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Stage-specific inhibition of mammary carcinogenesis by 1α-hydroxyvitamin D5

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Abstract

Active metabolites of vitamin D are well recognised as cancer chemopreventive and chemotherapeutic agents. However, they are toxic at effective concentrations. Earlier, we reported that a non-toxic analogue of vitamin D, 1α-hydroxyvitamin D5(1α(OH)D5), inhibited carcinogen-induced mammary lesion formation in mouse mammary organ cultures (MMOC) and in *N*-methyl-*N*-nitrosourea (MNU)-induced rat mammary carcinogenesis. In the present study, we determined if 1α (OH)D5 action is selective during the initiation or promotion phases in MMOC and *in vivo*. In MMOC, 1 μM 1α (OH)D5 suppressed both ovarian hormone-dependent and -independent mammary lesions by more than 60%. Inhibition of alveolar lesions was observed only during the promotion stage ($p = 0.0016$). In a 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary carcinogenesis experiment, 1α (OH)D5 (40 μg/kg diet) inhibited cancer incidence by 37.5% ($p < 0.05$) if 1α (OH)D5 was present in food during the promotion phase (+1 to end). However, a D5-supplemented diet during the initiation phase (−2 to +1 week) did not provide any protection. These results clearly show, for the first time, that the effects of vitamin D may be mediated selectively during the promotion or progression phases of carcinogenesis.

Keywords: Vitamin D analogue; D5; Mammary carcinogenesis; Chemoprevention; Organ culture; Preneoplastic lesions

1. Introduction

Vitamin D is a secosteroid and is classified into five major classes: ergosterol (D2), cholecalciferol (D3), 22,23 dihydroergocalciferol (D4), sitosterol (D5) and stigmasteroid (D6). The active form of vitamin D, 1,25(OH)2D3, is derived by the metabolic hydroxylation of cholecalciferol (D3) [1,2]. Toxicity studies have shown that the natural metabolite of vitamin D3 induces hypercalcaemia in animals at concentrations that provide protection against cancer formation or progression. This has led to the syntheses of analogues of vitamin D with the intention of retaining or enhancing the efficacy of vitamin D activity while reducing or eliminating its associated toxicity. More than 1000 analogues have been synthesised by various groups by modifying the side chain of the molecule, as well as introducing changes in the A and B rings. Changes in the C and D rings are not very common due to the rigidity of the structure [3,4]. Although many of these analogues have been evaluated in cell culture models for their antiproliferative activity, only a few have shown reduced toxicity and increased efficacy in *in vivo* mammary carcinogenesis models. These analogues include EB1089, KH1060, calcipotriol, RO24–5531, 22-oxa-calcitriol and 1α-24-ethylcholecalciferol (1α (OH)D5) [5–7]. More recently, another class of vitamin D analogues, 1α,25(OH)2-vitamin D3, with two side chains also termed as Gemini compounds, have received considerable attention since they are very active at very low concentrations [8],

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although no *in vivo* chemoprevention studies have been reported.

Cancer chemoprevention has traditionally been considered as a process that suppresses the initiation of cancer development or delays its onset [9,10]. However, there is no clear separation to suggest where prevention ends and therapy begins. It has always been generalised that chemopreventive agents are effective at non-toxic concentrations, whereas chemotherapeutic agents are often toxic. In recent years, chemopreventive agents have been evaluated both as chemopreventive agents and as possibly non-toxic chemotherapeutic agents [11]. Vitamin D analogues, like other chemopreventive agents, have been evaluated in both these settings. Results have shown that vitamin D analogues can only inhibit the growth of cells with vitamin D receptors (VDR+), indicating that the action of vitamin D is mediated by nuclear VDRs [12–14]. It has been reported that the effects of vitamin D analogues are brought about by affecting the VDR that mediates signalling, which results in a suppression of growth accompanied by either apoptosis or cell differentiation [15,12,16].

