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Vitamin D and cancer

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Abstract

Vitamin D, a steroid hormone and exerts its biological effects through its active metabolite 1α, 25 dihydroxyvitamin D3 [1,25(OH)2D3]. Like steroid hormones, 1,25(OH)2D3 is efficacious at very low concentrations and serves as a ligand for vitamin D receptors (VDR), associating with VDR very high affinity. Despite its potent property as a differentiating agent, its use in the clinical practice is hampered by the induction of hypercalcemia at a concentration required to suppress cancer cell proliferation. Therefore nearly 400 structural analogs of vitamin D3 have been synthesized and evaluated for their efficacy and toxicity. Among these analogs, relatively less toxic but highly efficacious analogs, EB1089, RO24–5531, 1α-hydroxyvitamin D5 and a few others have been evaluated in a preclinical toxicity and in Phase I clinical trials for dose tolerance in advanced cancer patients. Clinical trials using vitamin D analogs for prevention or therapy of cancer patients are still in their infancy. Vitamin D mediates its action by two independent pathways. Genomic pathway involves nuclear VDR and induces biological effects by interactions with hormone response elements and modulation of differential gene expressions. Evidence also suggests that vitamin D analogs also interact with steroid hormone(s) inducible genes. The non-genomic pathway is characterized by rapid actions of vitamin D. It involves interactions with membrane-VDR interactions and its interactions with protein kinase C and by altering intracellular calcium channels. Thus, the development of nontoxic analogs of vitamin D analogs and understanding of their molecular mechanism(s) of action are of significant importance in the prevention and treatment of cancer by vitamin D.

Keywords: Vitamin D; Analogs of vitamin D; VDR; Carcinogenesis; Metabolism; Mechanism of action

1. Background

Vitamin D was discovered by Edward Mellanby in 1919 during his classic experiments with rickets [1]. It is a family of compounds consisting of 9,10 secosteroids, which differ, in their side-chain structures. They are classified into five forms [2]; vitamin D2, ergosterol; D3, cholecalciferol; D4, 22,23 dihydroergoalciferol; D5 sitosterol (24-ethylcholecalciferol) and D6 stigmasterol (Fig. 1). Vitamin D is derived from a cholesterol-like precursor, 7-dehydrocholesterol. When human skin is exposed to sunlight, the UV-B photons (between 290–315 nm) interact with 7-dehydrocholesterol causing photolysis and cleavage of the B-ring of the steroid structure, which upon thermoisomerization results into a secosteroid [3,4]. In order to produce physiological activity, vitamin D has to be metabolized. Numerous in-depth reviews focusing on the metabolism of vitamin D have been published. Since the metabolism of vitamin D is not the primary focus of this article, a simplistic overview of D-metabolism is briefly discussed here. The pro-hormone vitamin D gets metabolized to 25-hydroxyvitamin D in liver by 25-hydroxylase. This metabolite is present in the circulation at a concentration of more than 0.05 μM (20 ng/ml). The active metabolite of vitamin D, however is generated by hydroxylation of 25-hydroxyvitamin D at 1α-position in kidney. The enzyme 1α-hydroxylase has also been shown to be present in keratinocytes and prostate epithelial cells, suggesting that the fact that target organs may also be able to generate 1,25 dihydroxyvitamin D3 from 25-hydroxyvitamin D3 [5]. More recently mRNA for 25-hydroxvitamin D-1α-hydroxylase has been reported in normal and malignant colon tissue [6,7]. The active metabolite 1α,25-dihydroxyvitamin D is present in the human plasma at a concentration of 0.05–0.15 nM (20–60 pg/ml) [8,9]. In addition to 1α-hydroxylation of 25-hydroxyvitamin D3, many metabolites have been identified. These metabolites are side chain modifications with no definitive function assigned to them. The overall path of metabolism of vitamin D2 is similar to vitamin D3 with a few differences [10]. Both 25-hydroxyvitamin D2 and 1α,25-dihydroxyvitamin

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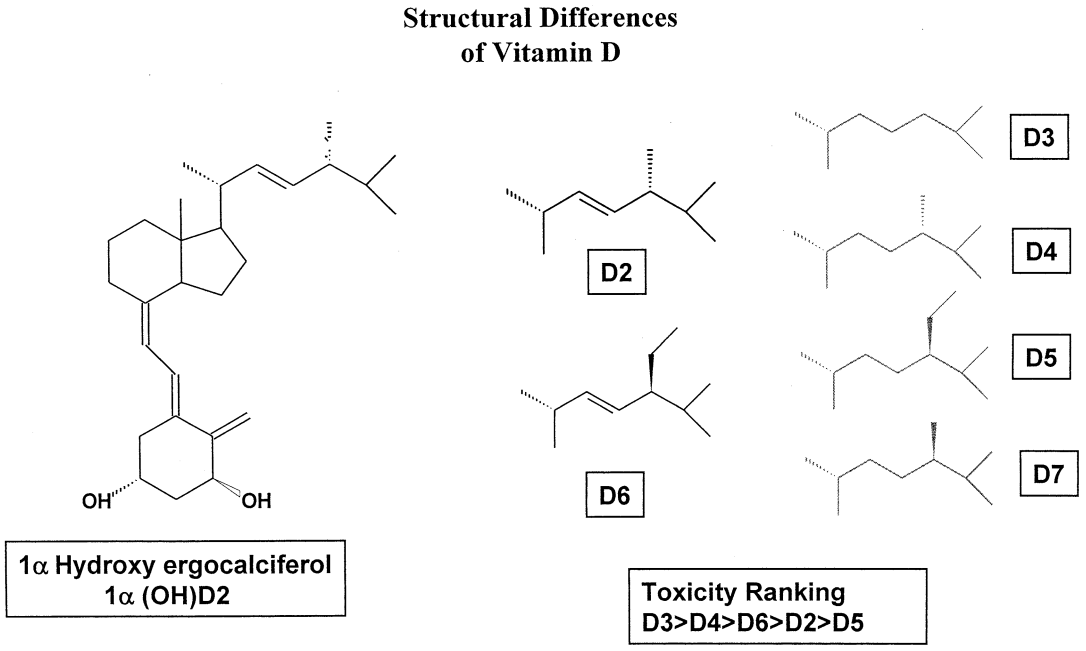

Fig. 1. Structural differences of vitamin D. Vitamin D has been classified into various classes of D2 through D7. Ergocalciferol is classified as vitamin D2 and vitamin D6 is a 24-ethyl analog of vitamin D2. On the other hand vitamin D3 or cholecalciferol is modified by either methyl or ethyl group on C-24 position. These vitamin D molecules are further classified as D3, D4, D5 and D7.

