Gemini Vitamin D Analogues Inhibit Estrogen Receptor–Positive and Estrogen Receptor–Negative Mammary Tumorigenesis without Hypercalcemic Toxicity

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Abstract Numerous preclinical, epidemiologic, and clinical studies have suggested the benefits of vitamin D and its analogues for the prevention and treatment of cancer. However, the hypercalcemic effects have limited the use of 1α,25$(OH)_2D_3$, the hormonally active form of vitamin D. To identify vitamin D analogues with better efficacy and low toxicity, we have tested >60 novel Gemini vitamin D analogues with a unique structure of two side chains for growth inhibition of breast cancer cells. Our initial studies found that some Gemini analogues are 5–15 times more active than 1α,25$(OH)_2D_3$ in growth inhibition assay. *In vivo* experiments were designed to study the inhibitory effect of selected Gemini vitamin D analogues against mammary carcinogenesis by using (*a*) an N-methyl-N-nitrosourea–induced estrogen receptor (ER)-positive mammary tumor model and (*b*) an MCF10DCIS.com xenograft model of ER-negative mammary tumors. Among vitamin D analogues we tested, Gemini 0072 [1α,25-dihydroxy-20S-21(3-trideuteromethyl-3-hydroxy-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-19-nor-cholecalciferol] and Gemini 0097 [1α,25-dihydroxy-20R-21(3-trideuteromethyl-3-hydroxy-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-19-nor-cholecalciferol] administration inhibited by 60% the NMU-induced mammary tumor burden compared with the NMU-treated control group, but these compounds were devoid of hypercalcemia toxicity. In an ER-negative xenograft model, Gemini 0097 significantly suppressed tumor growth without hypercalcemia toxicity. We found that the inhibitory effect of Gemini 0097 was associated with an increased level of cyclin-dependent kinase inhibitor p21 and the insulin-like growth factor binding protein 3 in both ER-positive and ER-negative mammary tumors. Our results suggest that Gemini vitamin D analogues may be potent agents for the prevention and treatment of both ER-positive and ER-negative breast cancer without hypercalcemia toxicity.

In the United States, breast cancer remains the most frequently diagnosed cancer and the second leading cause of cancer death in women according to Cancer Statistics from the American Cancer Society. Because of the complexity and heterogeneity of mammary carcinogenesis (1), many pharmacologic agents have been studied for their effects on the prevention of breast cancer. For example, selective estrogen receptor (ER) modulators such as tamoxifen and raloxifene have achieved significant reduction of breast cancer incidence in women at high risk (2, 3). However, selective ER modulators are not effective in preventing ER-negative breast cancer, which corresponds to at least one third of the breast cancer cases (4). The vitamin D receptor (VDR), a member of the nuclear receptor superfamily, has been suggested as a target for both ER-positive and ER-negative breast cancer prevention (5, 6) because it is present in most breast tumors (4), and *VDR* ablation in mice was reported to enhance carcinogen-induced formation of mammary tumors (7). These results suggest a role of vitamin D signaling in the regulation of mammary tumorigenesis.

The ligand for VDR, 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$; the key hormone in calcium/phosphate homeostasis) is a hormonally active metabolite synthesized from vitamin D_3 predominantly through hydroxylation by a 25-hydroxylase in the

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liver and a 1α-hydroxylase in the kidney (6, 8). It has been reported that several nonrenal tissues including breast, colon, and prostate also express 1α-hydroxylase, which can produce $1\alpha,25(OH)_2D_3$ from 25-hydroxyvitamin D_3. The formation of $1\alpha,25(OH)_2D_3$ in these tissues may result in inhibition of cell proliferation by (*a*) arrest in the G_1 phase of the cell cycle, (*b*) induction of apoptosis, and (*c*) promotion of cell differentiation (9–12). However, hypercalcemic toxicity has limited the use of $1\alpha,25(OH)_2D_3$ for cancer prevention, and numerous synthetic vitamin D analogues have been developed for better efficacy and less calcemic effects.

Classic synthetic vitamin D analogues have been tested in different animal models to determine their *in vivo* efficacy. For example, synthetic 1α-hydroxyvitamin D_5 reduced not only the formation of azoxymethane-induced aberrant crypt foci in CF-1 mice (13) but also the incidence and multiplicity of N-methyl-N-nitrosourea (NMU)-induced mammary tumors in Sprague-Dawley rats (14, 15). Treatment with the vitamin D analogue Ro26-9114 also caused a significant decrease in total tumor load over the entire gastrointestinal tract in APC^{min} mice (16). In a xenograft model, treatment with 1,25-dihydroxy-16-ene-23-yne vitamin D_3 attenuated the growth of retinoblastoma tumors formed by the s.c. injection of Y79 cells, and apoptosis was increased (17). Furthermore, administration of a combination of the vitamin D analogue EB1089 with fractionated doses of radiation was significantly more effective for tumor inhibition and apoptosis promotion than either treatment alone for MCF-7 tumor xenografts in athymic mice (18).

To identify vitamin D analogues with better anticancer efficacy and lower toxicity than $1\alpha,25(OH)_2D_3$, we tested novel Gemini vitamin D analogues, which have a unique structure of two six-carbon chains with a C-20-normal and a C-20-epi side chain (see Fig. 1). They have considerably less toxicity than $1\alpha,25(OH)_2D_3$ in animals (19, 20), and certain Gemini vitamin D analogues had a greater inhibitory effect on cell proliferation than $1\alpha,25(OH)_2D_3$ in MCF10 breast epithelial cells (21). In animal studies of mouse colorectal cancer cells (MC-26) implanted in BALB/c mice, administration of BXL-01-0072 (see Fig. 1 for the structure) significantly reduced tumor growth at a 10-fold lower dose than did $1\alpha,25(OH)_2D_3$ (19). This same deuterated Gemini analogue also greatly diminished the invasive spread of the tumor into the surrounding muscle compared with $1\alpha,25(OH)_2D_3$ (20). We also reported that certain Gemini analogues decreased mammary tumor burden in the NMU-induced rat mammary carcinogenesis model (22).

