0.04% increase) in the group receiving 0.05 μg to 55% inhibition ($P = 0.0056$). The effect was most profound in the group receiving 0.35 μg (55% inhibition), followed by the group receiving 0.5 μg (42% inhibition; $P = 0.36$) and the group receiving 0.75 μg (30% inhibition; $P = 0.1$). Microscopic examination revealed no difference in the histopathological appearance of the tumors.

DISCUSSION

In this study, we evaluated the toxicity of 16,23-D_3 in normal mice and the dose-response characteristics of a LHβ-Tag transgenic model of retinoblastoma. Although data have been reported comparing the relative antineoplastic and hypercalcemic effects of this vitamin D analogue, the toxicity in mice and the dose-response curves in endogenous tumor models have not been previously described. The toxicity data establishes 1.5 μg (approximately 60 μg/kg/day) as the toxic threshold in mice when 16,23-D_3 is given by i.p. injections, and mice are fed a nonrestricted diet. At this dose, there is hypercalcemia, weight loss, and 100% mortality. The survival of only 29% of the animals in the treatment group (Table 2) indicates that this dose is also toxic in tumor-bearing mice. Higher nontoxic doses have been achieved with alternate-day administration and/or calcium-restricted diets in mouse models of prostate cancer and leukemia (18, 22).

Our study used a protocol consisting of administration of the compound 5 days/week and an unrestricted diet. The dietary status is significant, because the hypercalcemic effects of vitamin D metabolites are attributed to increased intestinal absorption of calcium rather than to the liberation of calcium from the bone (23–25). Comparing our results with those of a previous study of tumor control using the same protocol and a calcium-free diet, we found that the tumors in our untreated controls were significantly smaller than the tumors in the controls of that study [34.6 *versus* 112 μm^2 ($P = 0.001$) or, using the square root scale, 497.5 *versus* 779.8 μm ($P < 0.001$)]. The possibility exists that this dietary difference may influence tumor size.

The pharmacokinetics of this analogue have not been fully elucidated, and the toxicity that we observed may be related to the dosing schedule. It is not known how long emulsified 16,23-D_3 persists in the peritoneal cavity before being absorbed. However, with variation in the dose and scheduling of 16,23-D_3 administration, serum calcium can be maintained at normal levels. This suggests that the absorption pattern of this compound may be manipulated to achieve overall higher drug levels without causing the toxic effects associated with hypercalcemia (see Table 3).

In a previous experiment, animals treated with 0.05 μg of 16,23-D_3 were found to have a significantly smaller tumor cross-sectional area compared with that of animals in the control group ($P = 0.02$; Ref. 21). The 0.05 μg dose was not active in the present study (see Table 2). The only apparent difference in the experiments is the diet, which was calcium-free and vitamin D-free in the previous experiment and was unrestricted in the present study. The dose-response data indicate a peak effect at 0.35 μg. The toxic effects in the transgenic mice, however, were seen at lower doses than in their normal, nontransgenic littermates. In the dose-response study, decreased weight gain and 17% mortality were observed at the 1 μg (40 μg/kg/day) dose level.

The degree of tumor growth inhibition in this model suggest that 16,23-D_3 may be effective in inhibiting the growth of human retinoblastoma. It was of interest that two of the animals in the high-dose (0.5 μg) group were found not to have tumors on gross and microscopic examination. Because no animals in the control group in the present experiment or in previous studies showed an absence of tumor formation, we believe this represents a "response" as well as a slowing of growth, and these animals were considered to have a tumor cross-sectional area of 0. The design of the present experiment did not permit us to distinguish between regression of tumors and slowing of their evolution. If the level of 16,23-D_3 can be raised, we may be able to achieve greater inhibition of tumor growth. Alterations in the dosing regimen have also been effective in avoiding the toxic effects at moderate doses (up to 2.0 μg; Ref. 22). At low-to-moderate doses, 16,23-D_3 has shown promise in the treatment of both hematological and solid tumors (15, 18, 27). In most of the previous studies involving 16,23-D_3, tumor cells were evaluated *in vitro* in xenografts in immunocompromised models. Transgenic models offer the opportunity to evaluate 16,23-D_3 in the treatment of an endogenous malignancy. Models with endogenously arising tumors are advantageous, because the tumors are native to the host, genetically identical, and arise and progress in a predictable, reproducible manner.