Chemoprevention can be ideally studied by inducing transformation of mammary epithelial cells and by evaluating whether or not the potential chemopreventive agent would inhibit such transformation. This has been carried out in cell cultures by transforming normal mammary epithelial cells by either Simian Virus 40(SV40) or carcinogen [17]. In organ cultures, this can be achieved by inducing transformation of mammary structures by carcinogens [18,19]. The suppression of 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced precancerous lesions in mouse mammary gland organ cultures (MMOC) has been extensively used as a model for evaluating potential chemopreventive agents [20,21]. In most cases, results have shown a correlation between chemopreventive agents efficacious in this model and their *in vivo* response [18]. We previously reported that 1α (OH)D5 suppressed mammary alveolar lesions (MAL) induced by DMBA by >60% [22]. However, whether it acts selectively against the initiation or the promotion stage of lesion formation is not known. In addition, previous *in vivo* studies showed an inhibition of carcinogen-induced mammary tumorigenesis by 1,25(OH)2D3, EB1089, RO24–5531, KH 1060 and 1α (OH)D5 at non-toxic concentrations [5,6]. However, the requirements for each analogue vary considerably. The maximum tolerated doses (MTDs) correlate well with their efficacy in carcinogenesis models. For example, in relation to 2.9 nmoles/kg 1,25(OH)2D3, the MTDs for EB1089 and RO24–5531 are 5 and 10 nmoles/kg, respectively. In comparison, 1α (OH)D5 can be tolerated at more than 100 nmoles/kg diet (42.8 μg/kg diet), without any systemic toxicity and hypercalcaemia. In the *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinogenesis model, 1α (OH)D5 inhibited the incidence of mammary tumour development in Sprague Dawley rats [23]. However, it has not been examined whether there is a selective suppression of mammary carcinogenesis during either the initiation or promotion phase. There are two animal models for experimental mammary carcinogenesis which are the most widely used [24]. Adenocarcinomas are induced in rats either by MNU or by DMBA. MNU is a direct acting carcinogen, whereas DMBA needs to be metabolised to an active carcinogen species. Both carcinogens are tissue-specific and do not induce tumours at other sites. The tumours histopathologically resemble human cancers and respond to hormonal manipulations. Since DMBA has to be metabolised to be active, one can differentiate whether the chemopreventive agent is selectively active during the initiation phase of carcinogen activation or during the promotion phase, i.e. after the cells have been transformed. In this report, we describe the stage-specific efficacy of 1α (OH)D5 in MMOC and in DMBA-induced mammary carcinogenesis *in vivo*.

2. Materials and methods

Two separate models were used to evaluate the stage-specific chemopreventive activity of 1α (OH)D5 against mammary carcinogenesis.

2.1. Mouse mammary organ cultures

There are two protocols to induce precancerous mammary lesions in the mammary glands of immature mice. The glands can either develop MAL or mammary ductal lesions (MDL), depending upon the steroid hormone combination present in the medium. If the glands are incubated in the presence of oestradiol 17β and progesterone, then they develop MDL [25], whereas if oestrogen and progesterone are replaced with glucocorticoids, then MAL are formed [18,20].

The procedure for the induction of mammary lesions has previously been described in detail in [18,21,22]. Briefly, thoracic pairs of mammary glands from Balb/c mice pre-treated with 1 μg oestradiol and 1 mg progesterone for 9 days were dissected free of muscles and explanted in culture dishes containing serum-free Waymouth's medium 752MB/1 supplemented with 5 μg insulin, 5 μg prolactin, 1 μg aldosterone and 1 μg hydrocortisone per ml of medium (for MAL) and incubated for 10 days. For MDL instead of aldosterone and hydrocortisone, the medium contained 0.001 μg oestradiol 17β and 1 μg progesterone. In order to induce the development of precancerous lesions, the glands are incubated with 2 μg/ml DMBA for 24 h on day 3 of the culture. This 10 day growth proliferative phase allows the glands to undergo structural differentiation and they appear similar to those from pregnant mice.

After 10 days, the glands are transferred to a medium containing insulin (5 μg/ml) alone for an additional 14 days. This interval compels the glands to undergo a structural regression back to the morphological appearance of glands resembling virgin mice. The glands for MDL are fixed in formalin for 24 h and processed for histopathological evaluation. The glands for MAL are fixed and stained with alum carmine for evaluation of unregressed areas and evaluated for the incidence (number of glands with lesions/total number of glands). For MDL, the glands are divided into several microscopic fields and each field is analysed for ductal sections; the ducts containing lesions are compared with the total number of ductal fields counted to determine the incidence of MDL.

In order to determine whether the effects of 1α (OH)D5 were selective for the initiation or promotion phases of lesion formation,10 glands per group were incubated with the vitamin D analogue for the first four days (0–4 days) of culture, which includes 3 days prior to DMBA treatment of the glands and one day post-DMBA treatment. For determining the antipromotional effects of 1α (OH)D5, the glands were incubated with 1 μM 1α (OH)D5 from day 4 to day 10 of the growth-promoting phase of epithelial cells.

