

## 20-Hydroxyvitamin D<sub>3</sub> Inhibits Proliferation of Cancer Cells with High Efficacy while Being Non-toxic

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**Abstract.** Aim: To define the potential utility of 20-hydroxyvitamin D<sub>3</sub> (20(OH)D<sub>3</sub>) as a tumorostatic agent, we assessed its *in vitro* antiproliferative activity and its *in vivo* toxicity. Materials and Methods: The antitumor activity of 20(OH)D<sub>3</sub> was tested against breast and liver cancer cell lines using colony formation assays. To assess *in vivo* toxicity, mice were injected with 5-30 µg/kg 20(OH)D<sub>3</sub> intraperitoneally each day for 3 weeks. Blood and organ samples were collected for clinical pathology analyses. Results: 20(OH)D<sub>3</sub> displays similar tumorostatic activity towards MDA-MB-453 and MCF7 breast carcinomas, and HepG2 hepatocarcinoma, in a dose-dependent manner. This compound is not hypercalcemic, does not cause detectable toxicities in liver, kidney, or blood chemistry in mice at a dose as high as 30 µg/kg. In contrast, both 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> caused severe hypercalcemia at a dose of 2 µg/kg. Conclusion: 20(OH)D<sub>3</sub> possesses high efficacy for inhibiting cancer cell proliferation *in vitro* and is non-toxic *in vivo*, supporting its further development as a potential anticancer therapeutic agent.

It is well established that vitamin D has a broad spectrum of benefits to human health, including regulation of bone formation, and antiproliferative and anti-inflammatory effects (1-3). However, the pharmacological use of vitamin D or its analogs is limited because of hypercalcemic effects (elevated levels of calcium in the blood stream) which can result in organ failure or even death (1).

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Key Words: 20-Hydroxyvitamin D<sub>3</sub>, antiproliferative activity, hypercalcemic effect, colony formation, HepG2 cells, MCF7 cells.

Extensive efforts to make new vitamin D analogs which retain the desired biological efficacy without the hypercalcemia effect have been pursued by both academia and industry, but the FDA has not yet approved any such drugs for cancer treatment. We recently reported the identification of a biologically active set of novel vitamin D metabolites that are initially generated from the action of Cytochrome P450scc (4-11). Out of the many metabolites identified, 20(OH)D<sub>3</sub> has consistently shown strong antiproliferative activity towards human keratinocytes cultured *in vitro* (5, 12, 13). Previous studies have shown that it is non-calcemic at doses up to 3 µg/kg in rats, while the native ligand for the vitamin D receptor, 1α,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), showed strong hypercalcemic effects at only 0.1 µg/kg, which caused secondary damage to the kidney and the heart (12).

The major obstacle in using 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs for disease prevention or treatment is their small therapeutic window (14). Given that in cell culture 20(OH)D<sub>3</sub> displays comparable potency to 1,25(OH)<sub>2</sub>D<sub>3</sub>, but in rats does not raise calcium levels at doses well above that of a calcemic dose of 1,25(OH)<sub>2</sub>D<sub>3</sub> (12), it is likely that 20(OH)D<sub>3</sub> has a substantially greater therapeutic window than 1,25(OH)<sub>2</sub>D<sub>3</sub>. To further evaluate this, we here report on the effects of 20(OH)D<sub>3</sub> in inhibiting cancer cell colony formation using three well-established cancer cell lines (HepG2, MCF7 and MDA-MB-453). We also performed *in vivo* studies designed to test the blood chemistry and clinical pathological responses at progressively higher doses of 20(OH)D<sub>3</sub> that are likely to be higher than the effective dose required for pharmacological benefit. Our study reveals that even at very high doses (30 µg/kg), 20(OH)D<sub>3</sub> is non-toxic and non-calcemic. Collectively, our results strongly support the further development of 20(OH)D<sub>3</sub> for potential cancer prevention or treatment, either as a single agent or in combination with other agents.

## Materials and Methods

**Production of 20(OH)D<sub>3</sub>.** 20(OH)D<sub>3</sub> was produced by the enzymatic hydroxylation of vitamin D<sub>3</sub> catalyzed by cytochrome P450scc in a manner similar to that described elsewhere (6, 15). A stock solution of vitamin D<sub>3</sub> was prepared by dissolving it in 45% 2-hydroxypropyl-β-cyclodextrin to a final concentration of 10 mM. Incubations (12.5 ml) were carried out in a buffer comprising 20 mM HEPES (pH 7.4), 100 mM NaCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, 2 μM human cytochrome P450scc, 10 μM adrenodoxin, 0.3 μM adrenodoxin reductase, 2 mM glucose 6-phosphate, 2 U/ml glucose 6-phosphate dehydrogenase, and 50 μM NADPH. Stock vitamin D<sub>3</sub> solution (200 μl) was added to the incubation mixture to give a final vitamin D<sub>3</sub> concentration of 200 μM and a 2-hydroxypropyl-β-cyclodextrin concentration of 0.9%. Samples were pre-incubated for 8 min, reactions started by the addition of NADPH and incubations were carried out for 3 h at 37°C with shaking. Reactions were stopped by the addition of 20 ml of ice-cold dichloromethane and products were extracted with dichloromethane (15). Purification of 20(OH)D<sub>3</sub> by preparative thin-layer chromatography was carried out as described elsewhere (15) using 20 cm × 20 cm × 0.2 mm silica gel 60 plates which were developed three times in hexane/ethyl acetate (3:1 v/v). The 20(OH)D<sub>3</sub> was further purified by preparative reverse-phase high-performance liquid chromatography (HPLC) using a Brownlee Aquapore C18 column (25 cm × 10 mm, particle size 20 μm) with an isocratic mobile phase of 85% methanol in water at a flow rate of 1.5 ml/min. The typical yield of purified 20(OH)D<sub>3</sub> from a 12.5 ml incubation was 0.3 mg. The 20(OH)D<sub>3</sub> was divided, dried and stored at -80°C until use.