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REFERENCES

1. Wardrop, J. Observations on the Fungus Haematodes. Edinburgh: Constable, 1809.

2. Verhoeff, F., and Jackson, E. Minutes of the proceedings: Sixty-second Annual Meeting. Trans. Am. Ophthalmol. Soc., *24:* 38, 1926.

3. Knudson, A. G., Jr. Mutation and cancer: statistical study of retinoblastoma. Proc. Natl. Acad. Sci. USA, *68:* 820–823, 1971.

4. Friend, S., Bernard, R., Rogelj, S., Weinberg, R. A., Rapaport, J. M., Albert, D. M., and Dryja, T. P. A human DNA sequence with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature (Lond.), *323:* 643–646, 1986.

5. Ferris, F., and Chew, E. A new era for the treatment of retinoblastomas. Arch. Ophthalmol., *114:* 1412, 1996.

6. Gallie, B., Budnig, A., DeBoer, G., Thiessen, J. J., Koren, G., Verjee, Z., Ling, V., and Chan, H. S. Chemotherapy with focal therapy can cure intraocular retinoblastoma without radiotherapy. Arch. Ophthalmol., *114:* 1321–1328, 1996.

7. Murphree, A., Villablanca, J., Deegan, W., III, Sato, J. K., Malogolowkin, M., Fisher, A., Parker, R., Reed, E., and Gomer, C. J. Chemotherapy plus local treatment in the management of intraocular retinoblastoma. Arch. Ophthalmol., *114:* 1348–1356, 1996.

8. Murray, T., O'Brien, J., Steeves, R., Smith, B. J., Albert, D. M., Cicciarelli, N., Markoe, A. M., Tompkins, D. T., and Windle, J. J. Radiation therapy and ferromagnetic hyperthermia in the treatment of murine transgenic retinoblastoma. Arch. Ophthalmol., *114:* 1376–1381, 1996.

9. Verhoeff, F. H. Retinoblastoma undergoing spontaneous regression. Calcifying agent suggested in treatment of retinoblastoma. Am. J. Ophthalmol., *62:* 573–574, 1966.

10. Saulenas, A., Cohen, S., Key, L., Winter, C., and Albert, D. Vitamin D and retinoblastoma: the presence of receptors and inhibition of growth *in vitro*. Arch. Ophthalmol., *106:* 533–535, 1988.

11. Cohen, S., Saulenas, A., Sullivan, C., and Albert, D. Further studies of the effect of vitamin D on retinoblastoma inhibition with 1,25 dihydroxycholecalciferol. Arch. Ophthalmol., *106:* 541–543, 1988.

12. Albert, D. M., Marcus, D. M., Gallo, J. P., and O'Brien, J. M. The antineoplastic effect of vitamin D in transgenic mice with retinoblastoma. Investig. Ophthalmol. Vis. Sci., *33:* 2354–2364, 1992.

13. Reichel, H., Koeffler, H., and Norman, A. The role of the vitamin D endocrine system in health and disease. N. Engl. J. Med., *320:* 980–991, 1989.

14. Bouillon, R., Verstuyf, A., Verlinden, L., Allewaert, K., Branisteanu, D., Mathieu, C., and van Baelen, H. Non-hypercalcemic pharmacological aspects of vitamin D analogs. Biochem. Pharmacol., *50:* 277–283, 1995.

