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CANCER CHEMOPREVENTION USING NATURAL VITAMIN D AND SYNTHETIC ANALOGS

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■ **Abstract** Substantial epidemiologic data support a role for vitamin D in cancer prevention. However, dose-limiting hypercalcemic effects have proved a major obstacle to the development of natural vitamin D as a cancer chemopreventive. Structure-activity studies have sought to disassociate the toxicities and chemopreventive activities of vitamin D, and a number of synthetic deltanoids (vitamin D analogs) have shown considerable promise in this regard. Several such compounds have chemopreventive efficacy in preclinical studies, as does natural vitamin D. Data supporting further development of agents of this class include in vitro and in vivo evidence of antiproliferative, proapoptotic, prodifferentiating and antiangiogenic activities. Ongoing studies are aimed at further defining the molecular mechanisms through which vitamin D and synthetic deltanoids affect gene expression and cellular fate. Additional efforts are focused on establishing the chemopreventive index (efficacy vs toxicity) of each synthetic deltanoid.

INTRODUCTION

Cancer is the second leading cause of death in the United States. An estimated 552,200 Americans, more than 1500 per day, are expected to die from cancer in the year 2000 (1). While both therapeutic and preventive approaches against cancer have been developed, prevention is likely to be less costly and more effective for alleviating the pain and suffering—and ultimately the mortality—associated with the disease (2). Cancer chemoprevention entails intervention with micronutrients or other chemicals to block or delay the carcinogenic process (2, 3). Although carcinogenesis can occur relatively quickly, most cancers develop over decades. During this protracted time period, a process of increasing disorganization and

heterogeneity unfolds: as genetic mutations accumulate, a progressive loss of control over normal cellular processes ensues. While initially thought to require a stepwise progression of particular molecular lesions, cancer is now believed to arise from myriad different combinations of mutations in the array of pathways that control cellular proliferation and apoptosis. This progressive nature of carcinogenesis underscores the advantage of chemoprevention—to intervene when mutations are fewer, while some normal controls persist (4).

The strategy of early and chronic chemopreventive drug intervention against cancer has parallels in cardiovascular disease prevention (4). Indeed, as evidenced by FDA endorsement of prophylactic aspirin use in individuals at high risk for cardiovascular disease, this approach has gained increasing credibility (5). Notwithstanding significant progress to date, the successful development of drugs for cancer chemoprevention nevertheless faces several major obstacles. Because chemopreventive drugs will be administered chronically to large populations at relatively low risk of developing cancer, only agents with minimal toxicity can be considered for chemoprevention. Diet-derived compounds are of particular interest as potential chemopreventive agents because of their expected safety for long-term administration to healthy people. Moreover, epidemiologic studies support the chemopreventive efficacy of certain dietary components including vitamin D (6). The focus of this article is to review the chemopreventive properties of natural vitamin D and synthetic deltanoids. As presented below, substantial epidemiologic data support the possibility that vitamin D has a role in cancer prevention. Based on these findings, the chemopreventive efficacy of vitamin D has been explored in several animal models. Although the vitamin has been found to be efficacious in preclinical studies, the National Cancer Institute is no longer exploring vitamin D in chemoprevention or chemotherapeutic clinical trials (7). Dose-limiting calcemic effects have proved a major obstacle to the development of the natural vitamin as a chemotherapeutic or chemopreventive agent (8, 9). Structure-activity studies have sought to disassociate the toxicities and chemopreventive activities of vitamin D with the aim of producing analogs with improved therapeutic indices for chemoprevention. Several synthetic analogs of vitamin D have demonstrated chemopreventive efficacy in animals and show considerable promise for further development.

An overview of the metabolism and mechanisms of action of vitamin D that pertain to its potential use as a chemopreventive is presented in Figure 1. Following cutaneous production or intestinal absorption from dietary sources, vitamin D must undergo two hydroxylation steps to attain biological activity. After transport to the liver via serum vitamin D binding protein, the vitamin undergoes hydroxylation at the twenty-fifth carbon to yield the major circulating metabolite, 25-hydroxyvitamin D_3 [$25(OH)D_3$], which has a 19-day half-life. Subsequent hydroxylation in the kidney at the 1-α position produces the hormonally active 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$). The activity of $1\alpha,25(OH)_2D_3$ is thought to be primarily mediated through its binding to vitamin D receptors (VDRs), members of the steroid/thyroid receptor superfamily (10). The ligand-VDR complex heterodimerizes with the retinoid X receptor and binds, in turn, to

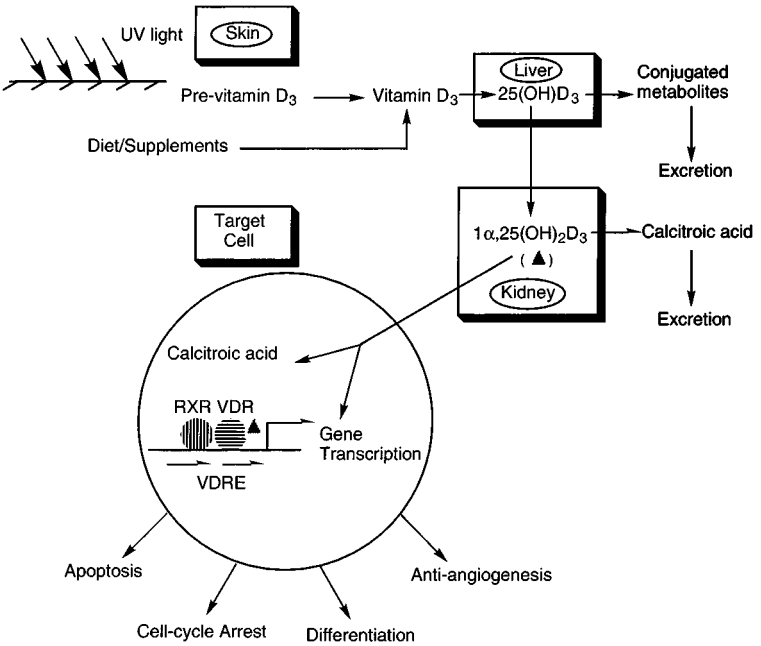

Figure 1 $1\alpha,25(OH)_2D_3$ biosynthesis and function.