2.2. Mammary carcinogenesis experiment

Fifty-day-old Sprague Dawley female rats were used for the study. All procedures were carried out under institutional guidelines and an approved protocol. Animals were randomised by weight into four groups of 20 animals each and received 15 mg of DMBA in 1 ml of corn oil intragastrically. The groups' diets included: (1) a placebo diet (the control group); (2) a diet supplemented with 1α (OH)D5 from 2 weeks prior to carcinogen treatment until the end of the study (initiation + promotion phases, −2 to end of study); (3) a 1α (OH)D5 supplemented diet from 2 weeks prior to the carcinogen treatment to the week after the carcinogen treatment (initiationphase only −2 to +1 week); and (4) a 1α (OH)D5-diet beginning one week after the carcinogen treatment until the end of the study (promotion phase only, +1 to end of study). Two additional groups were also included with 10 rats per group receiving no carcinogen and either the placebo or the 1α (OH)D5 supplemented diet. The concentration of 1α (OH)D5 in the diet was kept at 40 μg/kg diet. Beginning 3 weeks after the carcinogen treatment and continuing until the end of the study, the animals were weighed once a week and examined weekly by palpation. All animals were sacrificed 150 days post-carcinogen treatment. Tumours were removed and processed for histopathology and a portion of the tumour was saved for biochemical analyses.

2.3. Statistical analysis

Statistical significance between groups in MMOC was determined by χ^2 analysis. Tumour incidence in the carcinogenesis experiment was evaluated by unpaired Students *t*-test and χ^2 analysis. Latency for tumour appearance was determined by an analysis of variance test(ANOVA).

3. Results

As shown in Fig. 1, the basic difference between the natural active metabolite of vitamin D, 1,25 dihydroxyvitamin D3(1,25(OH)2D3), and 1α-hydroxyvitamin D5(1α(OH)D5) is that there is no hydroxylation at the 25 position in the D5 analogue, instead there is an ethyl group in the C-24 position of the vitamin D3 molecule. Both of these molecules are different in their retention properties on a high-performance liquid chromatography(HPLC) column. 1,25(OH)2D3 separated with a retention time of 5.2 min compared with 34.0 min for 1α (OH)D5. In the presence of insulin, prolactin, aldosterone and hydrocortisone MAL were induced in response to DMBA. In three experiments with15 glands per experiment, MAL were observed in 30 glands out of 45 (67% incidence). Incubation of glands in the presence of 1 μM 1α (OH)D5 resulted in a >60% suppression of MAL incidence. Out of 45 glands, 11 exhibited MAL, a 63% suppression of MAL development ($P < 0.001$). A representative photograph showing MAL morphology in a DMBA-treated gland compared with a control gland not treated with DMBA and a chemopreventive agent-treated gland is shown in Fig. 2. The ductal lesions were induced by including 0.001 μg/ml oestradiol and 1 μg/ml progesterone in the medium. These steroid hormones replaced aldosterone and hydrocortisone in the medium. 22 of 32 ductal sections examined in the

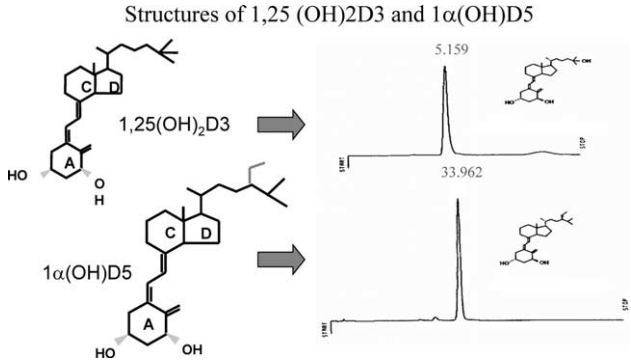


Fig. 1. Chemical structures of vitamin D analogues. Structural differences between 1,25 dihydroxyvitamin D3 (1,25(OH)2D3) and 1α-hydroxyvitamin D5(1α(OH)D5) are shown. High-performance liquid chromatographic (HPLC) analyses to show different retention times are shown as HPLC profiles for these two agents.