D2 have been evaluated for their biological functions. The catabolism of vitamin D occurs by further hydroxylation of 25-hydroxyvitamin D3 by 24 hydroxylase to yield 24,25 dihydroxyvitamin D3. The enzyme 24-hydroxylase is ubiquitous and is expressed in all the cells expressing vitamin D receptors (VDR). The enzyme is regulated by PTH and 1α,25-dihydroxyvitamin D3. The major significance of 24-hydroxylation is inactivation of vitamin D3 [11,12]. The inactivated vitamin D metabolites are nonfunctional. The overall metabolism of vitamin D is outlined in Fig. 2.

2. Experimental basis for vitamin D and cancer

For the past 20 years it has been consistently reported and well established that the active metabolite of vitamin D, 1,25(OH)2D3 exhibits potent cell differentiating property in leukemia cells as well as much cancer cells [13,14]. The antiproliferative and differentiation-inducing effects can be of clinical significance in prevention or treatment of cancer of several target organs. One of the main limitations in this modulation is the fact that the concentration required for being efficacious for 1,25(OH)2D3, is also very toxic. The effective concentration of 1,25(OH)2D3 induces dangerously high levels of serum calcium in experimental animals resulting in body weight loss and could be occasionally lethal [15]. This has resulted in the synthesis of analogs of vitamin D molecule with the hope of generating an analog that is effective in prevention of cancer or suppressing growth of cancer cells in culture and in vivo models without expressing any toxic adverse effects. Typically, the vitamin D structure is divided into four parts. The A ring, B ring, CD ring and the side chain. The alterations can be made at all these four sites, except the modification of the CD ring is not very common due to the rigid structure. The maximum alterations, on the other hand, are made from the open side chain. Nearly 400 analogs of vitamin D have been synthesized and many of them have been evaluated [16,17]. As far as the efficacy in in vitro or in vivo cancer models are concerned, where the risk benefit ratio related to toxicity and efficacy is determined, only a handful of vitamin D-chemicals have been successfully utilized [16,18]. The most widely studied analogs besides 1,25-dihydroxy D3, include 22-oxa-calcitriol [19,20] (Chugai Pharmaceuticals, Japan), EB1089 [21] (Leo Pharmaceuticals, Denmark), calcipotriol, KH1060 [22] (Leo Pharmaceuticals, Denmark), R024–5531 [23,24] (Hoffman la Roche, Nutley, NJ) and recently synthesized analog from our laboratory, 1α-hydroxy-24 ethyl-vitamin D3 [25] (1α(OH) D5, OncQuest, Chicago, IL). The side chain modifications of vitamin D3 molecule to result in these selective structures is shown in Fig. 3. All these analogs have been evaluated in a variety of cancer cell culture models, in vivo carcinogenesis models and in xenograft models using athymic mice. The main selection criteria here is to adopt a compound that does not induce hypercalcimia or other undesirable side effects at the effective dose level. The criteria for selection of vitamin D agents for other conditions such as bone disease, immunomodulation or hormonal therapy or nutrition can be very different and will not be discussed here since it will not be within the scope of this review.

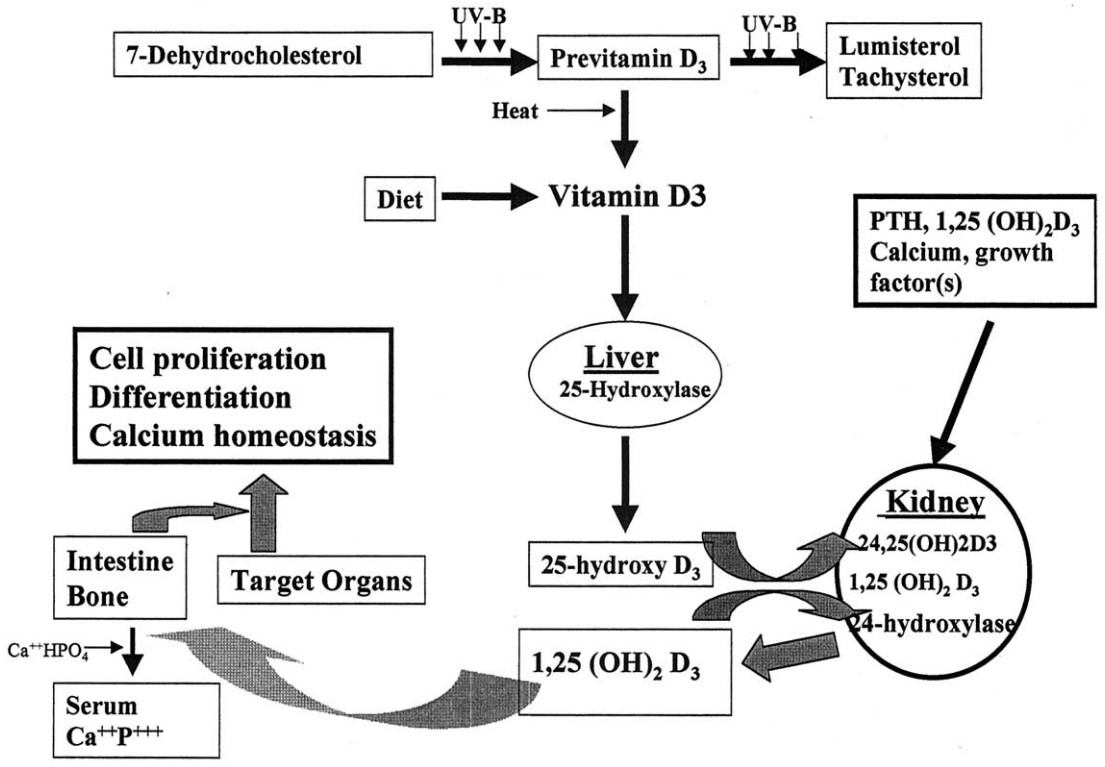

Fig. 2. Metabolism of vitamin D. Conversion of 7-hydrocholesterol to previtamin D3 by UV light and its subsequent processing to vitamin D3 and active metabolite 1,25-dihydroxyvitamin D3 is schematically shown. Vitamin D metabolism by liver and its processing by kidney is also shown in this diagram.