the specific DNA sequences that constitute vitamin D response elements. At least 50 genes are regulated in this manner by VDRs. VDRs are expressed in bone and intestine, in addition to approximately 30 other tissues including mammary glands, colon, prostate, hematopoietic cells and skin (11). This wide tissue distribution of VDRs underscores the ability of vitamin D to exert pleiotropic actions throughout the body. Well-known to serve as a regulator of calcium homeostasis, this metabolite also exerts effects analogous to steroid hormones on processes such as mononuclear cell maturation and cytokine production. $1\alpha,25(OH)_2D_3$ also appears to act through nongenomic mechanisms independent of VDR-mediated gene transcription (12). As discussed in detail below, the ability of vitamin D and its analogs to exert anti-angiogenic activity and effects on cellular proliferation, differentiation, and apoptosis may underlie the chemopreventive efficacy of this class of compounds.

EPIDEMIOLOGIC EVIDENCE OF CANCER PREVENTIVE ACTIVITY

A potential role for vitamin D in cancer prevention was first suggested in the 1930s and 1940s by the discovery of the inverse association between sun exposure, which enables cutaneous production of the vitamin, and cancer rates. Rates of skin

and internal cancers—and overall cancer death rates—were found to be inversely related to distance from the equator (13, 14). Enhanced sunlight exposure has since been associated with lower prostate, breast and colon cancer death rates, while the historical geographic distribution of rickets parallels that for these cancer deaths (15–20). Furthermore certain risk factors for prostate cancer, including advanced age and African-American ethnicity, are associated with reduced vitamin D levels (21). As detailed in the following paragraphs, a number of epidemiologic studies have sought to ascertain whether high intake or serum vitamin D levels are predictive of or associated with reduced colon, breast, or prostate cancer risk. While many studies have supported the hypothesis that the vitamin is inversely associated with risk, this finding has not been definitively elucidated.

The strongest epidemiologic evidence supporting a protective role for the vitamin is from prospective studies of dietary or total (dietary and supplemental) vitamin D intake and colorectal cancer development. Four such studies have reported inverse associations for vitamin D intake and colon or colorectal cancer with relative risks ranging from 0.33–0.74 (22). A 19-year prospective study of 2107 Western Electric employees found a significant association between reported dietary vitamin D intake and subsequent development of colorectal cancer (23). The Iowa Women's Health Study and the Health Professionals Follow-Up Study each found an inverse association between total vitamin D and colon cancer risk, but these findings were not significant after multivariate adjustment (24–26). An inverse correlation between total vitamin D intake and colorectal cancer risk was also suggested in a prospective study of 89,448 female nurses (27). The analysis based intake upon dietary questionnaires conducted in 1980, 1984, and 1986 and excluded women who reported a substantial change in milk consumption prior to 1980. The study found relative risks for colorectal cancer in the highest versus the lowest intake categories of 0.72 and 0.42 for dietary and total vitamin D, respectively.

Results from case-control studies have been inconsistent regarding the association between vitamin D intake and colorectal cancer risk. A case-control study in Sweden found that increasing levels of dietary vitamin D were inversely associated with colorectal cancer risk, but only after adjustment for age, sex, and total caloric and protein intake (28). The associations found in this Swedish cohort were stronger for women. However, a case-control study of Wisconsin women found that although higher vitamin D intake was weakly associated with reduced colon and rectal cancer risk, a consistent, dose-responsive effect was lacking (29). A French case-control study found that vitamin D was inversely related to the risk of small adenomas in women; no such association was found for men, and no significant correlation with colorectal cancer risk was noted (30). Evidence of a potentially protective effect of vitamin D intake on breast cancer development is limited but intriguing. For example, the National Health and Nutrition Examination Survey (NHANES I) Epidemiologic Follow-up Study examined the effect of sunlight exposure and vitamin D intake on breast cancer risk in a multivariate analysis that controlled for age, education, age at menarche, age at menopause, body mass index, alcohol consumption, and physical activity (31). Several measures of sunlight exposure and dietary vitamin D intake each found a correlation with

reduced breast cancer risk. The association was strongest for women living in regions of high solar radiation, for whom relative risks were 0.35–0.75; no risk reductions were found for women residing in regions of low solar radiation. Although limited by small case numbers, the study suggests a dependency of the protective effect of sun and dietary vitamin D intake against breast cancer on high residential solar radiation.