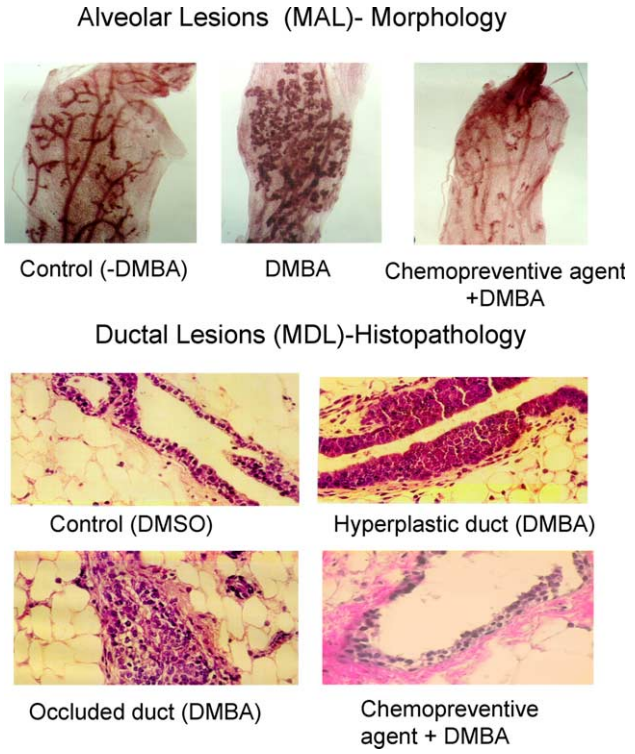


Fig. 2. Representitative examples of mammary alveolar and mammary ductal lesions in response to 7,12-dimethylbenz(*a*)anthracene (DMBA) and efficacy of chemopreventive agent. Thoracic pairs of mammary glands were dissected from oestrogen-and progesterone-pretreated mice. The glands were incubated with either aldosterone and hydrocortisone (for ovarian hormone-independent mammary alveolar lesions (MAL)) or with oestrogen and progesterone (for ductal lesions) for 10 days. The carcinogen treatment was for 24 h on day 3. The growth-promoting hormones were removed from the medium, leaving only insulin for additional 14 days. The chemopreventive agent was present for the first 10 days along with the growth promoting hormones. Glands were either stained with alum carmine (for MAL) or sectioned for histopathological processing and stained (for mammary ductal lesions (MDL)). The ovarian hormone-independent alveolar lesions are shown in the upper panels. The representative photographs of ovarian hormone-dependent ductal lesions and response to chemopreventive agent are shown in histopathological sections.

control glands contained hyperproliferative and atypical ductal lesions (Table 1). Treatment of the glands with 1 μM 1α (OH)D5 resulted in the suppression of these ductal lesions and only 6 of 24 ductal sections showed the presence of MDL. Representative photographs showing MDL and effects of a chemopreventive agent are shown in Fig. 2. These results indicated that there was a 64% inhibition of MDL formation by 1 μM D5 treatment ($P < 0.001$). These results suggest that 1α (OH)D5 inhibited the development of both ovarian hormone-independent (MAL) and hormone-dependent (MDL) mammary lesions. (Table 1)

In order to evaluate the stage-specific efficacy of 1α (OH)D5 on the development of DMBA-induced MAL formation, 15–20 glands per group were incubated with a MAL-promoting hormone combination, with or without 1 μM 1α (OH)D5. The D5-analogue was included in the medium during either the initiation phase from 0 to 4 days of culture (DMBA on day 3) or the promotion phase from day 4 to day 10 of the culture period. The control glands in this series of experiments developed MAL in 60% (18 out of 30 glands) of the glands. Compared with controls, treatment of glands during the initiation phase resulted in 12 out of 30 (40%) glands developing lesions. An inhibition rate of 33% [1 − (40% treated glands/60% controls) × 100]. This anti-initiation effect of 1α (OH)D5 was not statistically significant ($P > 0.1$). On the other hand, anti-promotional effects resulted in 6 glands with MAL out of 30 in the culture (20%). Comparison of treated and control lesion incidence indicated that there was a 67% ($P < 0.001$) inhibition in the promotion stage of MAL formation (Table 1).