2.1. Efficacy of vitamin D analogs on breast cancer in vitro

Effects of vitamin D analogs on cell proliferation has been studied in a number of breast cancer cell lines as well as on the cells derived from many other target organs. The breast cancer cell lines expressing estrogen receptor (ER+) as well as ER-status have been utilized. All the analogs evaluated thus far have shown antiproliferative effects on ER+ breast cancer cells [26]. However, the effects of vitamin D on the ER- cells are not consistent. 1α(OH)D5 is effective against ER- BCA-4 cells whereas it is ineffective against ER-BCA1 and MDA-MB 231 and MDA-MB-468 cells [27,28,29]. The MDA-MB cell lines express vitamin D receptor poorly. Presence of low expression of VDR and absence of VDR in these cells has been reported. On the other hand, all ER+ cell lines express VDR and are responsive to vitamin D analogs (Table 1). We and others have shown that except for some ER+ breast cancer cells such as MCF-7 and BT474 cells, vitamin D analogs do not induce apoptosis [30,31]. The majority of the cells respond to vitamin D by induction of cell differentiation. Induction of cell difefrentiation is analyzed by cell morphology, flow cytometry, lipid expression and expression of casein and integrin α2 in breast cancer cells [27]. Table 1 summarizes the effects of all the commonly used analogs of vitamin D. The majority of the analogs showed efficacy against ER+ cells at noncalcemic concentrations that are greater than 1,25(OH)2D3. Among the agents effective against ER- cells, KH1060 and 22-oxa-calcitriol appeared to be very effective against MDA-MB-231. KH1060, however was not effective against MDA-MB-435. This is especially interesting since MDA-MB-231 cells are reported to have either no VDR or very low expression of VDR. Both KH1060 and 22-oxa-calcitriol have similar chemical alteration at C-22 position and both are effective against MDA-MB-231 cells. Thus may provide an altered mechanism of action that may not involve VDR or estrogen responsiveness. Other efficacious analogs including 1α(OH)D5 are not effective in VDR- breast cancer cells (Table 1).

In addition to cell culture models experiments have been carried out in mammary gland organ culture model. Mouse mammary gland responds to carcinogen in the presence of growth promoting hormones and form precancerous alveolar or ductal lesions [32]. It has been shown that transplantation of epithelial cells prepared from these glands form adenocarcinoma in syngeneic mice [33]. This model has been used for studying efficacy of chemopreventive agents and understanding mechanism of their action. Comparison of 1,25-dihydroxyvitamin D3, RO24–5531 and 1α-hydroxyvitamin D5 indicated that the D5 analog exhibited similar activity compared to dihydroxy D3 at a log molar higher concentration. RO24–5531 and EB 1089 were toxic at concentration higher than 1 μM [25], whereas 1α-hydroxyvitamin D5 can be tolerated at higher concentrations. The analog 1α(OH)D5 induced VDR and TGFβ in the

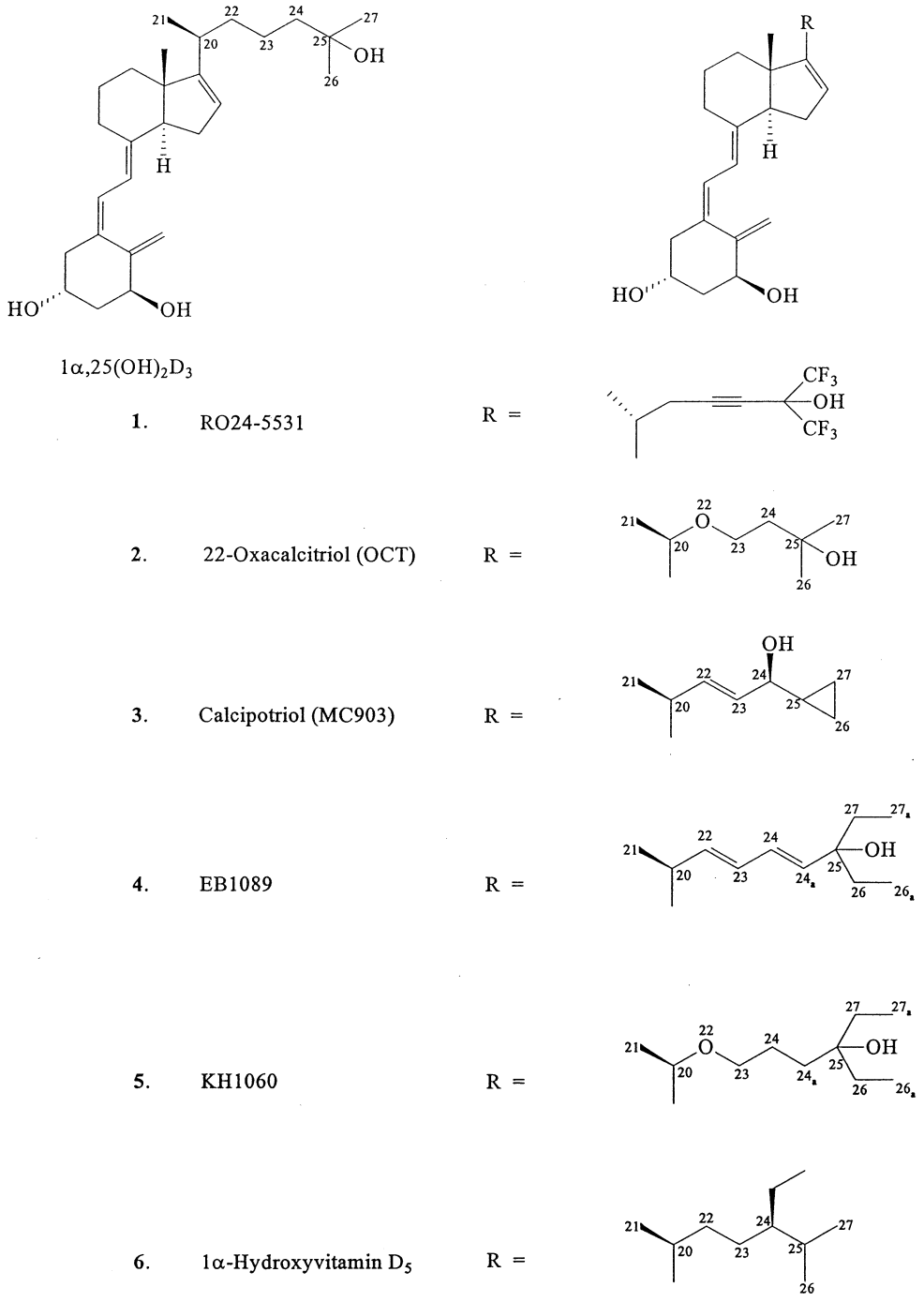

Fig. 3. Chemical structures of some of the active analogs of vitamin D.

mammary glands. These results suggested that the inhibitory effect of vitamin D analog 1α(OH)D5 be mediated by VDR.