Several epidemiological studies have demonstrated an inverse association between serum $25(OH)D_3$ levels and prostate cancer risk, but this finding has not been universally confirmed. For example in a Northern California cohort of European or African-American ethnicity, the 181 men who subsequently developed clinical prostate cancer had 1.8 pg/ml lower serum levels of total $1\alpha,25(OH)_2D_3$ than did age-matched controls. This inverse association between prediagnostic serum $1\alpha,25(OH)_2D_3$ levels and clinical prostate cancer was strongest in men aged >57 years (32). However, Braun et al (33) conducted a similar study in Maryland and observed no association with either $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$ in 61 men diagnosed with prostate cancer. A nested case-control study in a cohort of 3737 Japanese-American Hawaiian men also failed to find a strong association between serum vitamin D levels and the subsequent development of prostate cancer (34). After a surveillance period of 23 years, the serum levels of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ among 136 incident cases of prostate cancer were comparable to those of age-matched controls. Likewise, Gann et al (35) reported in 1996 that the 232 diagnosed cases of prostate cancer in a cohort of 14,916 US physicians were not associated with significantly lower serum $1\alpha,25(OH)_2D_3$, $25(OH)D_3$ or vitamin D binding protein. Nonetheless a nonsignificant inverse association for $1\alpha,25(OH)_2D_3$ was present for older men with low $25(OH)D_3$ levels.

A further study in the same cohort of US physicians demonstrated that VDR polymorphisms *Bsm*I and *Taq*I, which are not associated with altered receptor sequence or function, are not strong independent predictors of prostate cancer risk. It is interesting to note, however, that for men with plasma $25(OH)D_3$ levels below the median, risk was reduced for those homozygous for the absence of the *Bsm*I site (36). Increased risk of advanced prostate cancer in African-Americans was associated with a haplotype in which the *Bsm*I site is absent and a long poly-A microsatellite is present (37). In a separate study, men homozygous for the presence of the *Taq*I site were shown to have one third the risk of developing prostate cancer requiring prostatectomy compared to men who were heterozygous or homozygous for its absence (38).

PRECLINICAL EVIDENCE OF CHEMOPREVENTIVE EFFICACY OF VITAMIN D

Although vitamin D has demonstrated chemopreventive efficacy in several experimental models of carcinogenesis, the strongest evidence is for colon cancer prevention. As reviewed by Lipkin et al (39), deficient dietary vitamin D in a high fat diet induced adverse changes in the colon as well as in the mammary gland

and other organs. Several reports have shown that the vitamin or its active metabolite inhibits colon carcinogenesis induced by 1,2-dimethylhydrazine (DMH) and high fat diet. For example a high fat (20% corn oil) diet increased DMH-induced colon and small intestine tumor incidence in male F344 rats, while supplemental vitamin D (1000–2000 IU/kg-diet) reduced the level to the incidence seen in DMH-treated rats fed a low fat diet. However, the vitamin did not further suppress DMH-induced colon carcinogenesis in animals fed a low fat diet (40). In this study, dietary vitamin D was administered for 2 weeks before and 20 weeks after the 10-week carcinogen treatment. Although the total incidence of benign and malignant lesions was reduced by vitamin D, the most prominent effect was a reduction in adenoma incidence from 57% to 13%. Tumor number and size were not significantly affected by vitamin D intake. A less striking reduction in tumor incidence was found in a study of similar design in which vitamin D was administered with calcium (41). Supplementation with vitamin D (0.1 mg/kg-diet) in combination with calcium (15 g/kg-diet) inhibited colon tumor incidence by 45% in F344 male rats fed a high fat diet for 32 weeks following a single DMH administration. The decrease in tumor incidence was not statistically significant, but a significant reduction by the vitamin and calcium in kinetic indices of colonic proliferation was observed.

$1\alpha,25(OH)_2D_3$ (400 ng/rat) markedly reduced the multiplicity of colon adenocarcinomas when administered prior to DMH treatment of Charles River rats (42). The chemopreventive effect was associated with a reduction in ornithine decarboxylase levels. Elevated serum calcium levels were observed in treated animals. Inhibition of colon carcinogenesis was also engendered by 1α-hydroxyvitamin D_3 ($1\alpha(OH)D_3$) (0.04 μg) treatment in female F344 rats exposed to *N*-methyl-*N*-nitrosourea alone or with lithocholic acid (43). Serum calcium was significantly elevated by treatment. Additional evidence of the chemopreventive effects of vitamin D metabolites in the colon was obtained against tumors induced by azoxymethane (AOM). Male Wistar rats were given weekly AOM injections for 10 weeks concurrently with a 45-week, every-other-day administration of either 1α-hydroxyvitamin D_3 ($1\alpha(OH)D_3$) (0.06 or 0.12 μg/kg-body weight) or $1\alpha,25(OH)_2D_3$ (0.03 or 0.06 μg/kg-body weight). The higher doses of $1\alpha(OH)D_3$ and $1\alpha,25(OH)_2D_3$ inhibited the incidence of benign and malignant colon tumors but did not affect tumor multiplicity (44). A more prominent reduction in adenoma incidence was engendered by either form of the vitamin.

A chemopreventive effect of vitamin D has also been observed in models of cheek pouch, gastrointestinal, and skin carcinogenesis. Additionally $1\alpha,25-(OH)_2D_3$ (0.025 or 0.05 μg/mouse) inhibited retinoblastoma formation in transgenic SV40 T-antigen mice, although significant toxicity was manifested by hypercalcemia, weight loss, and death (45). Topical administration of vitamin D_3 or D_2 (approximately 0.2 ml of a 0.8% solution) significantly inhibited cheek pouch tumors induced by 7,12-dimethylbenz[*a*]anthracene (DMBA); 13/20 male Syrian golden hamsters administered DMBA for 8–10 weeks developed carcinomas, compared with 2/12 animals co-administered vitamin D_2 or 1/10 animals

co-administered D_3 (46). The 1-αhydroxylated metabolite (0.04 μg) has also been shown to inhibit gastrointestinal tumorigenesis in female Wistar rats when administered for 24 weeks after treatment with *N*-methyl-*N*′-nitro-*N*- nitrosoguanidine (47). The metabolite significantly reduced tumor incidence from 53% to 27% and tumor multiplicity from 16 to 8, in the stomach and small intestine of carcinogen-treated animals. Topical 1α,25$(OH)_2D_3$ (1 μg/week) also inhibited the multiplicity and enhanced the latency of skin tumors promoted by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) alone, or TPA and mezerin, in DMBA-initiated female Sencar mice (48, 49). However, dietary administration of vitamin D (200–4000 IU/kg-diet) did not affect DMBA-initiated, TPA-promoted skin tumor carcinogenesis (50).