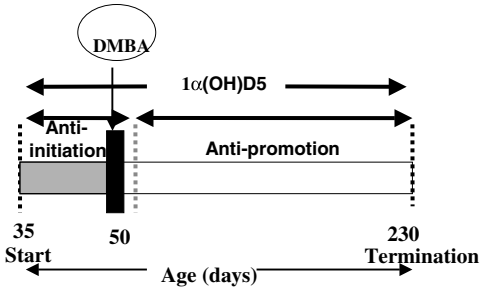
Previously, we showed that 1α (OH)D5 (50 μg/kg diet) inhibited both the incidence and multiplicity of MNU-induced mammary carcinogenesis in rats [23]. In this study, we evaluated the efficacy of 1α (OH)D5 at a 40 μg/kg diet concentration on DMBA-induced mammary carcinogenesis during the initiation and promotion phases. As shown in Table 2, DMBA induced mammary tumours in 16/20 animals, resulting in a tumour incidence of 80%. When the chemopreventive agent was present in the diet beginning 2 weeks before the carcinogen treatment and continued throughout the experimental period (initiation plus promotion), there was a reduction in tumour incidence from 80 to 50 percent (a 37.5% inhibition of incidence). This reduction in tumour incidence was compared with the initiation and promotion phases individually. In the group where the vitamin D analogue was included in the diet for a short period of 3 weeks, from 2 weeks prior to DMBA treatment to one week after, tumour incidence was 70%, a 12.5% reduction. These results suggested that 1α (OH)D5 had very little effect on the initiation of carcinogenesis in this model. Results also showed that when the chemopreventive agent was present in the diet from one week after the carcinogen treatment until the end of the study, tumour incidence was again 50%, a reduction of 37.5% ($p < 0.05$). This reduction was the same as when the treatment period included both the initiation and promotion phases. These results suggest that the effect of vitamin D may be selective during the promotion phase of carcinogenesis. Moreover, the median time for the appearance of the first tumour (latency) was compared amongst all groups and statistically analysed using ANOVA. The time to the first tumour appearance after the carcinogen treatment was 92.5 ± 5.9 days for the control group as compared with 120.7 ± 9.4 days for the promotion group (+1 to end). These results once again suggest a trend towards increased latency when 1α (OH)D5 was included during

Table 1
Chemopreventive efficacy of 1α-Hydroxyvitamin D5 in organ cultures

Treatment (DMBA + D5)	Days	Number of glands with lesions (% incidence)	% Inhibition	Ductal sections with lesions (% multiplicity)	Percent inhibition	Significance (P values[a])
Mammary alveolar lesions (MAL)						
None	N/A	30/45 (67)	–	N/A	N/A	
10–7M D5	0–10	14/30 (47)	30	N/A	N/A	0.085
10–6M D5	0–10	11/45 (24)	63	N/A	N/A	0.001
None	N/A	18/30 (60)		N/A	N/A	
10–6M D5	0–4	12/30 (40)	33	N/A	N/A	0.12
10–6M D5	4–10	6/30 (20)	67	N/A	N/A	0.0016
Mammary ductal lesions (MDL)						
None	N/A	N/A	N/A	22/32 (69)	–	
10–6M D5	0–10	N/A	N/A	6/24 (25)	64	0.0012

N/A, Not applicable.
[a] χ^2 analysis, comparision with controls.

Table 2
Effects of 1(OH)D5 in DMBA-induced mammary carcinogenesis in rats



Treatment	N	Schedule (wks)	Incidence (%)	Latency (days)	Final BW (g)
Control	20	–	16/20 (80)	92 ± 5	265 ± 10
D5 (40 μg/kg)	20	−2 to End	10/20 (50)*	106 ± 11	254 ± 13
D5 (40 μg/kg)	20	−2 to +1	14/20 (70)	114 ± 8	266 ± 8
D5 (40 μg/kg)	20	+1 to End	10/20 (50)*	121 ± 9	255 ± 11

wks, weeks; BW, body weight.
* $p < 0.05$ (compared with controls).

the post-carcinogen treatment phase. However, latency for the appearance of the first tumour amongst groups was not statistically significant. There was no difference observed in body weight gains. The initial weight was similar in all animals since they were randomised in groups based on their average weights. The final body weights for all groups are shown in Table 2. There was no difference in serum calcium or phosphorous concentrations, indicating no hypercalcaemic activity (data not shown).

4. Discussion

Although vitamin D has been considered as one of the most effective differentiating agents in leukaemic cells and as an antiproliferative agent against many cancer cells including breast, prostate and colon cancers [5,6], its clinical use has been limited due to the known hypercalcaemic activity of the natural metabolite of vitamin D3, 1,25(OH)2D3. Recognising the possible translational value of vitamin D, more than 1000 analogues of vitamin D3 have been synthesised in the past 15 years and many of them have been evaluated for their possible antiproliferative activity at non-toxic concentrations. We synthesised 1α (OH)D5 as an analogue of the D5 class of vitamin, which is also a modification of cholecalciferol [22], and evaluated its efficacy as an antiproliferative agent against breast cancer cells in culture. Results showed that it was efficacious against both ER+ and ER− breast cancer cells so long as they are VDR positive (VDR+) [26]. Simultaneously, we also