2.2. Vitamin D and other cancers

Effects of vitamin D analogs have been evaluated in a number of cell types. The majority of cancer cell types, including HL60 leukemia, Coco and HT29 human colon cancer cells and a variety of prostate cancer cells including LnCap cells are all responsive to vitamin D analogs [34]. More recently it was noted that the incubation of prostate cancer cells as well as normal prostate epithelial cells express 1α-hydroxylase activity which is responsible for converting 25-hydroxyvitamin D3 to the active metabolite 1,25-dihydroxyvitamin D3 [35,36]. Since 25-hydroxyvitamin D3 is less calcemic and less toxic compared to the dihydroxyvitamin D3, it may be more suitable for prostate cancer prevention and therapy. Moreover, all prostate can-

Table 1
Summary of efficacy of vitamin D analogs in cancer cell prolifration

Target organ	Cells	Vitamin D analogs	Efficacy	Comments
Breast	ER+			
	MCF-7, ZR75-1, T47D BT474, BT20, SK-BR-3	22-oxa-calcitriol, 1α(OH)D_5EB-1089, KH11060, MC903, RO24-5531, 22-oxa-Calcitriol	All effective	VDR+
	ER-			
	MDA-MB-231, MDA-MB-436	1α-(OH)D_5, 22-oxa-calcitriol, KH(1060, RO24-5531	Ineffective	VDR+/−
	UISO-BCA-4	1α(OH)D_5	Effective	VDR+
	UISO-BCA-1	1α-(OH)D_5	Ineffective	VDR−
	MDA-MB-231,	22-oxa-calcitriol	Effective	VDR+/−
Prostate	LnCap, PC-3	1α(OH)D_5, EB1089, RO24-2637, 22-oxa-calcitriol, MC903	All Effective	VDR+
	Du-145	1,25$(OH)_2D_3$, RO23-7553	Ineffective	VDR+/−
	Du-145	RO24-5531, RO26-2198	Effective	
Colon	HT-29, CaCo-2	1,25$(OH)_2D_3$, RO24-5531	Effective	VDR+

cer cells expressing positive efficacy for viamin D analogs are VDR positive. It has also been reported that the low VDR expresser PC3 and DU 145 cells poorly respond to the vitamin D as compared to LnCap cells. However, transfection of VDR cDNA was sufficient to establish growth responsiveness in PC3 and DU 145 cells. These results suggest that the presence of VDR is essential for the responsiveness of vitamin D, however the content may not directly correlate with the efficacy of the analog in prostate cells [37]. At the same time, the efficacy of vitamin D analogs did not correlate with the affinity of binding to VDR [38]. The majority of the analogs express lower affinity for VDR as compared to 1,25-dihydroxy D3, and yet they inhabited cell proliferation as effectively as the active metabolite. As with other compounds, 1α(OH)D5 also inhibited LnCap cell growth at 10−7 M concentration (unpublished). These results indicate that besides VDR, other factors may also influence action of vitamin D in cancer cells (Table 1).

2.3. Efficacy of vitamin D analogs in vivo

In order to establish possible clinical significance of vitamin D in preventing or treating cancer, it is essential to evaluate its activity in experimental models. Although over the past several years there is a considerable effort diverted towards evaluating chemopreventive effects of analogs of vitamin D in carcinogen induced experimental tumor models, very little mechanistic studies have been carried out. Unlike homogenous cell type in tissue culture, in vivo studies are much more complex and there is heterogeneity of cell types and presence of tissue interactions. Nonetheless, it is extremely important to establish the role of a chemopreventive agent in carcinogenesis models prior to understanding its mechanism(s) of action. Here, we have summarized current literature regarding the protective effects of analogs of vitamin D. The prerequisites for chemoprevention experiment are to ascertain that the agent is effective at a non-toxic concentration [29]. One of the primary side effect of vitamin D is hypercalcimia because of vitamin D treatment [39,40]. Therefore, the agent has to be active at non-hypercalcimic concentration. It is also important to mention that some analogs may be non-calcemic and yet can not be tolerated at high concentrations. Therefore, in such cases it is necessary to monitor the toxicity of the agent in a dose response study. This is usually achieved by establishing a maximum tolerated dose for each chemopreventive analog of vitamin. So far, there are only a handful of analogs evaluated in vivo for their efficacy in chemoprevention. These include RO24−5531 (Hoffman-LaRoche), EB 1089, CB 966, MC903 (Leo Pharmaceuticals), 22-oxacalcitriol (Chugai Pharmaceuticals Japan) and 1α(OH)D5 (OncQuest Inc.). Although experimental models for carcinogenesis are available for several target organs, effects of vitamin D analogs have been studied mainly in mammary and colon carcinogenesis with sparse reports on a few other organs. The results are summarized in Table 2.

2.4. Mammary carcinogenesis

The most widely utilized models are 7,12-dimethylbenzanthracene (DMBA) and N-methyl-N-nitrosourea (MNU). Both carcinogens induce mammary adenocarcinoma in rats with nearly 100% incidence. The time course of appearance of tumors and their response to ovarian hormones is well worked out [41]. Nearly all the tumors induced by MNU are ovarian hormone dependent whereas 80% of the tumors developed in response to DMBA are hormone dependent. The other 20% tumors induced by DMBA are fibroadenoma. The histopathological evaluations reveal very close similarities between tumors induced by these carcinogens in rats and human breast cancer pathology. These tumor models are extensively used for evaluation of chemopreventive agents for their efficacy [42]. In earlier studies it was ob-

Table 2
Summary of efficacy of vitamin D analogs in chemical carcinogenesis models