An inhibitory effect of vitamin D against cancer cell growth has been observed in several xenograft models. For example, significant growth inhibition was engendered by 1α(OH)D_3 or 1α,25$(OH)_2D_3$ (2.5 or 5 nmol/kg) treatment beginning the day after inoculation of female euthymic hairy or athymic nude Balb/c mice with murine renal cancer Renca cells (51). 1α,25$(OH)_2D_3$ (0.75 μg daily for 1 or 3 days) also reduced tumor volume when administered 14 days after implantation of squamous cell carcinoma cells into C3H/HeJ mice (52). The growth of EMT-6 murine mammary carcinoma cells injected into male or female Balb/c mice immediately after topical treatment with 1α,25$(OH)_2D_3$ (0.3 to 0.6 μg/mouse) was also inhibited (53).

PRACTICAL LIMITATIONS OF THE CHEMOPREVENTIVE APPLICATION OF NATURAL VITAMIN D

The primary dose-limiting toxicity associated with acute or long-term administration of excessive amounts of vitamin D is altered calcium metabolism (8). In humans, vitamin D stimulates an increase in calcium absorption from intestines and bone that results in elevated blood calcium levels. At pharmacological doses, the most common adverse effects are hypercalcemia and hypercalciurea; soft tissue calcification and nephrocalcinosis also occur. Such effects limited dose escalation in Phase II clinical trials of 1α,25$(OH)_2D_3$ for chemotherapy of advanced prostatic cancer despite some evidence of efficacy (9, 54). In preclinical toxicity studies, the primary manifestations of the vitamin's toxicity were hypercalcemia, weight loss, and tissue calcification (6). Unfortunately, these calcemic effects have been noted at doses required to achieve chemopreventive efficacy.

Based on this limitation, structure-activity analyses have been undertaken in an effort to produce analogs with a chemopreventive index superior to the natural vitamin (55, 56). Toward this goal, well over 2000 deltanoids have been designed and synthesized, with most containing structural changes in the C,D-ring and the side-chain region (57). Outstanding examples of such therapeutically promising deltanoids include the following: Leo Pharmaceutical Company's KH-1060 (58), EB-1089 (59), and C18-attached side-chain analogs (60); Hoffmann-La Roche's

16-ene-23-yne and 16,23-diene series (61); Chugai Pharmaceutical Company's 22-oxa series (62); the Riverside group's arocalciferols (63); the Belgian C- and D-ring nor analogs (64, 65) and 14-epi analogs (66); and 24-ethyl analog 1α-hydroxyvitamin D_5 (67). Structural changes have also been made exclusively in the A-ring region, leading to separation of desirable antiproliferative and pro-differentiating activities from undesirable calcemic activity. Prominent examples include the following: the Madison (68), the Riverside (69), and the Providence (70) 3-epi analogs, DeLuca's 19-nor analogs (71), the Austrian aromatic analogs (72), and the Johns Hopkins University 1-hydroxyalkyl series (73). Structural changes in both the A-ring and the C,D-ring side-chain regions have produced transcriptionally potent, noncalcemic hybrid analogs (74). Despite the very large number of deltanoids having been prepared and evaluated biologically, it is still risky to predict the biological activities of any new deltanoid based on planned structural changes (57). Many of the new synthetic analogs are showing considerable promise in preclinical trials, and a few even in clinical trials (9), for cancer chemotherapy; relatively few such deltanoids, however, have been examined for cancer chemoprevention.

CHEMOPREVENTIVE ACTIVITY OF SYNTHETIC DELTANOIDS

As shown in Figure 2, prominent examples of the structural modifications associated with chemopreventive activity include the following: an extra ethyl group at position-24 [1α(OH)D_5]; an extra oxygen atom at position-22 (Chugai-OCT) plus 20-epimerization (Leo KH-1060); 16- and 23-unsaturation with (Ro 24-5531) or without (Ro 25-9022 and Ro 25-6760) a 19-methylene group and with metabolism blocking terminal trifluoromethyl groups; an A-ring calcemia-lowering modification plus a side-chain potentiating group (Hopkins QW-1624F2-2); and a side-chain conjugated 22, 24-diene (Leo EB-1089). Evidence supporting the efficacy of these compounds is presented in the following paragraphs.

Synthetic deltanoid 1α(OH)D_5, lacking the 25-hydroxyl group thought to be essential for effective ligand binding to the vitamin D receptor (VDR), is only very weakly calcemic. 1α(OH)D_5 (0.042 μg/kg/day) was four times less calcemic in vitamin D-deficient Sprague-Dawley rats than was 1α,25$(OH)_2D_3$ (75). Some toxicity (i.e. decreased body weight gain) was observed in Balb/c athymic mice injected three times/week with 1α(OH)D_5 at a dose of 200 ng, but not at 100 ng or less (67). In an in vitro cancer chemoprevention study (75), 1α(OH)D_5 inhibited the development of DMBA-induced preneoplastic lesions in mouse mammary organ culture; this effect was dose-related (0.01–10.0 μM) and without any observed toxicity. Although the natural hormone 1α,25$(OH)_2D_3$ was 10- to 100-fold more potent than 1α(OH)D_5 at preventing preneoplastic lesion development, it induced significant toxicity at concentrations of 1.0 μM or higher. Efficacy of both agents was associated with dramatically enhanced expression of both VDR and TGF-β1.