**Cell culture.** Hepatocellular carcinoma cells (HepG2) and breast carcinoma cells (MDA-MB-453 and MCF7) (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotic/antimycotic mixture.

**Colony forming assay.** The assay followed standard methodology as previously described (5, 16). Briefly, cells were plated in 24-well plates at a density of 20 cells/9.6 cm<sup>2</sup> in medium containing 5% charcoal-treated FBS, 1% antibiotic solution and 20(OH)D<sub>3</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub>, or 25(OH)D<sub>3</sub> at graded concentrations, or ethanol (vehicle control). Cells were cultured at 37°C for 7 days with media being changed every 3 days. At the end of the incubation, the colonies were fixed with 4% paraformaldehyde in phosphate buffer solution (PBS) overnight at 4°C, washed, stained with 5% crystal violet in PBS for 30 minutes, rinsed, and air-dried. The number and size of the colonies were measured using an ARTEK counter 880 (Dynex Technologies Inc., Chantilly, VA, USA). Colony-forming units (CFU) were calculated by dividing the number of colonies by the number of cells plated and then multiplying by 100.

MCF7 cells were grown in soft agar as previously described (17). The tumorigenicity of HepG2, MCF7 and MDA-MB-453 cells were determined by their ability to form colonies in soft agar. Cells were grown in monolayer, trypsinized, and re-suspended (1,000 cells/well) in 0.25 ml medium containing 0.4% agarose and 10% charcoal-stripped serum. Cell suspensions were added to a 0.8% agar layer in 24-well plates. 20(OH)D<sub>3</sub>, 25(OH)D<sub>3</sub>, or 1,25(OH)<sub>2</sub>D<sub>3</sub> were added from ethanol stocks to final concentrations ranging from 0.1-100 nM. An ethanol control was

included in the assay. Two weeks later, colonies formed in the soft agar were stained with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (0.5 mg/ml) and scored under a microscope. The number of units was calculated from the number of colonies formed divided by the number of cells seeded ×100.

**In vivo toxicity studies.** The potential toxicity of 20(OH)D<sub>3</sub> was evaluated using male C57/BL6 mice (Charles River Laboratories International, Inc., Wilmington, MA). Mice weighed approximately 25-26 g and were seven weeks of age. Two positive control compounds, 1,25(OH)<sub>2</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub>, were obtained from Sigma-Aldrich, Co. LLC. (St. Louis, MO, USA). 20(OH)D<sub>3</sub> in autoclaved sesame oil (Sigma-Aldrich) was administered by intraperitoneal (*i.p.*) injection (50 μl/mouse) once daily for three consecutive weeks to groups of five mice at doses of 5, 10, 20 and 30 μg/kg body weight. In the positive control group, 1,25(OH)<sub>2</sub>D<sub>3</sub> or 25(OH)D<sub>3</sub>, was given at the dose of 2 μg/kg body weight by *i.p.* injection following the same pattern. Another group of five mice was injected with autoclaved sesame oil 50 μl/mouse daily, which served as a vehicle control. Clinical signs of toxicity and body weight were assessed twice a week throughout the experimental period.

Terminal blood samples (800-1000 μl/mouse) were collected by cardiac puncture at the end of the three-week treatment. The serum (~300 μl/mouse) was immediately separated using BD Microtainer® tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and stored at -20°C. All animals were sacrificed by cervical dislocation immediately after the blood collection and the main organs (heart, lung, liver, spleen, kidney, adrenal and one piece of skin) of each mouse were collected and stored separately in 10% buffered formalin phosphate solution (Fisher Scientific, Fair Lawn, NJ, USA) for subsequent pathological analysis.

**Blood chemistry and clinical pathological analysis.** All the blood and serum samples were shipped to Charles River Research Animal Diagnostic Laboratory (Wilmington, MA, USA) within 24 h after collection. The complete pathology, chemistry and hematology [complete blood count (CBC) with differential] profile was assayed and results were provided by Charles River. The corticosterone concentration was measured with an ELISA kit (Enzo Life Sciences Int'l, Inc., Plymouth Meeting, PA, USA) and the amount of corticosterone was calculated from the standard curve prepared according to the manufacturer's instruction. The data are presented as ng/ml of serum.

For pathological analysis, formalin-fixed tissues were processed to paraffin blocks, and after sectioning tissues were stained with hematoxylin and eosin. The slides were scanned to create a digital replica of entire tissues on a glass microscopic slide using ScanScope®XT (Aperio Technologies, Inc., Vista, CA, USA) at 0.25 pixel/μm. The scanning process allowed the tissue images to be displayed and analyzed at different magnifications, closely emulating traditional viewing of tissues with a conventional microscope. All the images were stored on a 20TB server that can be accessed through the internet.