Organ	Models	Analog	Dose	Efficacy	Comments
Breast	MNU-induced adenocarcinoma	RO24-5531,	1,10 nmole/kg diet	Effective	No toxicity
		1α-Hydroxyvitamin D_5	58.4, 116.8 nmole/kg	Effective	No hypercalcemia
				Dose related effect	No loss of body weight
		1α-hydroxy D_3	0.25 nmole	growth inhibition	Treatment schedule
		$1,25(OH)_2D_3$	0.59-2.99 nmole/kg	No Effect	Hypercalcemia
		MC903	111 nmole/kg	Growth inhibition	Hypercalcemia
		EB1089	1.1-5.5 nmole/kg	Effective	Hypercalcemia
					Loss of body weight
Prostate	MNU-induced	RO24-5531	10 nmole/kg	Effective	No toxicity
					No effect on dorsal prostate
		1α(OH)D_5	58.4-116.8 nmol/kg diet	In progress	
Colon	AOM-induced	RO24-5531	2.5 nmole/kg ip	Effective	No toxicity
	DMH-induced	22-oxa-Calcitriol	72.5 nmole/kg ip	Effective	
	DMH-induced	24R,25 dihydroxyvitamin D_3	0-24 nmole/kg	Effective	Reduced aberrant crypt
	DMH, MNU, and nitrosamines	24R,25 dihydroxyvitamin D_3	0-12 nmole/kg	Effective	foci colon only

served that treatment with 1,25 dihydroxyvitamin D3 up to 3 nmole/kg BW of rat resulted in no protection against mammary carcinogenesis and yet increased calcium levels in blood was reported [43,44]. Two other vitamin D analogs studied in this report included EB1089 and MC 903. MC903 at a very high dose provided some prtection against mammary tumor growth whereas EB1089 was effective at all the doses evaluated [45,46]. However, there was hypercalcimia observed at <2 nmole (1 and 2.5 μg)/kg dose level. An in depth study to evaluate effects of R024–5531 against MNU-induced mammary carcinogenesis has also been reported. Anzano et al showed that this non-calcemic analog was effective against both the incidence and multiplicity of mammary tumor development at very low levels of 2.5 nmole per kg of diet [24]. However, this effect was observed only when low carcinogen dose was employed. At higher carcinogen dose, level there was no effect against the tumor incidence. Higher than 2.5 nmole per kg of diet dose level was not evaluated in this study, it is possible that it induces toxicity other than hypercalcimia at higher concentrations and 2.5 nmole may in fact be maximum tolerated dose in rats. In a more recent study, we evaluated effects of 1α-hydroxyvitamin D5 in MNU-induced mammary tumor model. The results showed that the animals could tolerate 116 nmole/kg (50 μg/kg) diet concentration of the analog during a six-week toxicity study without adversely affecting serum calcium levels. In older animals, dietary treatment with 0.116 μmole/kg diet 1α-hydroxyvitamin D5 reduced both the incidence and multiplicity of MNU-induced mammary tumors [47]. In this experiment, the vitamin D supplementation began two weeks prior to the carcinogen treatment and continued through out the experiment (Table 2). This meant that both initiation and promotion phases were not separated and the dietary modulation was included during both phases. The selectivity between these two stages in relation to 1α-hydroxyvitamin D5 effect is currently in progress.

2.5. Colon carcinogenesis

There are several well-established colon carcinogenesis models available for evaluating effects of chemopreventive agents. Carcinogens successfully utilized for induction of colon cancers are MNU, 1,2, dimethylhydrazine and azoxymethane. The time frame of induction of aberrant crypts and carcinomas of colon by DMH and AOM have been established [48]. Analogs 1α-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 have been used against DMH induced colon carcinogenesis. Rats received 20 weekly injections of 20 mg/kg DMH. 1,25, Dihydroxyvitamin D3 at a concentration of <0.3 nmole reduced the incidence of colon carcinomas from 46% to 11%. However, there was hypercalcimia associated with this efficacy. In a separate study, effect of R024–5531 was also studied in colon carcinogenesis. Dietary inclusion of R024–5531 for 34 weeks resulted in 40% reduction of colon cancer formation in treatment groups [49]. None of the tumors developed in vitamin D treated rats was adenocarcinoma, they were all benign. Thus, RO24–5531 appears to be very effective against colon cancers. In another study, Otoshi showed that the IP injections of 22-oxa-cxalcitriol also suppressed the development of aberrant crypt foci in rats [50]. The analog, 1α-hydroxyvitamin D5 has not been evaluated for its efficacy in colon carcinogenesis (Table 2).

2.6. Transplantable models

Unlike chemically induced carcinogenesis models, transplantable models are used to evaluate effects of test agents on the growth of the established tumor cells. Since these studies are largely conducted with human cancer cells growing in culture, athymic mice are used as animal of choice. Surprisingly, not all cancer cells form tumors in athymic mice Earlier we had reported that breast cancer cells mixed with matrigel in the ratio of 1:1 results in a remarkable

increase in the development of tumors. Since our original report [51], the use of matrigel for better response in athymic mice has become a common practice for breast cancer. On the other hand, melanoma, sarcoma, colon cancers and prostate cancers typically are not mixed with matrigel to grow in nude mice. Effects of 1,25 dihydroxyvitamin D3 and synthetic analogs of vitamin D3 have been evaluated for their anticancer efficacy on the growth of many cancer types. Studies from our laboratory have shown that 1α-hydroxyvitamin D5 inhibited growth of steroid receptor positive MCF-7 as well as ZR75A cells in vivo [27]. Both these cell lines are positive for both ER and VDR. Cell line established in our laboratory, BCA-4, which is positive for VDR but negative for estrogen and progesterone receptors also responded to the D5 analog of vitamin D. The responsiveness was observed at 0.3nmole i.p. injections or by dietary incorporation of 30 nmole D5-analog/kg diet. These results suggested that the presence of VDR was essential for the efficacy of vitamin D analogs and steroid receptors were of less significance. This was further confirmed by demonstrating lack of effect of 1α (OH) D5 in MDA-MB231 cells that either lack VDR or are relatively very low in expression of VDR expression [27,52].