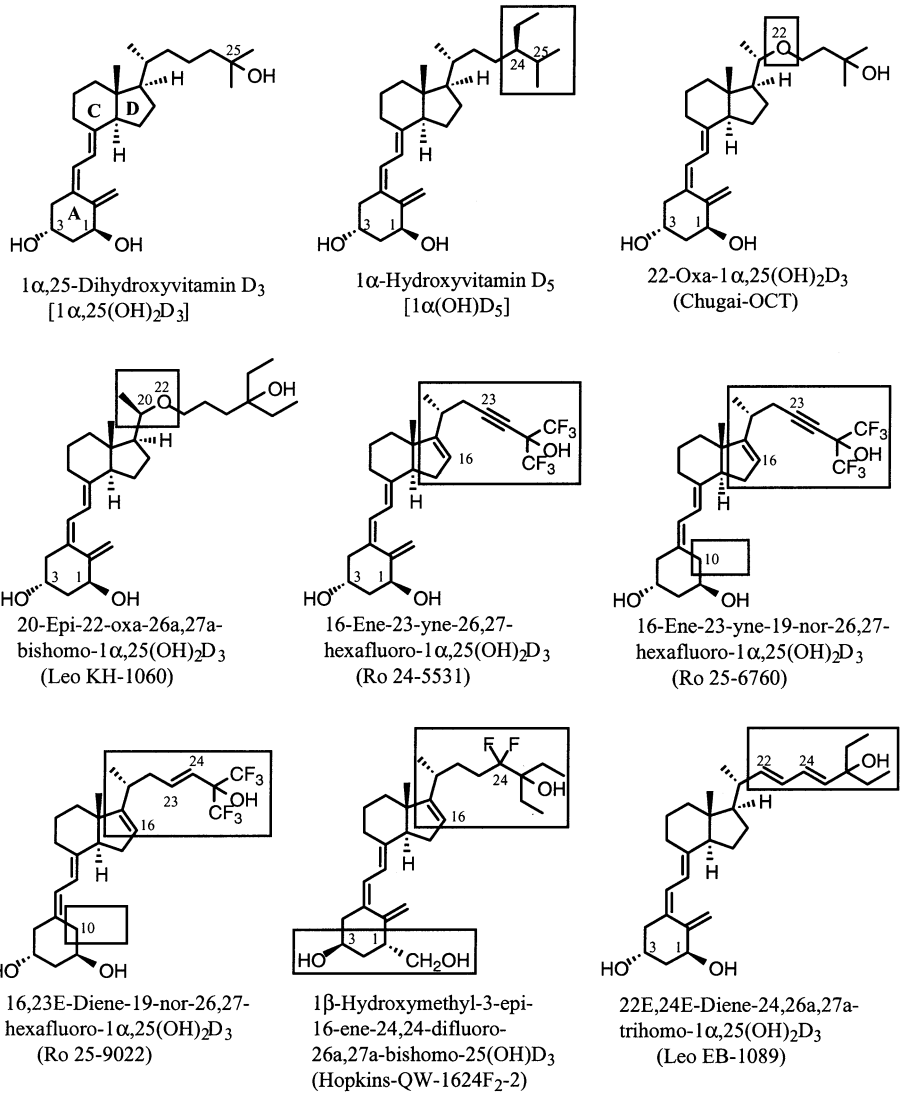

Figure 2 Deltanoids discussed in this chapter.

The deltanoid 1α(OH)D_5 was also efficacious in an in vivo xenograft model (67). Balb/c athymic mice were inoculated with human breast cancer U1S0-BCA-4 cells after which 1α(OH)D_5 (4 or 8 ng/animal, 25–50 times less than the toxic dose) was injected 3 times/week for 60 days. Tumors appeared at the injection site in 5/5 control animals, and lymph node metastases were detected in 2/5 of these animals. In contrast, only a scab-like structure was observed at the inoculation site

in 1/5 of the animals at each deltanoid dose. Histologically, this scab-like tumor showed few human breast carcinoma cells embedded in the stroma. No metastases were observed in the deltanoid-treated animals. Although this novel deltanoid is considerably less potent than Ro 24-5531 at inhibiting growth of U1S0-BC-4 cells (67), $1\alpha(OH)D_5$ may deserve further preclinical evaluation in a chemoprevention model of breast cancer.