Recently, breast cancer has been suggested to be classified into at least five different subtypes, such as luminal A, luminal B, basal A, basal B, and Her-2/neu (23, 24). Because of the heterogeneity of breast cancer, we selected two different animal models of breast cancer, a conventional chemoprevention model representing ER-positive luminal cell type and a basal type ER-independent model of breast cancer (24). In the present study, we tested a classic vitamin D analogue, Ro-26-2198, and several Gemini vitamin D analogues (Fig. 1) for their effects on NMU-induced ER-positive mammary carcinogenesis and on the *in vitro* growth of cultured ER-negative MCF10DCIS.com human breast epithelial cells. An ER-negative xenograft model with implanted MCF10DCIS.com cells was also used to investigate the effects of Gemini 0097 on the growth of ER-negative mammary tumors *in vivo*. Molecular mechanisms of action of Gemini vitamin D analogues that inhibited the growth of both ER-positive and ER-negative tumors were studied.

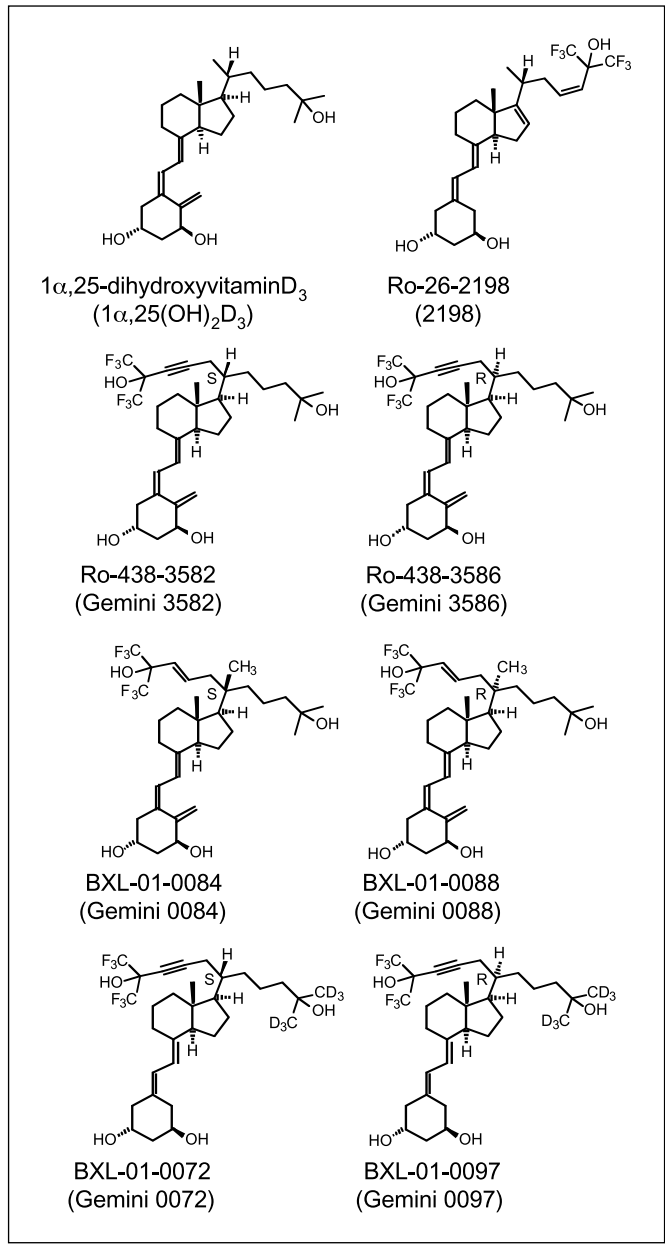

Fig. 1. Structures of 1α,25-dihydroxyvitamin D_3 [active vitamin D_3 metabolite, calcitriol or $1\alpha,25(OH)_2D_3$], the classic vitamin D analogue, Ro-26-2198 (2198, 1α,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor-cholecalciferol) and Gemini vitamin D analogues Ro-438-3582 [Gemini 3582, 1α,25-dihydroxy-20S-21(3-hydroxy-3-methyl-butyl)-23-yne-26,27-hexafluoro-cholecalciferol], Ro-438-3586 [Gemini 3586, 1α,25-dihydroxy-20R-21(3-hydroxy-3-methyl-butyl)-23-yne-26,27-hexafluoro-cholecalciferol], BXL-01-0084 [Gemini 0084, 1α,25-dihydroxy-20-methyl-20S-(4-hydroxy-4-methyl-pentyl)-23E-ene-26,27-hexafluoro-cholecalciferol], BXL-01-0088 [Gemini 0088, 1α,25-dihydroxy-20-methyl-20R-(4-hydroxy-4-methyl-pentyl)-23E-ene-26,27-hexafluoro-cholecalciferol], BXL-01-0072 [Gemini 0072, 1α,25-dihydroxy-20S-21(3-trideuteromethyl-3-hydroxy-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-19-nor-cholecalciferol], and BXL-01-0097 [Gemini 0097, 1α,25-dihydroxy-20R-21(3-trideuteromethyl-3-hydroxy-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-19-nor-cholecalciferol]. The different configuration of epimerization is indicated at the C_{20} position as either S or R.

Materials and Methods

Reagents. $1\alpha,25(OH)_2D_3$ and all Gemini vitamin D analogues (>95% purity; Fig. 1) were provided by BioXell, Inc. $1\alpha,25(OH)_2D_3$ and Gemini analogues were dissolved in DMSO. $1\alpha,25(OH)_2D_3$ and Gemini analogues were diluted in 5% horse serum/DMEM/F12 medium for [^{3}H]-thymidine uptake assay. For *in vivo* animal experiments, compounds were diluted in cremophor/PBS (1:8, v/v).