**Statistical evaluation.** Data are presented as means±SD and have been analyzed with the Student's *t*-test (for two groups) or with one-way ANOVA using Dunnett's Multiple Comparison post hoc test (for more than two groups) with Prism 4.00 (GraphPad Software, San Diego, USA). Statistically significant differences were considered when *p*<0.05.

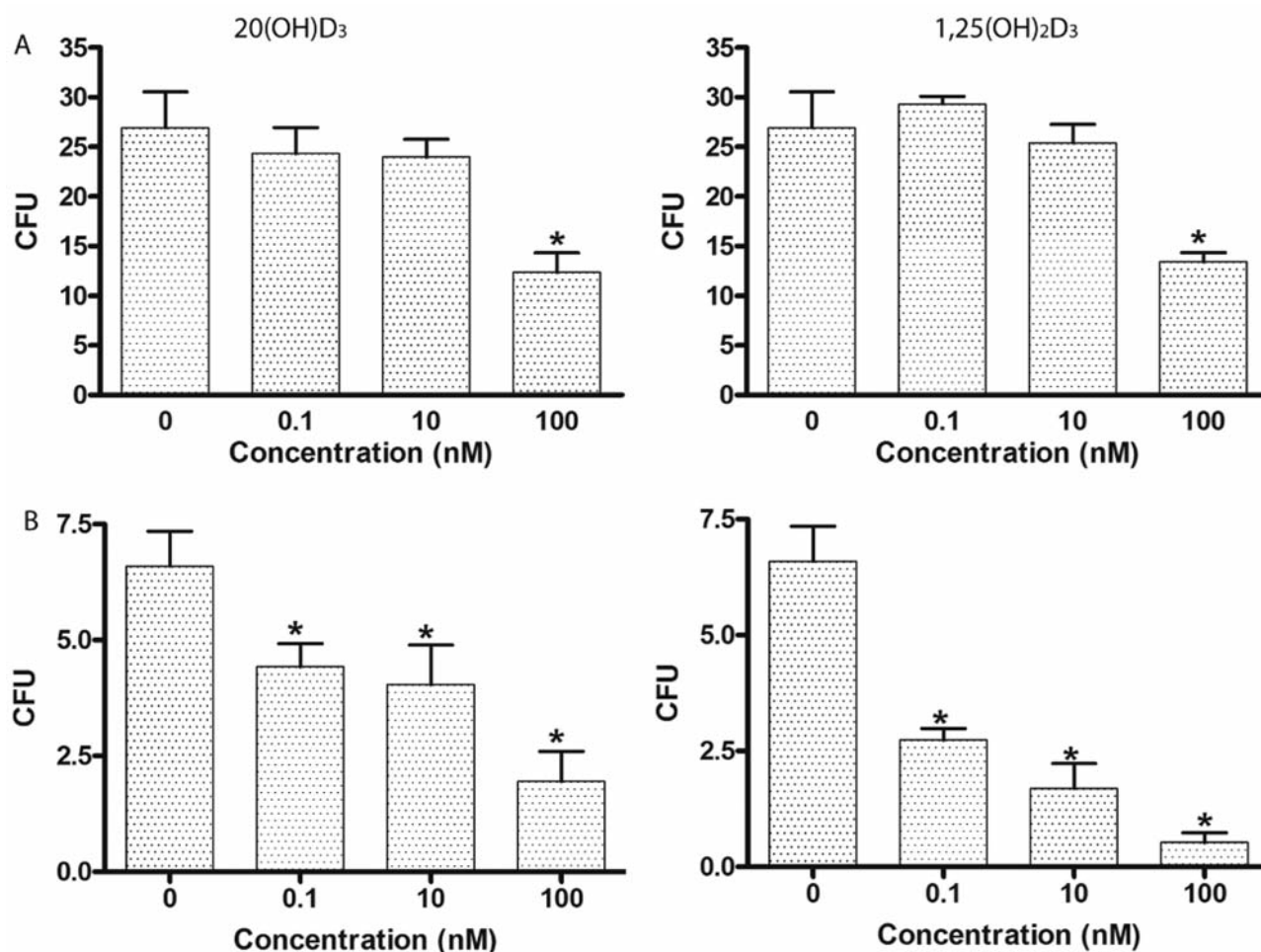


Figure 1. 20(OH)D<sub>3</sub> inhibits the proliferation of HepG2 hepatocellular carcinoma cells. HepG2 cells were grown in 6-well plates and were treated with different concentrations of 20(OH)D<sub>3</sub>, or with 1,25(OH)<sub>2</sub>D<sub>3</sub> as a positive control. 14 days after treatment, cells had formed colonies which were fixed in para-formaldehyde (PFA), stained with crystal violet and counted under a cell counter. The size and number of colonies were scored. Data representing colonies larger than 0.2 mm (A) and larger than 0.5 mm (B) in size following treatment with 20(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> are shown as the mean±SD (n=3). \*p<0.05 versus vehicle control. CFU: Colony-forming unit.

## Results

20(OH)D<sub>3</sub> inhibits cancer colony formation in monolayer and soft agar. The antitumor activity of 20(OH)D<sub>3</sub> was compared to that of 1,25(OH)<sub>2</sub>D<sub>3</sub> by measuring their abilities to inhibit cancer cell colony formation. Firstly, we tested the effect of these compounds on HepG2 hepatocellular carcinoma colony growth and found that 20(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibit HepG2 proliferation in a concentration-dependent manner. 20(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> at a concentration of 100 nM effectively reduced the CFU of colonies larger than 0.2 mm (54±14% and 50±6%, respectively) and larger than 0.5 mm (70±20% and 92±6%, respectively) in comparison to control (vehicle treated) cells (Figure 1A and B). The treatment with vitamin D<sub>3</sub>

derivatives at lower concentrations, 0.1 and 10 nM, had no significant effect on the total number of colonies larger than 0.2 mm (Figure 1A) but greatly prevented the formation of colonies larger than 0.5 mm (Figure 1B). 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibited formation of large colonies (>0.5 mm in size) to a greater extent than 20(OH)D<sub>3</sub> at all three concentrations tested.