The analog 22-oxa-1α,25$(OH)_2D_3$ (Chugai-OCT) is approved in Japan for treatment of secondary hyperparathyroidism associated with chronic renal failure. As reviewed in Brown & Slatopolsky (76), this deltanoid is less calcemic than 1α,25$(OH)_2D_3$. Chugai-OCT (1.0 μg/kg body weight) significantly inhibited the growth of established DMBA-induced mammary tumors in female Sprague-Dawley rats (77). The deltanoid did not, however, induce regression of the tumors. No elevation of serum calcium levels was noted, but Chugai-OCT did slightly retard weight gain. Chemopreventive activity in the colon was noted in male F344 rats treated with Chugai-OCT that had been exposed to multiple carcinogens (78). After four weeks of combined treatment with five different chemical carcinogens (*N*-diethylnitrosamine; *N*-methyl-*N*-nitrosourea, *N,N*'-dimethylhydrazine, *N*-buty l-*N*-(4-hydroxybutyl)nitrosamine, and di-*N*-propylnitrosamine), rats were maintained without any treatment for two weeks prior to initiation of a 30-week treatment with Chugai-OCT (30 μg/kg-body weight). Body weight gain was unaffected by Chugai-OCT, but plasma calcium concentrations were significantly elevated above those in controls (11.7 ± 0.6 vs 9.9 ± 0.4 mg/dL), as was also noted in rats receiving only 3 μg/kg body weight of Chugai-OCT. Remarkably, carcinoma incidence in the small intestine was 0% compared to 18% in carcinogen-treated controls. A lowering of large intestine carcinoma incidence (36% vs 12%) was also observed in the Chugai-OCT-treated rats. Chugai-OCT was without effect on carcinogenesis induced in a number of other organs, including lung, large intestine, kidney, urinary bladder, and thyroid gland.

Deltanoid Ro 24-5531 (1.25 or 2.5 nmol/kg-diet) was only moderately effective at inhibiting the development of carcinogen-initiated and androgen-promoted carcinomas of the seminal vesicle and anterior prostate of Lobund-Wistar rats (79, 80). These same doses of Ro 24-5531 significantly reduced the incidence as well as multiplicity of mammary carcinomas induced by a single injection of *N*-nitroso-*N*-methylurea in female Sprague-Dawley rats (81). The latency of palpable tumor incidence was also extended by deltanoid treatment, which began 1 week postcarcinogen and continued for 32 weeks thereafter. No effect on either body weight or serum calcium levels was observed with Ro 24-5531. The chemopreventive effect was synergistic with tamoxifen. The aim of a 1995 study (82) was to determine whether dietary supplementation with Ro 24-5531 inhibited colon cancer chemically induced by AOM. An Ro 24-5531 (2.5 nmol/kg-diet) treatment was given to the rats before and during AOM (initiation arm) or after AOM (postinitiation arm) and was terminated 34 weeks thereafter. Ro 24-5531 treatment during the initiation arm significantly reduced (by 70%) the incidence of AOM-induced

colonic tumors and abolished adenocarcinoma development. An observed trend for Ro 24-5531 treatment during the postinitiation arm to reduce colon tumor incidence did not reach statistical significance. Neither Ro 24-5531 regimen significantly influenced growth rates or serum calcium levels. Although the calcemic effects of Ro 24-5531 following acute administration were only 6.7%–10.4% of those engendered by the natural vitamin (83), a separate study revealed Ro 24-5531 to be significantly calcemic (84). In a recent long-term (55 week) study, Ro 24-5531 actually enhanced bone properties in Balb/c mice (85). Despite the immunosuppression (as evidenced by a profound decrease in serum IL-2) and decreased body weight seen in animals treated with Ro 24-5531 or Ro 25-6760, the analogs were fairly well tolerated at weekly doses of 0.0125 μg/mouse. However, a 50% dose reduction was necessitated at 16 weeks by hypercalcemia, which nevertheless redeveloped near the study's end. Blood chemistries and gross organ pathology were otherwise normal. The hybrid 19-nor deltanoid Ro 25-6760, as shown in a separate study, exhibits slightly higher calcemic activity than does 1α,25$(OH)_2D_3$ (84).

Efficacy of the hybrid 19-nor deltanoid Ro 25-6760 was demonstrated in a xenograft model using two different human colon cancer cell lines expressing either high (HT-29) or low (SW-620) levels of VDR (86). Injection of Ro 25-6760 (0.1 or 0.2 μg) three times per week for five weeks significantly inhibited the growth of HT-29 cells but not of SW-620 cells in female athymic nude mice without causing hypercalcemia. In 30% of mice treated with this deltanoid, the HT-29 tumors disappeared after the second injection, and tumor growth did not resume even after drug withdrawal. 1α,25$(OH)_2D_3$ was without inhibitory effect on the growth of either cell line.

Using the less calcemic hybrid 19-nor deltanoid Ro 25-9022,a dietary study of colon cancer prevention has recently been completed (87). Adult Sprague-Dawley rats were administered Ro 25-9022 (3.0 or 3.5 nmol/kg-diet) for 30 weeks with concurrent DMH weekly treatment during weeks 10–30. Compared with controls, tumor incidence was reduced by 32% and 28% with 3.0 or 3.5 nmol/kg Ro 25-9022, respectively. The development of metastases was significantly inhibited by the lower and completely abrograted by the higher deltanoid dose. Neither undesirable lowering of animal weight gain nor increase in serum calcium was observed.

Conventional wisdom requires the presence of both the 1α-OH and the 25-OH groups for expression of the high and diverse biological activities of 1α,25$(OH)_2D_3$ (57). We have demonstrated, however, that replacing the natural 1α-OH group by a 1β-CH_2OH unit produces antiproliferative analogs having severely diminished calcemic activity; the additional incorporation of potentiating side-chain structural modifications along with the 1β-CH_2OH unit produces hybrid analogs that are antiproliferatively and transcriptionally potent yet noncalcemic (88). One of the best of such hybrid deltanoids is QW-1624F2-2, which incorporates not only the calcemia-ablating 1β-CH_2OH group but also the potentiating C,D-ring 16-unsaturation, the side-chain 24,24-difluorination, and the 26,27-homologation