Cell culture. The human MCF10 breast epithelial cell line, MCF10DCIS.com, was provided by Dr. Fred Miller at the Barbara Ann Karmanos Cancer Institute (Detroit, MI). MCF10DCIS.com cells were maintained in DMEM/F12 medium supplemented with 5% horse serum, 1% penicillin/streptomycin, and 1% HEPES solution at 37°C, 5% CO_2. The cells were passed every 3 to 4 d.

NMU-induced mammary tumorigenesis in rats. Female Sprague-Dawley rats (ages 21 ± 1 d) were purchased from Taconic Farms. Rats were treated with a single i.p. injection (50 mg/kg body weight) of the carcinogen NMU (Sigma). One week after the NMU injection, rats were treated with vehicle or with $1\alpha,25(OH)_2D_3$ or Gemini vitamin D analogues in vehicle via i.p. injection daily, 5 d a week. The rat weight and tumor size of each animal were measured weekly. Nine weeks after starting vitamin D treatment, the rats were sacrificed and tumors were weighed at autopsy. Tumors and blood samples were collected for further analysis. Serum was collected after centrifugation of clotted blood samples. All animal studies were done in accordance with an institutionally approved protocol.

Determination of serum calcium level. The concentration of calcium in serum samples was determined using the calcium reagent set (POINTE Scientific, INC.), and we followed the procedure provided by the manufacturer. Briefly, serum (4 μL) was mixed with the appropriately diluted reagent (200 μL) in a 96-well plate. After incubating for 1 min, the plate was read at 550 nm using a spectrophotometer. To adjust for differences in hemolysis among samples, serum blanks (serum in water) were prepared and the absorbance reading was subtracted from the test reading. The calcium concentration was calculated from calcium standards provided by the manufacturer.

Animal experiment in xenograft model. MCF10DCIS.com cells were trypsinized and prepared in HBSS (Invitrogen). After the midline incision around the second teat in severe combined immunodeficiency mice, 1×10^6 cells were injected in the mammary fat pad. The incision was closed by wound clips and the clips were removed after 4 d. Vehicle control (0.1 mL) or Gemini vitamin D analogue 0097 (0.1 μg/kg body weight in 0.1 mL vehicle) was injected i.p. daily from day 4 until the termination of the experiment. Body weight and tumor size were measured every week. Six weeks after the injection of MCF10DCIS.com cells, tumors were weighed and collected at autopsy for further analysis. All animal studies were done in accordance with an institutionally approved protocol.

Western blot analysis. Tumor samples were homogenized in radioimmunoprecipitation assay buffer (10 mmol/L Tris-HCl, 5 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.1 mmol/L Na_3VO_4, 1% phenylmethylsulfonyl fluoride, 1% aprotinin, and 0.1% leupeptin) using a Dounce homogenizer (Wheaton), and the protein extracts were electrophoresed in 4% to 15% gradient gels (Bio-Rad) and transferred to a polyvinylidene difluoride membrane (PALL). The primary antibodies against VDR (Affinity BioReagents), PARP, cleaved PARP, cleaved caspase-3, pSmad1/5 (Cell Signaling Technology, Inc.), p21, p27, apolipoprotein A-1 (Santa Cruz Biotechnology), insulin-like growth factor binding protein 3 (IGFBP-3; R&D Systems), actin (Sigma), and secondary antibodies (Santa Cruz Biotechnology) were used. The band intensity was quantified using TableLab software (ver.1.10) and normalized with actin.

Sample preparation and two-dimensional gel electrophoresis. Rat mammary tumor tissue (1-2 grams) was grossly freed of necrotic areas and homogenized in 2 volumes of 50 mmol/L Tris-HCl (pH 7.4) at 4°C, 1.15% KCl, 1 mmol/L EDTA and 0.2 mmol/L phenylmethylsulfonyl fluoride using a glass-teflon Potter-Elvehjem tissue homogenizer. Cytosolic fractions were isolated as described previously (25) and prepared for two-dimensional gels by precipitating proteins for 1 h on ice with 2 volumes of ice cold methanol/acetonitrile (50:50). Samples (500 μg) were applied by cup loading to buffer hydrated (overnight) 24 cm Immobiline IPG strips (pH 4-7; Amersham Biosciences). Isoelectric focusing was done at 25°C on an Ettan IPGphor II and the remainder of the two-dimensional gel electrophoresis was as described previously (26). Protein-stained gels were scanned for data analysis using a Molecular Dynamics laser densitometer. Spot patterns were analyzed with Non-Linear Phoretix two-dimensional software. Selected protein stained spots were excised and in-gel–digested with trypsin and peptides extracted as described previously (27). Tryptic peptides were resolved on a microcapillary column (50 × 0.3 mm ID, Magic C18) and introduced into a ThermoFinnigan LCQ DECA XP ion trap mass spectrometer through an Electrospray Ionization interface operated in positive ion mode by a ThermoFinnigan Surveyor LC system. Data-dependent MS/MS spectra were generated and used for a protein identification database search using the ThermoFinnigan TurboSEQUEST program (Bioworks 3.1) after automatic removal of prominent MS/MS peaks due to digestion enzymes and other experimental background compounds.

Quantitative PCR analysis. These procedures have been previously reported (21). Labeled primers, including glyceraldehyde-3-phosphate dehydrogenase, *CYP24A1,* and *IGFBP-3,* were obtained from Applied Biosystems.

Immunohistochemistry. Tumor samples from each group were harvested at autopsy and fixed in 10% formalin for 24 h. They were sectioned, paraffin embedded, and microtomed into 4 μm-thick tissue sections. The slides were incubated overnight at room temperature with antibody to proliferating cell nuclear antigen (PCNA; 1:1,000 diluted; BD Pharmingen), or cleaved caspase-3 (1:200 diluted; Cell Signaling Technology, Inc.). The slides were incubated with biotinylated secondary antibody, and then with avidin/biotinylated peroxidase complex for 30 min at room temperature (Vector Labs) and were then incubated with 3′-diaminobenzamine substrate. The sections were then counterstained with Modified Harris Hematoxylin. The images were taken randomly at using a Zeiss AxioCam HRc camera fitted to a Zeiss Axioskope 2 Plus microscope.