Next we tested the effect of 20(OH)D<sub>3</sub> on MDA-MB-453 human breast carcinoma cell growth in soft agar. 20(OH)D<sub>3</sub> and 25(OH)D<sub>3</sub> at a concentration of 100 nM effectively inhibited the formation of MDA-MB-453 colonies larger than 0.2 mm (54±4% and 43±7% of CFU, respectively) in comparison to control (vehicle-treated) cells (Figure 2A and B). Colonies larger than 1.5 mm were not detected in either of the two 100 nM treatment groups. At 10 nM, 20(OH)D<sub>3</sub>



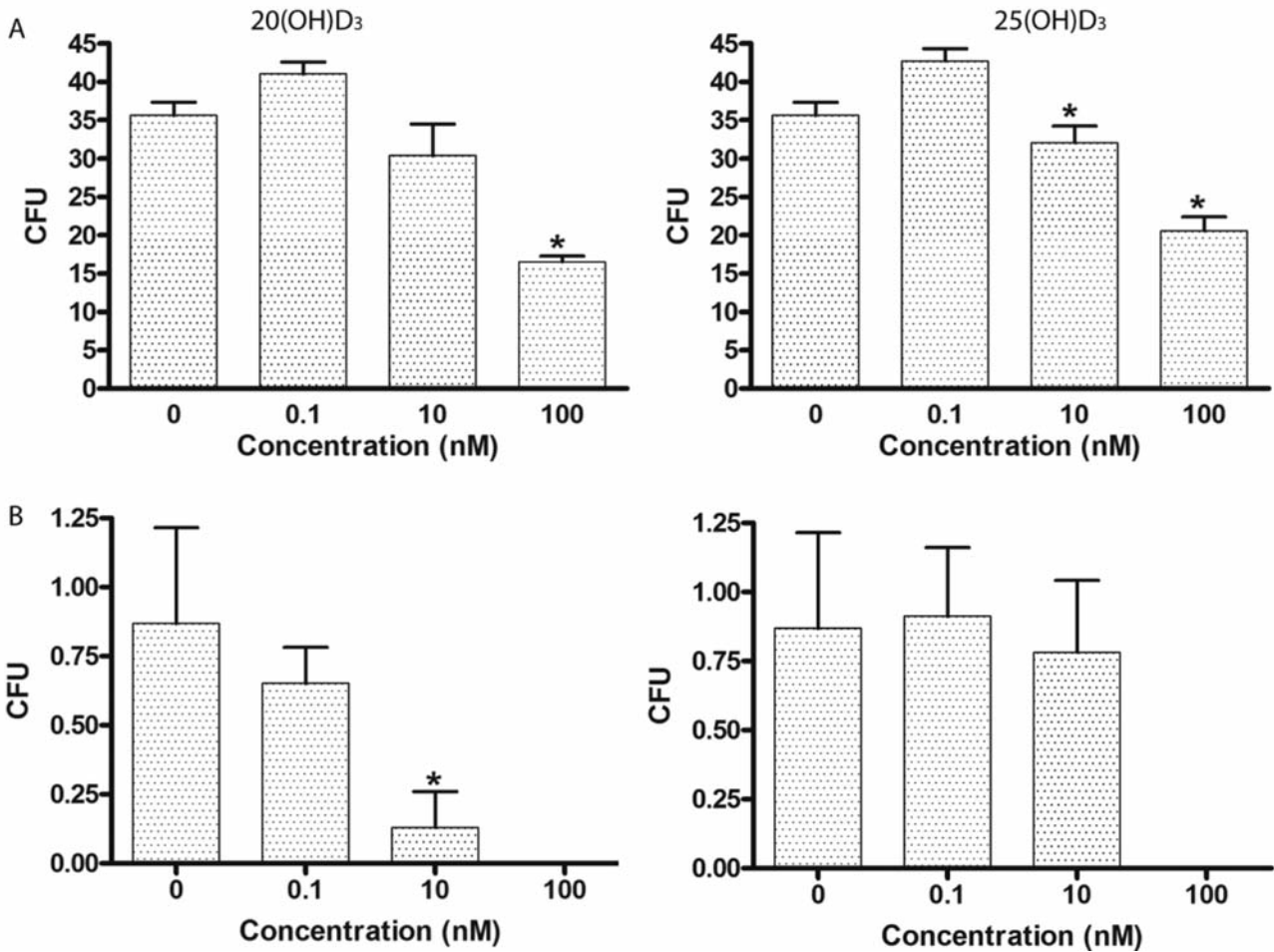


Figure 2. 20(OH)D<sub>3</sub> inhibits the proliferation of MDA-MB-453 human breast carcinoma cells. MDA-MB-453 cells were grown in 6-well plates and were treated with different concentrations of 20(OH)D<sub>3</sub>, or with 25(OH)D<sub>3</sub> as a positive control. By 14 days after treatment cells had formed colonies which were fixed in PFA, stained with crystal violet and counted under a cell counter. The size and number of colonies were scored. Data representing colonies larger than 0.2 mm (A) and larger than 1.5 mm (B) in size following treatment with 20(OH)D<sub>3</sub>, and 25(OH)D<sub>3</sub> are shown as the mean±SD (n=3). \*p<0.05 versus vehicle control. CFU: Colony-forming unit.