Effects of 1,25-dihydroxyvitamin D3 was evaluated and compared with EB1089 in transplantable prostate tumor model using androgen-insensitive metastatic rat prostate model. MAT LyLu cells were injected in Copenhagen rats and appropriate groups were treated with low (0.5 μg/kg) and high (1 μg/kg) doses. Both these analogs reduced the metastatic foci in lungs in these rats, however the effect was accompanied by hypercalcemia and loss of body weight at higher dose [53]. More recently, we evaluated effects of 1α(OH) D5 on the growth of LnCap cells in athymic mice (unpublished). Results showed that 55 nmole/kg (25 μg/kg) of diet of the vitamin D analog for 60 days resulted in reduced tumor volume as compared to the control LnCap tumors. At 55 nmole/kg diet concentration, the D5 analog did not elevate concentration of serum calcium levels. Thus, the experimental evidence indicates that not only vitamin D analogs are effective as chemopreventive agents in experimental carcinogenesis models but they also suppress the growth of human cancer cells in athymic mice. Not many studies have been reported to establish the role of vitamin D analogs in preventing or retarding the metastasis of cancer cells to a distant organ, however a couple of reports as described above clearly hint that the selective analogs may be very influential against the cancer cell metastasis. Again, mechanistic studies have not been carried out in these models.

3. Clinical application of laboratory research

As described above vitamin D and its analogs have been examined for their efficacy in numerous in vitro and in vivo models to identify the most potent and yet non-toxic chemical forms of vitamin D. Many of these synthetic analogs have been evaluated in one or multiple models [16]. The reason for lack of analogs, which qualify for further evaluations, possibly in the clinical trials and subsequently as a chemopreventive or chemotherapeutic agent, is the toxicity to efficacy relationship. If a compound were toxic at a concentration which is effective in preventing experimental carcinogenesis or in suppressing cancer growth in experimental models then that analog would be of little value. This is one of the major reasons why 1α,25-(OH)2 D3, the active metabolite of vitamin D has not been employed in cancer prevention or treatment schedules. As discussed previously, the concentration, at which 1α,25 dihydroxy vitamin D3 is efficacious, is also sufficient to induce hypercalcemia in experimental animals. This was first observed by Koeffler and colleagues [54], by evaluating induction of cell differentiation of blast cells taken from the patients with acute myelogenous leukemia. Concentrations of 1μM induced cell differentiation but also was found to be toxic. The studies were extended by treating patients with myelodysplastc syndrome with 2 μg/day of 1,25(OH)2D3. Results also showed that 9 out of 18 patients developed hypercalcimia. In another study, safety and efficacy of both oral and topical treatments of 1α,25(OH)2D3 were evaluated for psoriasis. A study with 85 patients who received calcitriol (1α,25 (OH)2D3) showed that 88% of the patients showed some improvement in the disease. Among those responded to treatment, 26% showed complete protection from psoriasis. There was a significant increase in the calcium excretion, however renal function remained unaffected [55]. A similar mall clinical study was also carried out with 84 patients. The majority of the patients in this study responded to the topical treatment of 1.5 μg of calcitriol. No calcium metabolism abnormalities were observed. The study concluded that topical calcitriol was safe and effective for the treatment of psoriasis [56]. These early reports led to development of relatively nontoxic analogs of vitamin D. The agents that have received considerable attention include EB1089 (seocalcitol), MC903, RO24–5531, 1α-hydroxyvitamin D2, 25-hydroxyvitamin D3, 19-nor-1α,25-dihdyroxyvitamin D2 and 1α-hydroxyvitamin D5 [57,58,59]. These agents have been considered acceptable, based on the preclinical toxicity in animals under stringent experimental conditions. Early results with EB1089 confirmed low calcemic activity in a human maximum tolerated dose finding Phase I/II study [55]. Similar Phase I clinical trial with 36 patients with advanced breast cancer or colorectal cancers is also completed. The maximum tolerated dose of 16–24 nmole/m2 (total daily dose of 20–40nmole) for EB1089 was reported as compared to 2–4 nmole for 1,25-dihydroxyvitamin D3 [60]. Several reports in Japanese have also appeared for toxicity in experimental animals for MC903. Based on this clinical trials have been conducted for psoriasis using this analog, however the clinical trials for cancer patients have not been reported.

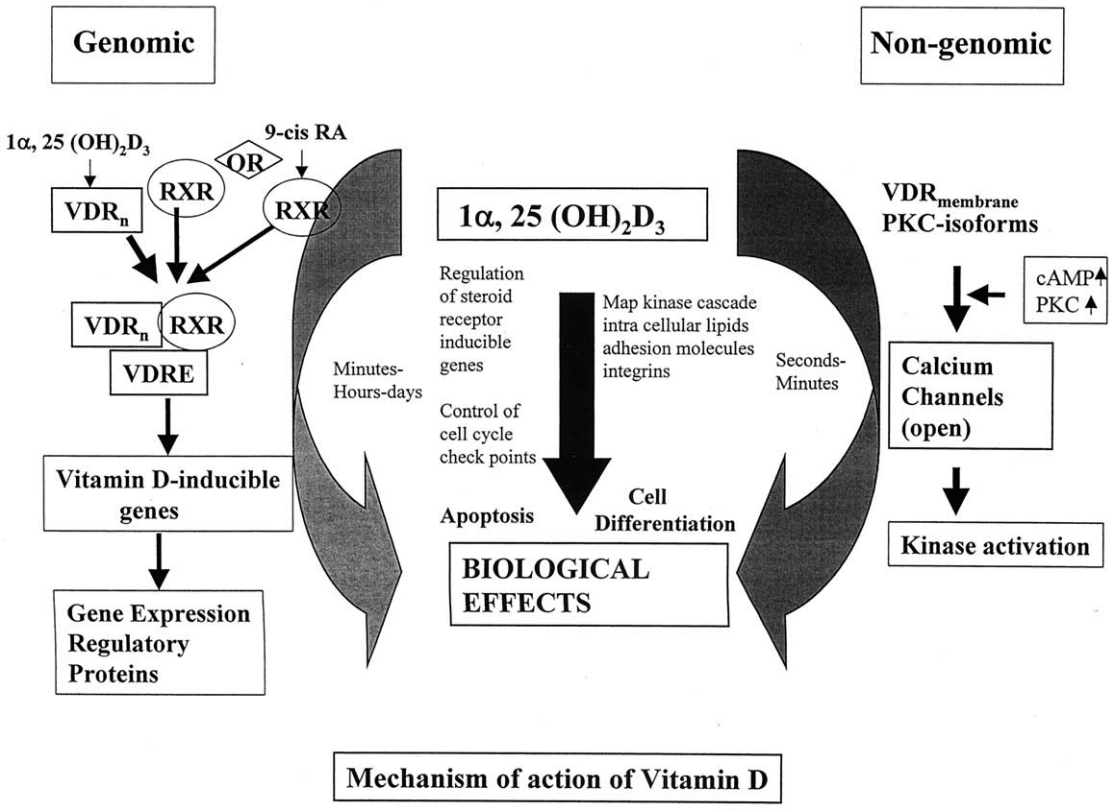

Fig. 4. Schematic diagram of potential mechanism of action of vitamin D. Vitamin D functions both via genomic and non-genomic pathways. Possible pathways of both these mediations of vitamin D action are shown in this diagram.