[^{3}H]Thymidine uptake assay. MCF10DCIS.com cells were incubated with compounds in 5% horse serum/DMEM/F12 medium for 3 d. One μCi of [^{3}H]thymidine was added to each well 3 h before the harvest. Incorporation of [^{3}H]thymidine into the cells was analyzed with a liquid scintillation spectrometer (Beckman Coulter).

Statistical analysis. Statistical significance for average tumor burden, average body weight, and serum calcium level was evaluated using the Student's *t* test.

Results

Gemini vitamin D analogues inhibit NMU-induced mammary tumorigenesis in rats. The efficacies of a classic vitamin D analogue and six different Gemini vitamin D analogues on NMU-induced mammary tumorigenesis were tested in rats. The tumors formed with this model are luminal ER-positive (28). The doses of vitamin D analogues in Table 1 were determined based on the maximum tolerated dose in CD-1 mice and our preliminary data (data not shown). As summarized in Table 1, body weights of animals treated with the vitamin D analogues were not significantly different from those in the control group throughout the study. Among 7 different vitamin D analogues, administration of 0072 and 0097 resulted in approximately a 60% reduction of the average tumor burden per rat. Gemini 0072 and 0097 have modification on both side chains with hexafloride or hexadeuterium functional groups, which may have contributed to better bioavailability

Table 1. Prevention of NMU-induced breast cancer by vitamin D analogues

Treatment*	Dose (μg/kg body weight)	No. animals	Body weight at autopsy (grams; mean ± SE)	Tumor burden† (grams; mean ± SE)
Control (Vehicle)	—	12	242.7 ± 6.5	9.0 ± 2.8
Ro-26-2198 (2198)	0.03	6	224.3 ± 16.0	8.4 ± 4.1
Gemini 3582 (3582)	0.1	6	248.3 ± 10.6	9.2 ± 2.3
Gemini 3586 (3586)	0.3	6	235.0 ± 2.6	5.0 ± 2.0
Gemini 0084 (0084)	0.03	6	247.6 ± 8.1	6.4 ± 3.0
Gemini 0088 (0088)	0.1	6	255.7 ± 9.9	8.9 ± 2.9
Gemini 0072 (0072)	0.3	6	250.8 ± 9.5	3.6 ± 1.7 ($P = 0.06$‡)
Gemini 0097 (0097)	0.3	6	262.7 ± 7.0	3.4 ± 1.7 ($P = 0.05$‡)

*All rats (ages 21d) received NMU (50 mg/kg body weight, i.p.) 1 wk before injecting vitamin D analogues i.p. daily, 5 d a week for 9 wk.
†Tumor burden; average tumor weight per rat at autopsy.
‡*P* is the value for the comparison of tumor burden in rats treated with vitamin D analogues when compared with control rats treated with vehicle alone.

and an increased half-life of these compounds in animals. Gemini 0072 and 0097 had stronger activity than 3586 (44% reduction) and 0084 (29% reduction). Other analogues, 2198, 3582, and 0088 had little or no effect on tumor burden (Table 1). Potential toxicities such as body weight loss, diarrhea, or blood in the feces were not observed in Gemini vitamin D treated animals.

Gemini vitamin D analogue 0097 inhibits tumor growth without hypercalcemia toxicity. Hypercalcemic toxicity has limited the use of naturally occurring $1\alpha,25(OH)_2D_3$ as a chemopreventive agent, although it has been shown to have a potent antiproliferating activity in different cell types (6, 10–12). Among the vitamin D analogues we tested (Table 1), Gemini 0097 exerted the strongest activity in inhibiting tumor growth (Table 1), and it was selected for a further dose dependent study. In Table 2, we compared the efficacy and toxicity of Gemini 0097 to those of $1\alpha,25(OH)_2D_3$. Table 2 shows that body weight in $1\alpha,25(OH)_2D_3$–treated groups was comparable with those in the control group, and tumor growth was inhibited 20% and 34% at doses of 0.1 and 0.3 μg/kg body weight, respectively. However, we observed that the average level of calcium in serum was increased to 10.9 and 11.7 mg/dL by $1\alpha,25(OH)_2D_3$ at tumor inhibitory doses (Table 2). The reference range of calcium in

Table 2. Effect of different doses of $1\alpha,25(OH)_2D_3$ (calcitriol) and Gemini 0097 on NMU-induced breast cancer

Treatment*	Dose (μg/kg body weight)	No. of animals	Body weight at autopsy (grams; mean ± SE)	Tumor burden† (grams; mean ± SE)	Tumor multiplicity‡ (mean ± SE)	Serum calcium level (mg/dL; mean ± SE)
Control (Vehicle)	0	30	241.3 ± 4.0	8.5 ± 1.7	2.9 ± 0.4	9.3 ± 0.9
Calcitriol ($1\alpha,25(OH)_2D_3$)	0.03	15	238.3 ± 4.2	8.8 ± 1.8	2.9 ± 0.5	9.3 ± 0.8
Calcitriol ($1\alpha,25(OH)_2D_3$)	0.1	15	241.9 ± 6.6	6.8 ± 1.5	2.3 ± 0.5	10.9 ± 1.5§
Calcitriol ($1\alpha,25(OH)_2D_3$)	0.3	14	225.7 ± 9.9	5.6 ± 1.5	2.6 ± 0.4	11.7 ± 1.4§
Gemini 0097 (0097)	0.03	15	251.2 ± 6.1	9.0 ± 2.6	2.9 ± 0.5	9.5 ± 1.0
Gemini 0097 (0097)	0.1	15	248.5 ± 6.1	4.2 ± 1.2 ($P = 0.02$‖)	1.8 ± 0.4 ($P = 0.03$‖)	9.6 ± 1.5
Gemini 0097 (0097)	0.3	15	252.5 ± 5.4	4.2 ± 1.4 ($P = 0.03$‖)	2.3 ± 0.4	9.9 ± 1.1