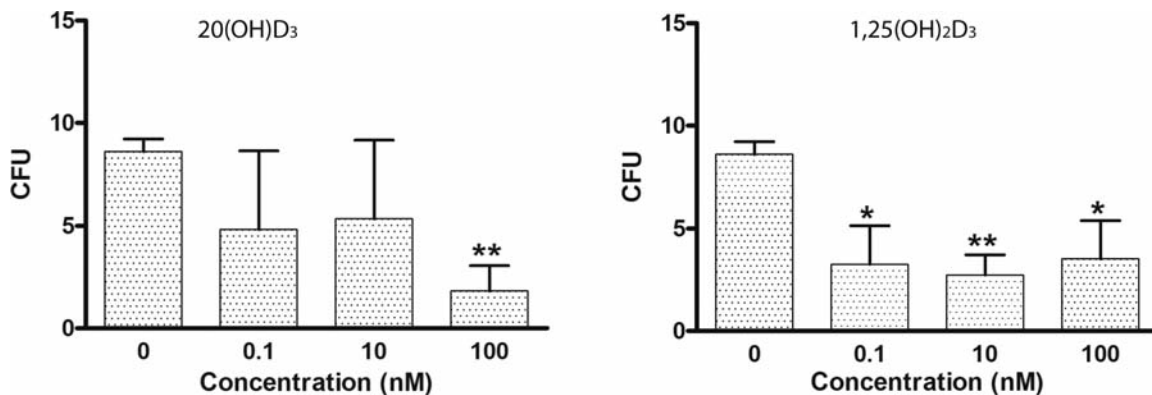


Figure 3. 20(OH)D<sub>3</sub> inhibits the proliferation of MCF7 breast cancer cells. MCF7 cells were grown in soft agar and were treated with different concentrations of 20(OH)D<sub>3</sub>, or 1,25(OH)<sub>2</sub>D<sub>3</sub> as a positive control. By 26 days after treatment cells had formed colonies which were stained with MTT reagent and counted under a microscope. The size and number of colonies were scored. Data representing colonies larger than 0.2 mm in size following treatment with 20(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> are shown as the mean±SD (n=3). \*p<0.05 or \*\*p<0.01 versus vehicle control. CFU: Colony-forming unit.

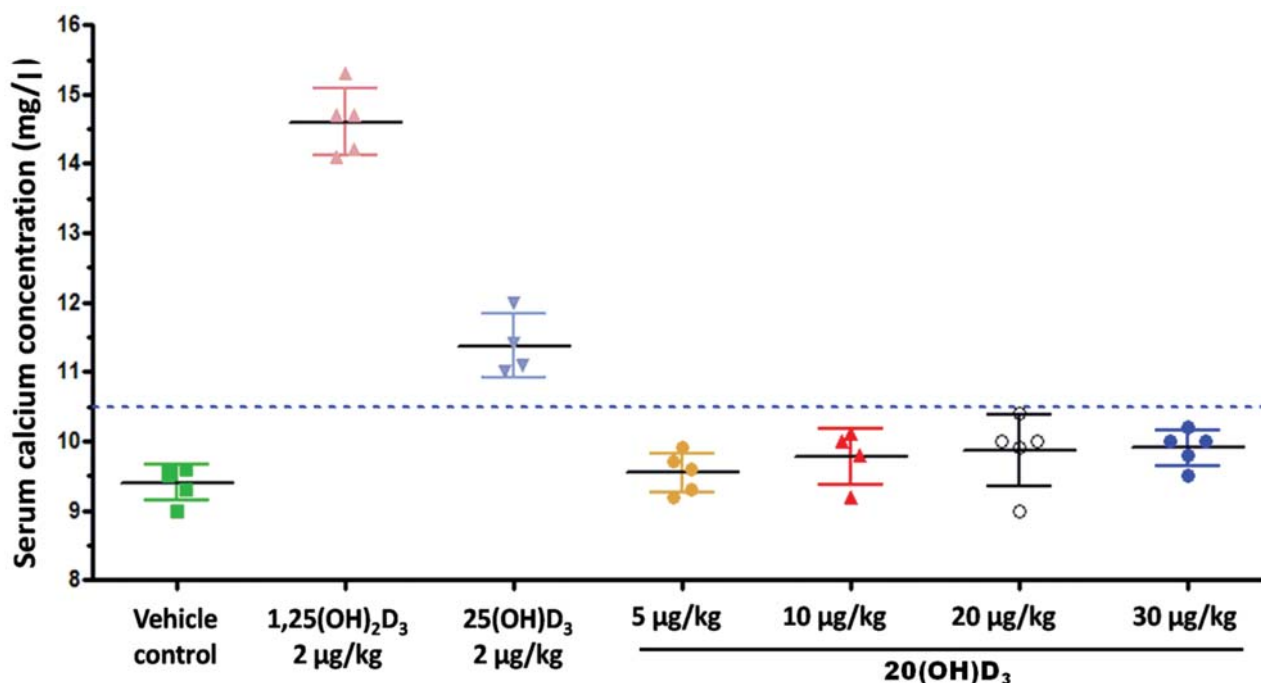


Figure 4. 20(OH)D<sub>3</sub> did not show hypercalcemic side effects at different high doses up to 30 µg/kg. Mice were treated with *i.p.* injection for 3 weeks (five mice per treatment group). Data are presented as the mean value of serum calcium concentration (mg/l) for each group. There were no significant differences between the vehicle control group and the 20(OH)D<sub>3</sub> treatment groups at all doses ( $p > 0.05$ ). There were significant differences between the positive control groups and vehicle control group, and between the positive control groups and the 20(OH)D<sub>3</sub> treatment groups ( $p < 0.01$ ). Dotted line: Upper range for normal calcium level in serum (10.5 mg/dl).