4. Mechanism of action of vitamin D and cancer

Vitamin D is classified as a steroid hormone [16]. The most unique feature for the steroid hormone has been its association with the specific nuclear receptors. The functional significance of the receptor-associated ligand is the initiation of a cascade of events involving signal transduction eventually leading to the biological function. As shown in Fig. 4, there are two distinct modes of action for vitamin D, one mediating vitamin D action via its binding with high affinity to its specific protein receptor (vitamin D receptor, VDR) and the second involving rapid functions using non-genomic membrane associated functions [61,62]. The non-genomic actions are generally very rapid, often the response could be within minutes as compared to genomic actions which may take longer period for the response. Vitamin D is unique in this respect since both these pathways have been well worked out and are supported by extensive evidence.

4.1. Genomic actions of vitamin D

Consistent with all other steroid hormones vitamin D mediates its action via VDR. Identification of VDR was initially made in chicken intestines by Haussler and Norman [63], followed by its preferential uptake by mammalian intestines and cell free binding of the cytosol to radioactive 1,25(OH)2D3, resulting in the saturable binding with a dissociation constant of 10−9 M. The sucrose density gradient studies of cytoplasmic VDR showed sedimentation of 3.5 S. Subsequently it was observed that the active metabolite associated with cytoplasmic VDR could bind to chromatin fractions [64]. Results have been reported over the years indicating localization of VDR in a variety of target organs and tissue types. These include digestive tract (esophagus and colon), mammary glands, prostate glands, lung alveolar cells brain neurons, connective tissues, fibroblasts, testes and ovaries as well as bone and osteoclasts. These results formed the basis for the establishment and future studies on VDR and its functional significance.

The VDR mRNA in human is a 4.8 kb whereas VDR is a 60 kD protein ranging from 400 to 27000 copies per cell yielding 10 to 100 femto (10−15)-moles/mg protein. Using anti-VDR antibody 9A7 cDNA, libraries derived from chicken intestine were screened in a viral expression system. Protein generated from a single clone from this screen reacted with the anti-VDR antibodies. Since then, VDR-cDNA has been sequenced using monoclonal antibody selection process [6]. Numerous reports collectively have concluded that there is a cluster of hormone receptors forming a family of steroid hormone receptor gene family [66]. Common structural motifs containing DNA binding do-

mains associated with regulatory domains are conserved during the evolution. These genes are under a direct control of transcription factors which regulate biological functions of cell proliferation, differentiation and death. As it is well established steroid hormone receptors are divided into five sections termed A through F. The segments A/B includes residues amino terminal to DNA binding domain, whereas C region contains highly conserved DNA binding domain. The ligand-binding domain at the carboxyl end is termed as either E or E/F region. The hinge on the other hand between C and E segments is termed as D region. While C region of the DNA binding domain is highly conserved, E section is the most flexible region. All regulatory controls reside in this region [67]. Within the ligand binding domain there are both homologies and structural differences among steroid hormones, which make them significantly different from other nuclear receptors including estrogen receptor, retinoic acid receptors, progesterone receptors, peroxisome proliferator activated receptor and thyroid hormone receptors [68]. In order for VDR to function, it needs to interact with vitamin D response element (VDRE) and bind to DNA. VDRE is a two identical hexanucleotide sequences separated by a spacer of 3 nucleotides. The spacer sequence is not conserved. Unlike estrogen receptors, this repeated sequence of two six-nucleotide segments, suggest that VDR must form a dimer for its action. Recent experiments have shown that VDR heterodimerizes with nuclear accessory factor (NAF) or retinoid X receptors (RXR). The natural metabolite 1,25(OH)2D3 transactivates VDRE in VDR positive cells but fails to show interaction in CV-11 (VDR-) cells. These results imply that the synthetic analogs transactivating VDR-VDRE interaction probably mediate their function via genomic pathway in a manner similar to dihydroxyvitamin D3 [69]. Results generated from our laboratory have shown that CV-1 cells transfected with VDR and VDRE when incubated with 1α(OH)D5 showed enhanced transactivation of VDR. Similarly, T47D and ZR75 ER+ VDR+ breast cancer cells express basal level of interaction with transient transfection of VDRE. However, co-transfection of VDR and VDRE significantly enhance the VDR-VDRE interaction when the cells are incubated with 1α(OH)D5 [29,70]. These results indicate that this analog of vitamin D mediates its action via genomic pathway.

Interactions among VDR and other receptors within the steroid receptor family have been a subject of a few investigations in recent years. Since the estrogen receptor positive and negative cells respond differently to vitamin D, in recent studies effects of 1,25-dihydroxyvitamin D3 on ER regulation have been investigated [71]. Results showed that all D-analogs evaluated, EB1089, KH-1060, R023–7553 down regulated ER levels when measured by western blot analysis as well as ligand binding assays. Moreover, this reduction was correlated with steady state levels of ER mRNA indicating direct down regulation of ER transcription by vitamin D analogs. In these studies, induction of progesterone receptors by estrogen was also reduced. More recently, in our laboratory we determined role of 1α-hydroxyvitamin D5 on cell cycle arrest and expression of progesterone receptors in BT474 cells. Results showed that cells were arrested in G1 phase accompanied with apoptosis down regulated estrogen inducible progesterone receptors [72]. Similar studies have also been conducted in prostate cancer cells to determine if vitamin D interacts with androgen receptors. Similar to ER+ breast cancer cells, androgen receptor (AR) positive LnCap cells respond better to vitamin D analogs compared to androgen resistant cells. Human glandular kallikerin (hK2) is an androgen regulated protein expressed in LnCap cells. Recent studies provide evidence for the role of vitamin D analogs for signaling pathways for androgen receptors [73].