*All rats (ages 21 d) received NMU (50 mg/kg body weight, i.p.) 1 wk before injecting vitamin D analogues i.p. daily, 5 d a week for 9 wk.
†Tumor burden; average tumor weight per rat at autopsy.
‡Tumor multiplicity; average number of tumors per rat at autopsy.
§Hypercalcemic. The reference range of calcium in serum is 8.8-10.4 mg/dL (19).
‖*P* is the value for the comparison of tumor burden in rats treated with calcitriol or Gemini 0097 when compared with control rats treated with vehicle alone.

serum is 8.8 to 10.4 mg/dL (19). As shown in Table 2, Gemini 0097 at the doses of 0.1 or 0.3 μg/kg body weight significantly inhibited tumor burden per rat as well as tumor multiplicity but did not show any changes in body weight or serum calcium level compared with the control group, indicating that Gemini 0097 had a better efficacy/toxicity profile than $1\alpha,25(OH)_2D_3$.

Gemini vitamin D analogues increase markers of cell cycle arrest and apoptosis in NMU-induced mammary tumors. In Western blot analysis, we showed that administration of Gemini 0072 and 0097 up-regulated the VDR and increased the level of the cyclin dependent kinase (CDK) inhibitor p21, a well-known inhibitor of cell proliferation, in NMU-induced mammary tumors (Fig. 2A). Another CDK inhibitor, p27, was not affected. In addition, administration of 0072 and 0097 increased the level of cleaved PARP and caspase-3, which are markers of apoptosis. These results suggest that administration of 0072 and 0097 inhibit tumor growth by arresting the cell cycle and inducing apoptosis.

Effect of administration of Gemini 0072 and 0097 on proteins in NMU-induced mammary tumors: two-dimensional gel electrophoresis studies. Proteins in the cytosol of mammary tumors of rats treated with vehicle or Gemini vitamin D analogues 0072 or 0097 were resolved on two-dimensional electrophoresis gels to determine if the inhibition in tumor growth by the analogues was reflected by changes in the proteome. The protein stained two-dimensional gels resolved ~700 to 900 distinct protein spots after subtracting background using Non-Linear Phoretix two-dimensional software. Comparison of the data derived from tumors of rats treated with Gemini vitamin D analogues 0072 or 0097 to those from tumors of vehicle-treated rats revealed relatively few protein spots that were increased or decreased >2.0-fold. Some of the protein spots were identified by liquid chromatography-mass