inhibited the formation of MDA-MB-453 colonies larger than 1.5 mm (Figure 2B) by 85±11% while the effect of 25(OH)D<sub>3</sub> was not statistically significant.

Finally we tested the effect of 20(OH)D<sub>3</sub> on colony formation by MCF7 breast cancer cells on soft agar. Due to the slow colony formation for this cell line, we prolonged the test period to 26 days in order to investigate the antiproliferative effect. 20(OH)D<sub>3</sub> at a concentration of 100 nM inhibited formation of colonies larger than 0.2 mm by 78±28% (Figure 3A). However, 1,25(OH)<sub>2</sub>D<sub>3</sub> demonstrated a relatively stronger effect in this model and inhibited colony formation by 62±6% at a concentration as low as of 0.1 nM (Figure 3B).

*High doses of 20(OH)D<sub>3</sub> have no calcemic effect on mice after 3 weeks of treatment.* We have reported that 20(OH)D<sub>3</sub> has no effect on systemic calcium levels at a dose of 3 µg/kg for 7 consecutive days in a rat model (12). In the current study, we assessed the potential toxicity of 20(OH)D<sub>3</sub> by administering a range of doses to C57BL/6 mice with the highest dose being 30 µg/kg. As positive controls, we used 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> because they are reported to generate hypercalcemia at a concentration ≥1 µg/kg in mice (18) or rats (12). As indicated in Figure 4, 20(OH)D<sub>3</sub> did not

display hypercalcemic activity (calcium=9.7±0.69 vs. 9.4±0.25 mg/dl for vehicle control) at even the highest dose tested (30 µg/kg) for the 3-week *i.p.* treatment. In contrast, 1,25(OH)<sub>2</sub>D<sub>3</sub> at a dose of only 2 µg/kg caused the expected dramatic rise in calcium up to 14.6±0.48 mg/dl. 25(OH)D<sub>3</sub> at a dose of 2 µg/kg showed a mild hypercalcemic effect elevating the serum calcium level to 11.5±0.48 mg/dl, beyond the 10.5 mg/dl upper range for normal serum calcium.

While there appeared to be slight dose-dependent increases in calcium concentrations for 20(OH)D<sub>3</sub>-treated groups compared with the vehicle control group, the differences were not significant ( $p > 0.05$ ). In addition, mice in the vehicle control and all 20(OH)D<sub>3</sub> treatment groups maintained a healthy weight (25±1 g), whereas those in both 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> groups lost body weight at the dose (2 µg/kg) tested (22±1 g and 21±1 g, respectively), which correlated with their status of hypercalcemia. Hematological testing and other clinical chemistry parameters (Figure 5A) e.g. alanine aminotransferase (ALT), alkaline phosphatase (ALK), aspartate aminotransferase (ASK), cholesterol (CHOL), which profile liver and kidney functions or lipid metabolism, did not produce any significant difference among all groups after 3 weeks of treatment. In addition, corticosterone levels were similar for mice in the vehicle