15. Jung, S. J., Lee, Y. Y., Pakkala, S., de Vos, S., Elsmer, F., Norman, A. W., Green, J., Uskokovic, M., and Koeffler, H. P. 1,25(OH)2–16-ene-vitamin D_3 is a potent antileukemic agent with low potential to cause hypercalcemia. Leuk. Res., *18:* 453–463, 1994.

16. Clark, J. W., Posner, M. R., Marsella, J. M., Santos, A., Uskokovic, M., Eil, C., and Lasky, S. R. Effects of analogs of 1,25(OH)2 vitamin D_3 on the proliferation and differentiation of the human chronic myelogenous leukemia cell line, RWLeu-4. J. Cancer Res. Clin. Oncol., *118:* 190–194, 1992.

17. Vandewalle, B., Hornez, L., Wattez, N., Revillion, F., and Lefebvre, J. Vitamin D_3 derivatives and breast tumor cell growth: effect on intracellular calcium and apoptosis. Int. J. Cancer, *61:* 806–811, 1995.

18. Schwartz, G. G., Hill, C. C., Oeler, T. A., Becich, M. J., and Bahnson, R. R. 1,25-Dihydroxy-16-ene-23-yne-vitamin D_3 and prostate cancer cell proliferation *in vivo*. Urology, *46:* 365–369, 1995.

19. Shabahang, M., Buras, R. R., Davoodi, F., Schumaker, L. M., Nauta, R. J., Uskokovic, M. R., Brenner, R. V., and Evans, S. R. Growth inhibition of HT-29 human colon cancer cells by analogues of 1,25-dihydroxyvitamin D_3. Cancer Res., *54:* 4057–4064, 1994.

20. Windle, J. J., Albert, D. M., O'Brien, J. M., Marcus, D. M., Disteche, C. M., Bernards, R., and Mellon, P. L. Retinoblastoma in transgenic mice. Nature (Lond.), *343:* 665–669, 1990.

21. Shternfeld, I. Antineoplastic effect of 1,25-dihydroxy-16-ene-23-yne-vitamin D_3 analog in transgenic mice with retinoblastoma. Arch. Ophthalmol., *114:* 1396–1401, 1996.

22. Zhou, J., Norman, A., Chen, D., Sun, G., Uskokovic, M., and Koeffler, H. 1,25-Dihydroxy-16-ene-23-yne-vitamin D_3 prolongs survival time of leukemic mice. Proc. Natl. Acad. Sci. USA, *87:* 3929–3932, 1990.

23. Mortensen, J. T., Brinck, P., and Binderup, L. Toxicity of vitamin D analogues in rats fed diets with standard or low calcium contents. Pharmacol. Toxicol., *72:* 124–127, 1993.

24. Zhou, L. X., Nemere, I., and Norman, A. W. 1,25-Dihydroxyvitamin D_3 analog structure-function assessment of the rapid stimulation of intestinal calcium absorption (transcaltachia). J. Bone Miner. Res., *7:* 457–463, 1992.

25. Norman, A. W., Sergeev, I. N., Bishop, J. E., and Okamura, W. H. Selective biological response by target organs (intestine, kidney, and bone) to 1,25-dihydroxyvitamin D_3 and two analogues. Cancer Res., *53:* 3935–3942, 1993.

26. Chambers, J. H. Statistical Models in S. Belmont, CA: Wadsworth, 1992.

27. Vandewalle, B., Wattez, N., and Lefebvre, J. Effects of vitamin D_3 derivatives on growth, differentiation and apoptosis in tumoral colonic HT 29 cells: possible implication of intracellular calcium. Cancer Lett., *97:* 99–106, 1995.

28. Pakkala, S., de Vos, S., Elstner, E., Rude, R. K., Uskokovic, M., Binderup, L., and Koeffler, H. P. Vitamin D_3 analogs: effect on leukemic clonal growth and differentiation, and on serum calcium levels. Leuk. Res., *19:* 65–72, 1995.

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