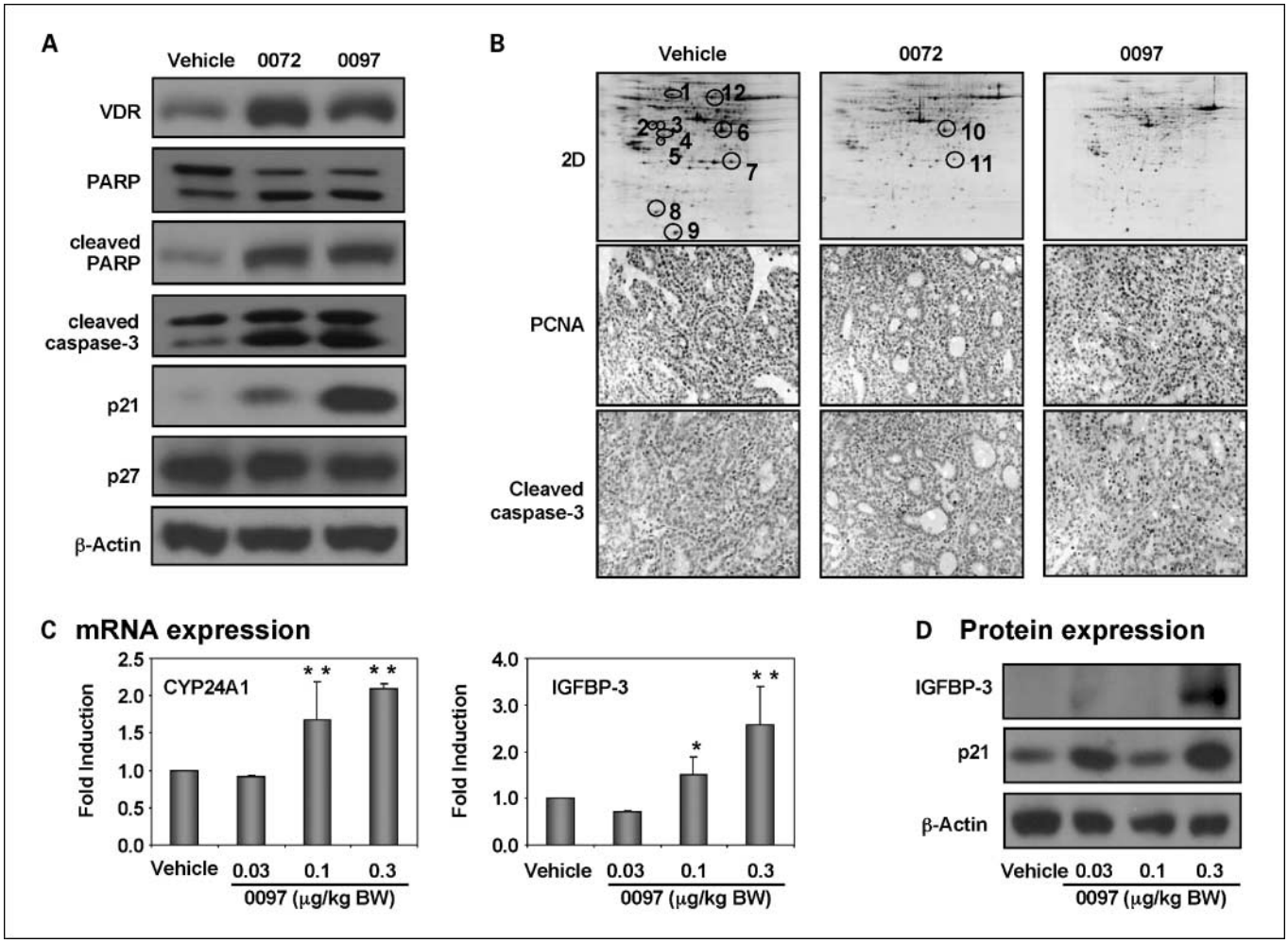

Fig. 2. Effect of Gemini vitamin D analogues on cell cycle, apoptosis, and vitamin D target genes in NMU-induced tumors. NMU-treated rats were given 0072 and 0097 as described in Tables 1 and 2, and tumor samples were analyzed. *A,* Gemini vitamin D analogues 0072 and 0097 (0.3 μg/kg body weight/d) enhance VDR, apoptosis, and a cell cycle arrest protein in mammary tumors induced by NMU in rats. *B,* Gemini vitamin D analogues 0072 and 0097 (0.3 μg/kg body weight/d) reduce the protein expression level of Apolipoprotein A-I. *1* and *3,* Krt-19 protein; *2,* cytokeratin; *4,* no protein match; *5,* Annexin V complexed with glycerophosphoserine; *6,* β-actin; *7* and *11,* apolipoprotein A-I; *8,* thioredoxin; *9,* S100 calcium binding protein A6 (calcyclin); *10,* actin; *12,* heat shock protein 70 cognate (*first row*). PCNA and caspase-3 staining of tumors are shown. Positive reactions for PCNA and cleaved caspase-3 are noted by a reddish brown precipitate in the cells (*second* and *third row*). *C,* the Gemini vitamin D analogue 0097 increases the mRNA level of *CYP24A1* encoding 24-hydroxylase and *IGFBP-3* in NMU-induced mammary tumors. Two separate analyses were done and combined (statistical significance: *, $P < 0.01$; **, $P < 0.001$). *D,* the Gemini vitamin D analogue 0097 increases the protein level of IGFBP-3 and p21 in NMU-induced mammary tumors.

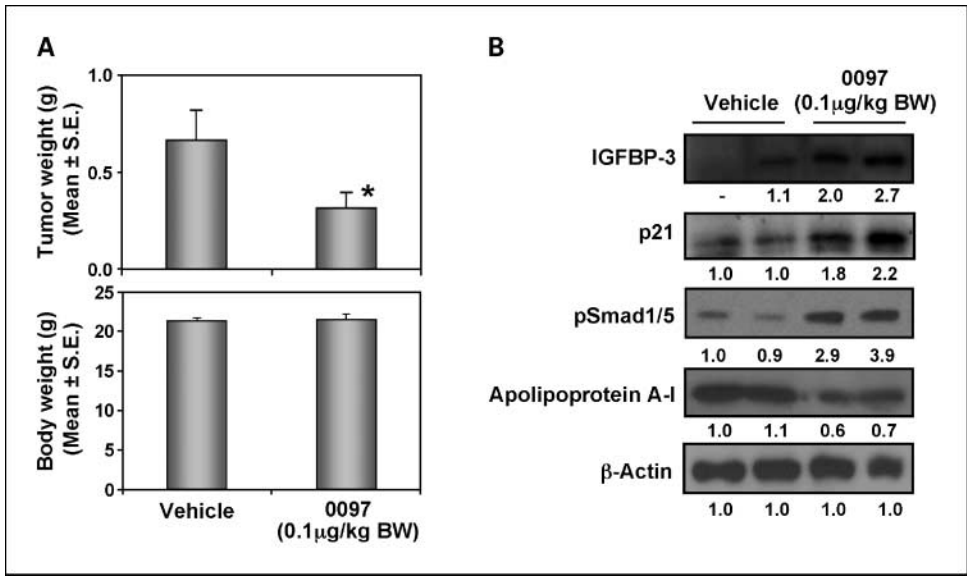

Fig. 3. The Gemini vitamin D analogue 0097 significantly inhibits tumor growth and regulates the level of IGFBP-3, the CDK inhibitor p21, pSmad1/5, and apolipoprotein A-I in ER-negative MCF10DCIS.com xenograft tumors in severe combined immunodeficiency mice. MCF10DCIS.com cells (1×10^6 cells) were injected into a mammary fat pad. Four days later, the mice received either vehicle (0.1 mL) or Gemini vitamin D analogue 0097 (0.1 μg/kg body weight in vehicle in 0.1 mL) i.p. daily. Six weeks later, tumors were weighed and collected at autopsy for further analysis. *A,* the average body weight and tumor weight per mouse were shown. The number of mice in vehicle control and 0097-treated group was 7 and 5, respectively (statistical significance: *, $P = 0.04$). *B,* the proteins from tumor samples were extracted with radioimmunoprecipitation assay buffer and the expression level of IGFBP-3, CDK inhibitor p21, apolipoprotein A-I, pSmad1/5, and β-actin was analyzed by Western blotting. The band intensity was quantified as described in Materials and Methods.

spectrometry/mass spectrometry of tryptic in-gel-digests of gel plugs (Fig. 2B). One of these proteins, apolipoprotein A-I, which was decreased by treatment with Gemini vitamin D analogues 0072 and 0097, was further investigated on Western blots (Fig. 3B). Apolipoprotein A-I was down-regulated in tumor-bearing rats treated with 0072 and 0097. Several other proteins were also identified using LC/MS/MS (Fig. 2B).