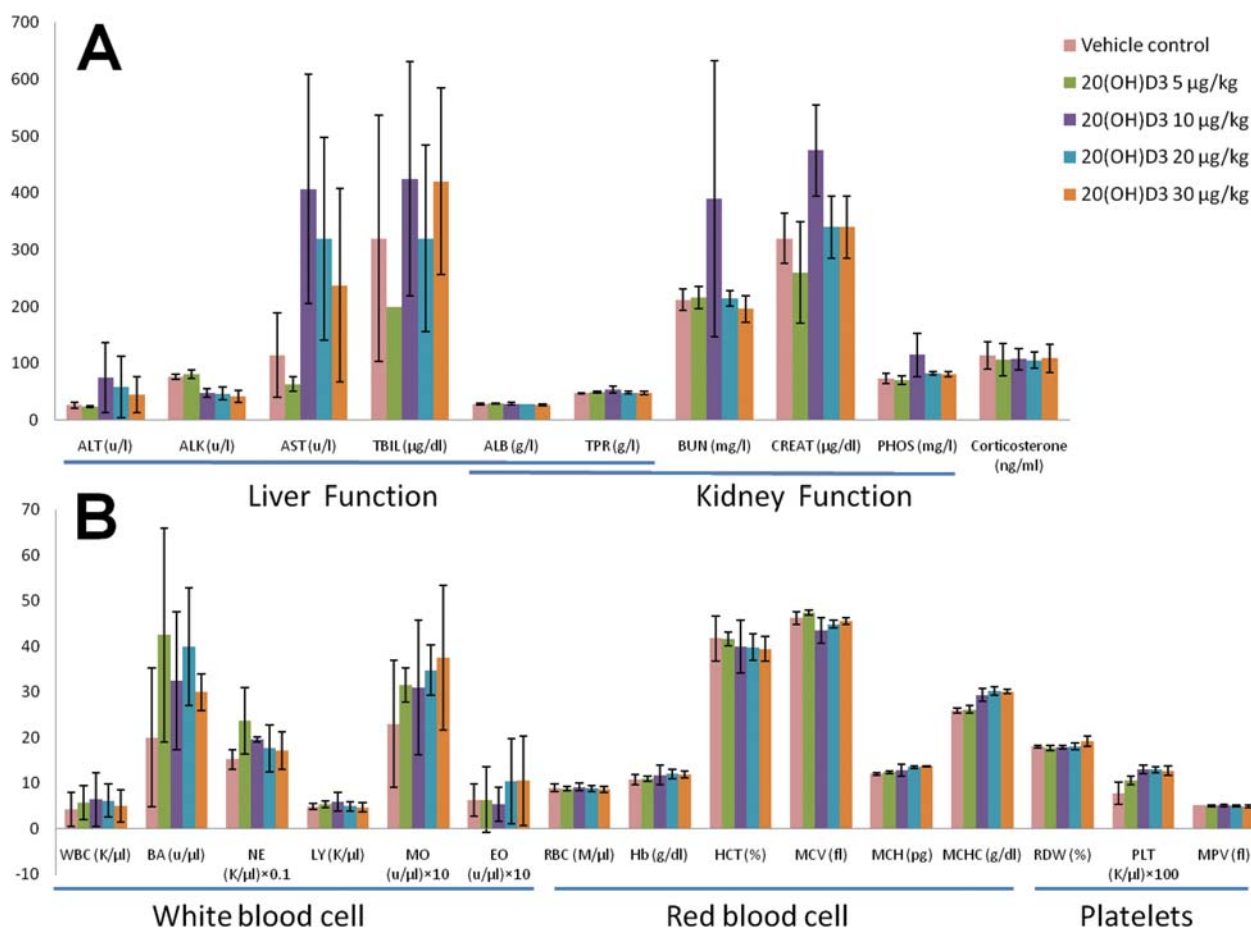


Figure 5. 20(OH)D<sub>3</sub> did not show toxicity in vivo at different high doses up to 30 µg/kg. A: Analysis of complete clinical pathology chemistry profiles testing liver/kidney functions and corticosterone levels. B: Complete blood count (CBC) with differential results for all treatment groups in the toxicity study. All data are well within the normal physiological range for mice and there are no statistically significant differences between mice in the vehicle control group and all treatment groups. ALT: Alanine aminotransferase; ALK: alkaline phosphatase; AST: aspartate aminotransferase; TBIL: total bilirubin; ALB: albumin; TPR: total protein; BUN: blood urea nitrogen; CREAT: creatinine; PHOS: inorganic phosphate; WBC: white blood cell count; BA: basophil granulocytes; NE: neutrophil granulocytes; LY: lymphocytes; MO: monocytes; EO: eosinophil granulocytes; RBC: red blood cell count; Hb: hemoglobin; HCT%: hematocrit percentage; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; PLT: platelet count; MPV: mean platelet volume.

control and those receiving all doses of 20(OH)D<sub>3</sub> (Figure 6A). Furthermore the hematology profile was similar for the control and all treatment groups (Figure 5B).

Consistent with the clinical pathological and hematological measurements, histopathological analysis of major organs (liver, heart, brain, spleen, kidney, and skin) did not show any abnormalities between vehicle control and 20(OH)D<sub>3</sub> treatment groups at the various doses tested. Figure 6 shows representative images of sections of organs from vehicle control and 20(OH)D<sub>3</sub>-treated groups at the highest dose (30 µg/kg). A lack of calcium deposits and other histologically identifiable signs of toxicity are evident.

### Discussion

1,25(OH)<sub>2</sub>D<sub>3</sub>, or calcitriol, is the hormonally active form of vitamin D<sub>3</sub> which plays a pivotal role in the regulation of calcium homeostasis and the normal mineralization of bone (19). It can raise the calcium level in the blood by increasing the calcium absorption from the intestine, enhancing the calcium uptake from bone and reducing the transfer to the urine (20). However, 1,25(OH)<sub>2</sub>D<sub>3</sub> has demonstrated several beneficial effects that are unrelated to bone or mineral metabolism, including antiproliferative, anti-inflammatory, pro-differentiating and immunomodulatory activities (21). It also acts additively or synergistically with other types of