(88). Despite the absence of the natural 1α-OH in hybrid deltanoid QW-1624F-2-2, its VDR-mediated transcriptional activity ($ED_{50} = 5 \times 10^{-11}$M) in rat osteosarcoma ROS 17/2.8 cells exceeds that of the natural hormone 1α,25$(OH)_2D_3$ ($ED_{50} = 3 \times 10^{-10}$M) (88). A skin cancer chemoprevention study using this hybrid deltanoid has been performed recently in female CD-1 mice initiated with DMBA and promoted biweekly for 20 weeks with TPA (89). Topical application of deltanoid QW-1624F2-2 (3 μg/mouse) 30 min before each TPA application significantly enhanced tumor latency in addition to reducing tumor incidence by 28% and tumor multiplicity by 63%. Unlike natural 1α,25$(OH)_2D_3$ at this dose, hybrid deltanoid QW-1624F2-2 did not adversely affect body weight gain in these animals. Moreover, no increase in urinary calcium excretion was observed following this chronic treatment with QW-1624F2-2, a result consistent with our observation that this deltanoid is about 100-fold less calcemic than calcitriol (88). In a separate experiment, the low in vivo calcemic activity of deltanoid QW-1624F2-2 was confirmed even when administered to rats at a dose 100-fold higher (20 μg/kg body weight) than that at which Leo Pharmaceutical's deltanoid CB-1093 (90) caused significant weight loss (D Somgen, A Kaye, & GH Posner 2000, unpublished results). Based on this high efficacy and safety of our deltanoid QW-1624F2-2, it is now being tested in a preclinical rat mammary cancer chemoprevention model. If, as expected, deltanoid QW-1624F2-2 performs well in this second rodent model of carcinogenic chemoprevention, then further preclinical testing of this hybrid deltanoid will be timely and appropriate, as will basic research into the fundamental biological mechanism(s) underlying the selective pharmacology and toxicology of this fascinating deltanoid.

Selecting a lead candidate for development from among the various synthetic deltanoids would be greatly facilitated by a series of cancer chemopreventive studies involving direct, side-by-side comparative performance. Only via such simultaneous comparisons of these deltanoids in the same experimental protocol will their relative prophylactic indices (chemopreventive potency vs toxicity) be reliably established. The following combination treatment experiment, albeit in tumor growth inhibition, illustrates such a side-by-side assessment. The combination of a deltanoid with paclitaxel (Taxol) was efficacious in inhibiting the growth of human breast cancer MCF-7 cells in BNX nude mice when administered for nine weeks after inoculation (91). Leo EB-1089, Ro 25-6760, or natural 1α,25$(OH)_2D_3$ administered alone or with Taxol, resulted in statistically smaller tumors than those in controls. The potency of Leo EB-1089 exceeded that of Ro 25-6760, which in turn was more potent than natural 1α,25$(OH)_2D_3$. Furthermore, Leo EB-1089 plus Taxol suppressed tumor growth more than either drug alone, causing a 30%–50% lowering of average tumor weight compared to tumor weight in controls. In combined Leo EB-1089 and Taxol treatment, no elevation in serum calcium levels was observed, nor was there a statistically significant reduction in animal weight gain. Leo EB-1089 was evaluated in a phase I clinical trial of advanced breast

and colorectal cancer; although less calcemic than $1\alpha,25(OH)_2D_3$, hypercalcemia limited the tolerable dose of Leo EB-1089 to 7 $\mu g/m^2$/day (92).

MECHANISMS OF ACTION OF NATURAL VITAMIN D AND SYNTHETIC ANALOGS

Prominent chemoprevention-related actions of vitamin D and synthetic deltanoids include antiproliferative, prodifferentiating, proapoptotic, and antiangiogenic effects that have been observed in a variety of tissues and cell lines. For example, vitamin D and its analogs inhibit the growth of cancer cell lines derived from skin, breast, endometrium, head and neck, lung, prostate, colon, and hematopoietic lineages (93). As reviewed in Kelloff et al (6), a number of mechanisms through which vitamin D may achieve these effects on cellular fate have been explored, including modulation of signal transduction and oncogene expression, and inhibition of ornithine decarboxylase induction, DNA synthesis, lipid peroxidation, and TGF-β expression. An expanding number of genes involved in cell growth and differentiation, including cytokines (interleukins IL-1β, IL-2, IL-8, IL-12, and GM-CSF), transcription factors, and tumor suppressor genes (BRCA1 and E-cadherin), appear to be targeted by vitamin D (94). Although some of the actions are independent of VDR (for example, changes in PKC isoform expression by Ro 24-5531 in AOM-treated rat colon (95), VDR is thought to play a prominent role in mediating the chemopreventive effects of vitamin D and its analogs. Indeed, the expression of VDRs in cancers of a number of tissues, including those of the colon, prostate, and breast, supports this view.

The ability of the vitamin and synthetic deltanoids to directly affect VDR half-life and transcriptional activity has been the subject of several recent investigations. For example, Leo KH-1060 caused a threefold increase in VDR half-life in human osteoblastic sarcoma MG-63 cells; EB-1089 maintained VDR levels for a longer time than did $1\alpha,25(OH)_2D_3$, although each doubled the half-life from 5 to 10 h (96). Leo EB-1089 may achieve this effect by stabilizing the high affinity ligand binding conformation of VDR (97). $1\alpha,25(OH)_2D_3$ and the deltanoid Chugai-OCT may enhance the efficiency with which VDR binds other necessary components of the transcriptional machinery, particularly the VDR interacting protein coactivator complex, and thereby they may increase the transactivation capacity of the receptor (98). These data suggest that upon binding of natural vitamin D or synthetic deltanoids, the VDR assumes a conformation that is permissive of transcriptional activation but which prevents binding of proteins mediating degradation (96). In human skin, $1\alpha,25(OH)_2D_3$ retards degradation of VDR via the ubiquitin/proteasome pathway by blocking ubiquitination of the receptor (99). Structure-activity analyses have provided insight into the VDR residues at which certain deltanoids may bind to achieve enhanced VDR stabilization and activation. For example, Peleg et al (100) demonstrated that some hybrid analogs with

an unnatural 1-hydroxymethyl substituent do not interact with the transcription activation function 2 domain of the VDR, but do exhibit increased ability to stabilize and transcriptionally activate the receptor.