The action of steroid hormone is regulated by various factors such as the receptor subtypes, regulation of hormone responsive gene promoters and the activation or suppression of function in response to steroid receptor complex. .Several cellular signaling pathways are involved in the regulation of gene expression by the steroid hormone receptors. The transcriptional activity of some hormone receptors is enhanced by protein kinase activators and growth factors. These proteins stimulate steroid receptor phosphorylation. These findings suggest that changes in steroid receptor phosphorylation are important in determining biological effects of these hormones and their receptors [74]. Alternatively, estrogen receptors could be activated by signals from tyrosine kinase-linked cell surface receptors. This process also involves phosphorylation of the kinases or the transcription factors [75]. Thus, either receptor phosphorylation or ligand bound receptor mediated phosphorylation of other factors is important for the receptor function. VDR like other receptors also get phosphorylated on the serine residues. The extent of phosphorylation is correlated to the extent of responsiveness of the cells to 1,25(OH)2D3 or calcitriol [76]. Furthermore, the phosphorylation is also correlated with VDR-VDRE interaction in transiently transfected cell system. These results suggest that 1,25(OH)2D3 mediated transcription may be dependent on VDR phosphorylation. Phosphorylation of human VDR has also been reported, however the extent of hVDR phosphorylation is significantly lower than the rat VDR phosphorylation [67]. In human, the majority of VDR phosphorylation is located at Serine 51. It has been fairly well established that the ser-51 phosphorylation is regulated by protein kinase C. The phosphorylatable residue at ser 51 is also observed for retinoic acid, thyroxin, and estrogen receptors. However, PKC-mediated phosphorylation is unique to VDR, the functional significance of PKC mediated phosphorylation is not conclusively demonstrated [77]. Both genomic activation by PKC-mediated phosphorylation and inhibition of VDR binding to DNA by this phosphorylation process has been reported [78]. In the later case, it is proposed that PKC dependent phosphorylation create a negative feed back loop that reduces availability of VDR for DNA binding. In addition to VDR phosphorylation by PKC, casein kinase II

and protein kinase A have also has been shown to phosphorylate VDR. Based on the working model for vitamin D action as proposed by Mark Hausler and colleagues [67], it is assumed that VDR resides in target cell nucleus are associated with DNA in a monomeric weak inactive confirmation. Upon binding with the ligand, VDR may become phosphorylated. Moreover, this complex allows dimerization of VDR with RXR. Phosphorylation of VDR and its heterodimerization allows inactivation of the repressor molecules. Haussler proposes that VDR-vitamin D complex dimerization with unliganded RXR makes it unresponsive to 9-cis retinoic acid. On the other hand, if RXR is preoccupied with its ligand then it can form homodimers as well as heterodimers. The homodimers may then disallow the vitamin D interaction with VDR and VDRE interaction. Needless to say that the understanding of genomic regulation of VDR mediated vitamin D function is far from complete. Yet, tremendous progress has been made to elucidate a delicate balance between receptors their interactions, phosphorylation of receptors and their regulatory proteins in order to understand molecular genomic mechanisms of vitamin D action.

4.2. Nongenomic rapid actions of vitamin D

While there is a wealth of information available and is constantly getting updated on the genomic actions of steroid receptors, not all actions of vitamin D can be explained by the genomic regulations. Anthony Norman and his colleagues have been studying the non-genomic action of vitamin D for the past 20+ years and have elegantly demonstrated that in addition to genomic actions of vitamin D there are rapid actions of the hormone largely mediated by membrane receptors of vitamin D and PKC [79,80]. The early studies demonstrated vitamin D mediated stimulation of calcium transport in chick duodenum called transcaltachia. Typically, the vitamin D induced initiation of responses in transcaltachia is not mediated by nuclear VDR directed signal transduction pathways. These responses occur within minutes unlike the genomic expression, which may take days prior to the modulation of endpoint markers. The rapid responses involve membrane receptors of vitamin D, and the pathways involved in induction of calcium channels leading to the exocytosis of calcium bearing vesicles from lysosomes. The ligand binding domain of the plasma binding protein, nuclear VDR and membrane VDR require unique shape of confirmationally flexible 1α,25(OH)2D3. The orientation and rigidity of the flexible side chain as well as the position of A ring in relation to C/D rings determine the vitamin D action. For example, the non-genomic responses including opening of the chloride channels, activation of PKC and MAP kinases require a planar 6-s-cis ligand shape which is recognized by the membrane-VDR as opposed to 6-s-trans bowl shaped 1α, 25(OH)2D3 required for nuclear-VDR interactions [81,82]. Involvement of non-genomic pathways for vitamin D action in carcinogenesis or prevention and therapy of cancer is not clearly defined, however in recent years increasing evidence for rapid effects of steroids that are incompatible with the classical genomic actions is accumulating. Norman and colleagues presented a Mannheim classification of nongenomic action at the first International meeting on the rapid actions of steroid hormones in Mannheim, Germany in 1998 [83]. According to this definition, the nongenomic action of steroid hormones is divided into six categories arbitrarily termed as A1, AIIa, AIIb, BI, BIIa, and BIIb. These differences in various types were according to the functional properties of the hormone. Type AI was classified as non-genomic direct action of steroids at high concentrations that does not require hormones. AIIa is direct action requiring classical receptor for example ERα induction of nitric oxide synthatase. Classification AII-b relates to a nongenomic rapid response transmitted by membrane receptors. Steroid hormones such as estradiol, mineralocorticoids, and vitamin D do function via membrane receptors. Finally, BII-b is the action of steroid hormone where the steroid functions as an agonist. There are several examples of such action in neuroendocrinological, function. However this would be out of scope for the current review and is not described in detail. The functional significance of BI and BII-a is not defined. Despite these developments, the rapid responses of vitamin D are not understood well in relation to the vitamin D action in cancer prevention or therapy.

In summary, in recent years role of synthetic analogs in the management of cancer patients has been extensively evaluated. Several hundred analogs of vitamin D have been synthesized and evaluated for their toxicity and efficacy in a variety of experimental models. To date only a few analogs have been considered for further development. Although it is generally accepted that the action of vitamin D is mediated via both genomic and non-genomic pathways, the major emphasis for the antiproliferative action of vitamin D analogs is placed on the VDR mediated action of the hormone. The VDR- breast and prostate cancer cells do not respond to vitamin D analogs. In steroid hormone receptor positive breast and prostate cancer cells the vitamin D acts by regulating steroid hormone inducible genes, whereas in the steroid receptor negative cells vitamin D induces cell differentiation. Understanding of the molecular mechanism of action of vitamin D will be crucial in generating more efficacious analogs of vitamin D in the prevention and treatment of cancer.

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