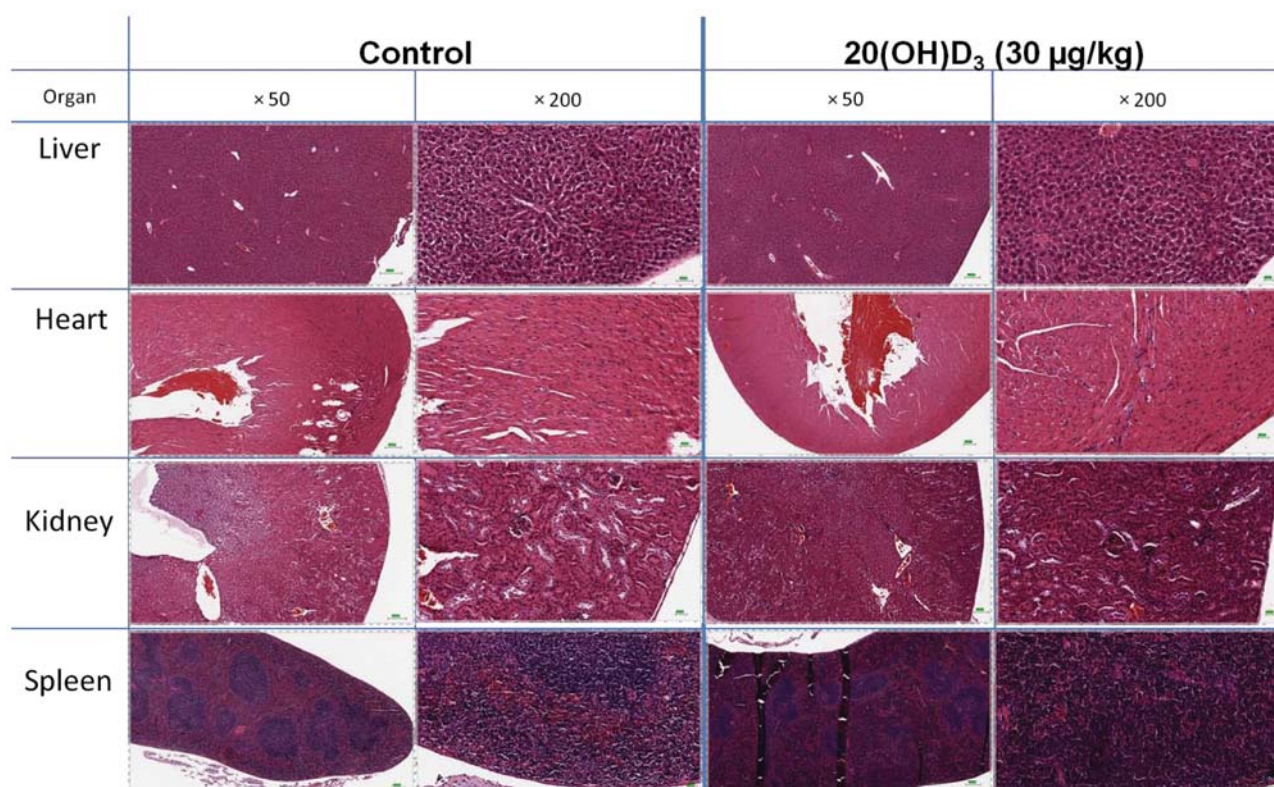


Figure 6. Histopathological analyses of representative organs taken from mice in the vehicle control group and mice receiving the highest dose of 20(OH)D<sub>3</sub>. No calcification or other abnormalities were observed in any of these organs. Organs were processed and slides were digitized and analyzed. Photographs of various organs were taken at different magnifications.

chemotherapeutic agents (22). Although 1,25(OH)<sub>2</sub>D<sub>3</sub>, or its combination treatment has been studied with respect to its antitumor activity in various clinical trials, the results are disappointing (19). The fundamental problem is the safety concern. The antitumor activity of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> depends on sufficient exposure to a high concentration while the dose-related hypercalcemia strictly limits the maximum dose that can be utilized clinically (22). Our study demonstrates that mice are able to safely tolerate a dose of 20(OH)D<sub>3</sub> at least 15-fold higher than for 1,25(OH)<sub>2</sub>D<sub>3</sub>. Furthermore, there were no detectable differences in blood chemistry, liver and kidney functional profiles, and organ pathology between mice in the vehicle control group and mice treated with very high doses of 20(OH)D<sub>3</sub>. Similarly, corticosterone levels did not change after treatment with 20(OH)D<sub>3</sub>, indicating an undetectable effect on the stress response system. Collectively, these data strongly support that 20(OH)D<sub>3</sub> may overcome the obstacle of the narrow therapeutic window associated with currently available vitamin D<sub>3</sub> analogs for cancer prevention or treatment.

We have previously demonstrated that 20(OH)D<sub>3</sub> has anti-proliferative activity similar to or more pronounced than 1,25(OH)<sub>2</sub>D<sub>3</sub> on cell lines including normal keratinocytes, melanoma cells and leukemia cells (5, 11-13,

16). The current study has further expanded the *in vitro* antitumor spectrum of 20(OH)D<sub>3</sub> to carcinoma cells including HepG2 hepatocellular carcinoma, and MDA-MB-453 and MCF7 human breast cancer cells. Antitumor activities were determined using a colony formation model, which represents an accepted *in vitro* measure of anti-tumorigenic activity, mimicking the therapeutic response of solid tumors *in vivo* (23). 20(OH)D<sub>3</sub> acted comparably to 1,25(OH)<sub>2</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub> at the highest concentration tested (100 nM) on the three cell lines examined. In summary, 20(OH)D<sub>3</sub> displays antiproliferative activity comparable to the one of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> but can be safely tolerated by mice at a high dose without generating hypercalcemia or other signs of toxicity or stress, making it a potentially safe and efficient antitumor agent.

#### Acknowledgements

This work was supported by NIH grant R01AR052190, 1R01CA148706-01A1 and a Technology Maturation Grant from the University of Tennessee Research Foundation. Additional support was provided from NIH grant 1R01AR056666-01A2 to AS. The Whole Slide Imaging System was obtained from an NCRR Shared Instrumentation Grant (1S10RR025665-01).

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Received January 3, 2012

Revised February 8, 2012

Accepted February 9, 2012