The antiproliferative actions of $1\alpha,25(OH)_2D_3$, Ro 24-5531, and Ro 25-6760 appear to be mediated by the induction of arrest of cell cycle progression at the G1 phase (101). This G1 arrest is associated with upregulation of the cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p27^{KIP1}$, decreased cyclin-dependent kinase activity, hypophosphorylation of retinoblastoma protein, and repressed E2F transcriptional activity in several cell types; as the $p21^{WAF1}$ gene promoter contains a VDR response element, this effect may in part be VDR-dependent (102–104). TGFβ-1 induction may also play a role in the induction of these genes and the resulting G1 arrest (105). $1\alpha,25(OH)_2D_3$ also enhances the expression of HoxA10, a homeobox protein that causes G1 arrest (104). It is interesting that forced overexpression of $p21^{WAF1}$ in myelomonocytic U937 cells results in cell-surface expression of differentiation-associated proteins (102). However, $p27^{KIP1}$ antisense treatment of HL60 cells abrogrates the G1 block but does not affect differentiation stimulated by $1\alpha,25(OH)_2D_3$, which suggests that $p27^{KIP1}$ plays a pivotal role in vitamin D-induced HL60 cell cycle arrest but not in differentiation (106).

The ability of vitamin D and analogs to induce apoptosis has been investigated in vivo and in several cell types in vitro. For example, apoptosis was detected in prostate sections of Leo EB-1089-treated rats, an effect associated with increased expression of IGF-binding proteins -2, -3, -4, and -5 and of IGF-I mRNA (107). The analog Leo EB-1089 also promotes breast cancer cell apoptosis in vitro and thereby enhances the sensitivity of these cells to radiation (108). Recent evidence suggests that vitamin D and Leo EB-1089 induce apoptosis in colon and breast cancer cells via a mechanism independent of p53 (109, 110). The Bak protein is suggested to mediate this effect in colon cancer cells (110).

Vitamin D and its analogs inhibit angiogenesis and metastasis in xenograft and transgenic mouse models, and they decrease the invasiveness of several cell types in vitro. A significant reduction in angiogenesis was engendered by daily administration of $1\alpha,25(OH)_2D_3$ (0.5 or 1 μg/kg) for five days prior to intradermal injection into immunosuppressed Balb/c mice of human tumor cell lines of different origin (cervical, vulval and breast) (111). As assessed by vessel counts, angiogenesis was also inhibited in transgenic murine retinoblastoma mice treated with vitamin D (0.025 or 0.05 μg five times/week for five weeks) (112). Both $1\alpha,25(OH)_2D_3$ and Leo EB-1089 (1.0 μg/kg) inhibited prostate cancer cell metastasis in the rat Dunning MAT LyLu model (113). In this study, rats treated with $1\alpha,25(OH)_2D_3$ or Leo EB-1089 developed significantly fewer lung tumor foci; although Leo EB-1089 was significantly less calcemic than $1\alpha,25(OH)_2D_3$, serum calcium levels were elevated by both agents. $1\alpha,25(OH)_2D_3$ also induced severe weight loss. In in vitro bioassays of cell invasion, $1\alpha,25(OH)_2D_3$ inhibited the invasiveness of human prostate cancer DU 145 cells (114) and mouse melanoma B16 cells (115). In both cases, reduced invasion was associated with a decrease in

secreted levels of type IV collagenases. In vivo treatment with $1\alpha,25(OH)_2D_3$ (0.5 μg/kg) for 28 days reduced the formation of lung metastases in mice inoculated with B16 cells (115).

SUMMARY AND PERSPECTIVES

Several deltanoids with considerable promise as chemopreventive agents are discussed in this article. The potential for clinical development of these compounds depends largely on their ability to prove safe upon chronic exposure. A major concern is calcemic side effects; although less potent than natural vitamin D at inducing hypercalcemia, several deltanoids (e.g. Ro 24-5531 and Ro 25-6760) do mediate such effects in a dose-dependent manner. One possibility for minimizing the dose of deltanoids in the chemoprevention setting is to administer the agent in combinations that would engender a synergistic effect. Because the VDR heterodimerizes with the retinoid X receptor to control transcription of target genes, retinoids constitute one class of compounds that could be considered for combination chemopreventive interventions with deltanoids. In support of this view is the fact that retinoids act synergistically with vitamin D and synthetic deltanoids to inhibit the growth and promote the differentiation of human myeloid leukemia, as well as prostate and breast cancer cells in culture (93, 116). The antiestrogen tamoxifen has also been shown to act synergistically with vitamin D in the inhibition of breast cancer cell growth in vitro (117); moreover, tamoxifen synergistically enhances the chemopreventive efficacy of Ro 24-5531 against carcinogen-induced rat mammary carcinogenesis (81). Selection of an appropriately safe and effective deltanoid for development, for application in either a single or combination chemopreventive regimen, should rely on direct comparisons among candidate compounds.

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