Evaluation of PCNA and caspase-3 expression in NMU-induced mammary tumors. All tumors from the control, 0072, or 0097-treated groups shown in Fig. 2B were evaluated and were determined to be adenocarcinomas, and the tumor type, differentiation/grade, and stromal responses among the control group and Gemini 0072 and 0097-treated groups were not different when evaluated after H&E staining (data not shown). However, there was a significant difference in PCNA and caspase-3 expression between the control group and the Gemini 0072 and Gemini 0097–treated groups, as shown in Fig. 2B. The PCNA staining in tumor tissues was much stronger in the control group (vehicle) than in the Gemini 0072 or Gemini 0097–treated groups. The percentage (±SE) of PCNA-positive cells in tumors from the control group was 62.6% ± 8.5%, whereas the percentages of PCNA-positive cells in tumors from the Gemini 0072 and Gemini 0097–treated groups were 32.5% ± 5.1% (significantly different from the control group, $P = 0.02$) and 45.3 ± 6.7% ($P = 0.1$), respectively. In addition, the expression of caspase-3 was evaluated in five to six different sections and representative sections of mammary tumors from the control group or the Gemini vitamin D analogues–treated groups are shown (Fig. 2B). The percentage (±SE) of caspase-3–positive cells in tumors from the control group was 1.8 ± 0.4, whereas the percentages of caspase-3 positive cells in tumors from the Gemini 0072 and Gemini 0097 treated groups were 13.1 ± 2.7 and 15.2 ± 4.3, respectively (both significantly different from the control group, $P < 0.01$), indicating that the Gemini vitamin D analogues strongly induced apoptosis in mammary tumors.

Effect of administration of Gemini 0097 on the expression of CYP24A1, IGFBP-3, and p21 in NMU-induced mammary tumors. *CYP24A1* encoding the 24-hydroxylase adds a hydroxyl group at the carbon 24 position in $25(OH)D_3$ or $1\alpha,25(OH)_2D_3$, which inactivates and leads to urinary excretion (8). *CYP24A1* is a well-known target gene of vitamin D and it has been shown to be induced by $1\alpha,25(OH)_2D_3$ or its analogue in different cells (6). In this study, the mRNA level for *CYP24A1* determined by quantitative PCR was increased by 0097 in tumor tissue dose dependently (Fig. 2C). The expression of insulin-like growth factor binding protein 3 (IGFBP-3), and CDK inhibitor p21 were enhanced by 0097, indicating that 0097 may regulate the insulin-like growth factor pathway to inhibit ER-positive mammary tumorigenesis in rats (Fig. 2C and D).

***Inhibitory effect of Gemini 0097 on MCF10DCIS.com growth inhibition* in vitro *and MCF10DCIS.com xenograft tumor growth* in vivo.** Because NMU-induced mammary tumors in rats are ER positive (28), we investigated the antitumor effect of Gemini vitamin D analogues on the growth of ER-negative MCF10DCIS.com cells. We first tested 60 Gemini vitamin D analogues for their growth inhibitory effects on cell growth of MCF10DCIS.com *in vitro*. As shown in Table 3, the growth inhibitory effects of the Gemini vitamin D analogues in the vitro assay were 5 to 15 times greater than that of $1\alpha,25(OH)_2D_3$ based on IC_{50} values. In an ER-negative MCF10DCIS.com xenograft model, we selected Gemini 0097 for further *in vivo* testing, and we found that administration of Gemini 0097 inhibited the tumor burden by 50% without a significant change in body weight (Fig. 3A) or serum calcium level (data not shown). Interestingly, the expression levels of IGFBP-3 and p21 protein were

Table 3. IC_{50} for the inhibitory effect of $1\alpha,25(OH)_2D_3$ and Gemini vitamin D analogues on the growth of MCF10DCIS.com breast cancer cells

Vitamin D and analogues	IC_{50} (nmol/L)*
$1\alpha,25(OH)_2D_3$	3.2
Gemini 3582 (3582)	0.2
Gemini 3586 (3586)	0.2
Gemini 0072 (0072)	0.4
Gemini 0097 (0097)	0.6

*MCF10DCIS.com cells were treated with vitamin D analogues (0.01, 0.1, 1, 10, and 100 nmol/L) in 5% horse serum/DMEM/F12 medium for 3 d. IC_{50} values were determined using TableCurve two-dimensional v5.1 software.

up-regulated by 0097 in MCF10DCIS.com tumors (Fig. 3B), suggesting that IGFBP-3 and p21 may act as common mediators of Gemini vitamin D analogues in inhibiting the growth of both ER-positive and ER-negative mammary tumors. Furthermore, expression of apolipoprotein A-I protein, which was suppressed by 0072 and 0097 in NMU-induced rat tumors (Fig. 2B), was also reduced by 0097 in MCF10DCIS.com xenograft tumors (Fig. 3B). The bone morphogenetic proteins (BMP) pathway was activated by 0097 treatment, which was measured by enhanced levels of phospho-Smad1/5 in tumors (Fig. 3B). Other Smad proteins (Smad1, 2, 3, and 5) were not changed by 0097 treatment (data not shown).

Discussion

Breast cancer in humans is complex involving different histopathologies, genetic changes, and clinical outcomes. These observations indicate that a single animal model may not mimic all features of human breast carcinogenesis (1). In the present study, we evaluated the inhibitory effects of Gemini vitamin D analogues on tumorigenesis in two different models: an ER-positive NMU-induced breast cancer model in rats and an ER-negative MCF10DCIS.com xenograft model in immunodeficient mice. NMU-induced mammary tumors showed similar gene expression profiles as were found in luminal type ER-positive, low- to intermediate-grade human breast cancer (28). Selective ER modulators, such as tamoxifen, raloxifene, or arzoxifene, have shown inhibitory effects in the NMU model (29), suggesting that this model may be a good ER-positive animal model of breast cancer for testing cancer preventive drugs.

As a xenograft model of breast cancer, we selected the MCF10DCIS.com cell line, which is a basal-like ER-negative cell type (24). MCF10DCIS.com human breast cancer cells are derived from *Ha-ras* oncogene transfection and further passages in mice (30). In addition to genetic *ras* mutation, MCF10DCIS.com cells significantly overexpressed the breast cancer associated marker, Her-2, compared with the parent cells, MCF10A,[5] and mammary tumors generated from MCF10DCIS.com cells were shown to dynamically interact with stroma during progression from *in situ* to invasive cancer (31). We used this cell line for both *in vitro* and *in vivo* studies. Although xenograft models may not be the best model for testing preventive effects of Gemini vitamin D analogues, MCF10DCIS.com model represents DCIS progression to invasive ductal carcinoma, which can be useful for testing new compounds for inhibition of breast cancer progression.

In this report, we showed that several Gemini vitamin D analogues had better efficacy for growth inhibition of MCF10DCIS.com cells than $1\alpha,25(OH)_2D_3$ (Table 3) and that certain Gemini vitamin D analogues significantly suppressed tumor growth in both an ER-positive NMU-induced breast cancer model and in an ER-negative MCF10DCIS.com xenograft model without hypercalcemic toxicity (Table 2; Fig. 3). Hypercalcemia is a well-established toxicity induced by $1\alpha,25(OH)_2D_3$ and many classic vitamin D derivatives. We found that $1\alpha,25(OH)_2D_3$ significantly increased the serum calcium level at doses showing tumor growth inhibition (Table 2). In contrast, Gemini 0097 exerted potent efficacy in preventing mammary tumor growth without causing hypercalcemia (Table 2). These results are in agreement with previous reports using a colon cancer model (19, 20). The lack of hypercalcemic effects of Gemini vitamin D analogues at anticancer doses may be because of the flexibility of the ligand binding pocket in the VDR (32). Recently, it was reported that structural rearrangement of the ligand binding pocket provides space to accommodate the second side chain of Gemini vitamin D analogues, which leads to expansion of the ligand binding pocket volume (33). This conformational flexibility of the ligand binding pocket to accommodate different ligands may result in different ligand-specific cofactor binding and/or selectivity of transcription of target genes (32, 34), thus suggesting that Gemini vitamin D analogues may show different regulation of vitamin D target genes when compared with $1\alpha,25(OH)_2D_3$.

In our mechanistic study, the expression levels of CDK inhibitor p21 and IGFBP-3 in tumor tissues from both models were enhanced by Gemini 0097 treatment (Figs. 2 and 3). Interestingly, however, the markers for apoptosis including cleaved PARP and caspase-3 were regulated by Gemini 0072 and 0097 in ER-positive mammary tumors induced by carcinogen (Fig. 2) but not in ER-negative tumors from the xenograft model (data not shown). It has been shown in several studies that $1\alpha,25(OH)_2D_3$ and its analogues suppress the mRNA and protein synthesis of ER through the negative VDR element in the ER promoter, which eventually leads to inhibition of cell proliferation through cell cycle arrest and apoptosis (35–37). The involvement of ER in inducing apoptosis by Gemini vitamin D analogues in NMU-induced mammary tumors needs to be further investigated.

IGFBP, a group of six different proteins, sequester free insulin-like growth factors by binding with high affinity, which inhibits the insulin-like growth factor signaling pathway having mitogenic activity (38). The expression of IGFBPs has been shown to be up-regulated by $1\alpha,25(OH)_2D_3$ and certain of its analogues in different cancer cells including prostate (39, 40), colon (41), and breast cancer (21). In breast cancer, several studies suggest that IGFBP-3 acts as a tumor suppressive factor (42, 43). Recently, the functional VDR element has been identified in the promoter region of the IGFBPs (44, 45),

[5] Unpublished data.

indicating that IGFBPs may be primary target genes of 1α,25 $(OH)_2D_3$ and its analogues. In the present study, we showed that Gemini vitamin D analogue treatment increased the expression of IGFBP-3 in breast tumors from both the carcinogen-induced model and the xenograft model, suggesting that IGFBP-3 induction may be mediated via direct transcriptional regulation. Interestingly, IGFBPs were shown to be involved in the regulation of the CDK inhibitor, p21 (46, 47). IGFBP-3 knockdown by siRNA reversed the induction of p21 and growth inhibition by synthetic androgen R1881 in LNCaP human prostate cancer cells, indicating that IGFBP-3 was upstream of p21 (46). These studies support our hypothesis that the Gemini vitamin D analogue 0097 inhibits tumor growth, at least in part, through the regulation of the link between IGFBP-3 and p21.

In several previous studies, vitamin D and its analogues have been involved in the regulation of transforming growth factor-β signaling, which is extensively reviewed in ref. (6). We have also reported that Gemini vitamin D analogues were shown to activate the bone morphogenetic protein/Smad signaling pathway via Ras/PKCα, which mediates the proliferation of the MCF10 breast epithelial cells (48, 49). In this report, we show for the first time that the Gemini vitamin D analogue 0097 activates Smad signaling *in vivo* in MCF10DCIS.com transplanted tumors (Fig. 3B), suggesting that the regulation of Smad signaling by vitamin D analogues may contribute to the suppression of mammary tumorigenesis. In addition, we showed that Gemini 0097 suppressed the apolipoprotein A-I protein expression in tumors from both animal models that we tested (Figs. 2B and 3B). Apolipoprotein A-I is one of the high-density lipoproteins, and 1α,25$(OH)_2D_3$ and other VDR modulators were shown to down-regulate the expression of apolipoprotein A-I in human hepatocytes and intestinal cells (50). Because a low serum level of high-density lipoproteins, such as apolipoprotein A-I, has been associated with breast cancer, it will be important to determine whether inhibition of apolipoprotein A-I by vitamin D analogues has an effect on the regulation of mammary tumorigenesis.

In conclusion, we investigated the *in vivo* effect of certain Gemini vitamin D analogues on mammary tumor growth in a chemically induced breast cancer model and in a xenograft model. We report here that Gemini vitamin D analogues significantly inhibit both ER-positive and ER-negative mammary tumor growth without increasing serum calcium levels. Mechanistic studies showed that the inhibitory activity was associated with the induction of IGFBP-3 and the CDK inhibitor p21 in both animal models. Taken together, these results suggest that Gemini vitamin D analogues may be promising agents for the prevention and treatment of breast cancer without hypercalcemic toxicity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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