showed that 1α (OH)D5 could be tolerated by rats and mice at higher concentrations than most of the other vitamin D analogues. Toxicity studies with 1α (OH)D5 in dogs and rats have been completed under good laboratory practice (GLP) guidelines and it will be evaluated in a Phase I study for breast cancer patients. It should also be noted that a high concentration of 1α (OH)D5 is required to produce protective effects. However, the analogue is non-calcaemic at an effective concentration. Compared with EB1089, RO24–5531 and 1,25(OH)2D3, 1α (OH)D5 requires a log molar higher concentration to provide equivalent effects in cell cultures. Most effective analogues are active at 10–7M concentrations, whereas D5 requires a 1 μM concentration to have antiproliferative effects. At 1 μM concentration, 1α (OH)D5 inhibited the development of MAL in MMOC.

More recently, we showed that if glucocorticoids in the medium are replaced with oestradiol 17β and progesterone, glands develop ductal lesions. These ductal lesions can be suppressed by tamoxifen; however, tamoxifen was ineffective against MAL, indicating these lesions have different properties in relation to hormonal sensitivity [27,25]. In the present study, we observed that 1α (OH)D5 is efficacious against both alveolar (ovarian hormone-independent) and ductal (oestradiol 17β-progesterone dependent) lesions. This is consistent with a prior report that indicated that the effect of 1α (OH)D5 was dependent on VDR in the cells. The protective role of VDR in the mammary gland has been recently evaluated by examining mammary gland development in VDR-knockout (KO) mice. Results showed that the glands from VDR–KO mice exhibited accelerated growth, and the regression of the gland during involution was observed at a reduced rate [28]. In MMOC, it was observed that VDR sensitised mammary glands to 1,25(OH)2D3, because the glands from VDR–KO mice did not respond to the vitamin D analogue [29]. Both ER+ and ER− breast cancer cells that were VDR+ responded to vitamin D analogues [26]. MMOC can be expanded to determine if the chemopreventive agent is selectively effective against either the initiation or promotion stage by exposing the glands to the test agent either before or after the carcinogen treatment. In the present study, we found that 1α (OH)D5 showed efficacy selectively against the promotion stage of lesion formation: there was more suppression of alveolar lesions during the promotion stage than during the initiation stage.

Numerous chemopreventive agents have been evaluated in MMOC and rat mammary carcinogenesis protocols [30]. Results have shown that there is a 75% correlation between efficacy observed in MMOC and efficacy *in vivo*. Several vitamin D analogues, including EB1089 [31], RO24–5531 [32] and 1α, 25(OH)2D3 [33], have been reported to have chemopreventive activity in mammary carcinogenesis. The question has been asked as to whether 1α (OH)D5 can be detected in mammary tissues subsequent to *in vivo* treatment. We determined the tissues level of 1α (OH)D5 in rats. Rats were treated with 50 μg (1.2 μmoles) of 1α (OH)D5 at 50 days of age for 24 h. Mammary glands were removed and extracted for vitamin D. The extract was separated on liquid chromatography-mass spectrometry (LC-MS) using 1α (OH)D5 as a standard. Results showed that there was 3.15ng (63 pmoles)/mg mammary gland. It is not possible to make a direct comparison between the amount of 1α (OH)D5 included in the medium (1 μM or 428ng/ml) and its *in vivo* uptake by the tissues. Nonetheless, 1α (OH)D5 by itself was detected in the mammary tissue.

Earlier, we reported that in an MNU-induced mammary carcinogenesis model, 1α (OH)D5 inhibited the incidence of mammary tumour development in adult (100 day old) rats. In the present experiment, we evaluated the effectiveness of 1α (OH)D5 in a DMBA-induced carcinogenesis model using 50-day-old rats. The results are comparable to those observed for MNU-induced cancers. At a 40 μg/kg D5 diet level, there was a reduction in the incidence of tumour development. There was a 37.5% ($p < 0.05$) reduction in tumour incidence in groups receiving D5 either during the promotion phase (+1 to end) alone or during the entire period of carcinogenesis (−2 to end). However, if the animals consumed D5 for a short time only (−2 to +1 weeks), the reduction in tumour incidence was marginal (12.5%). These *in vivo* results are consistent with the MMOC results described in this report. It would be of considerable importance if a chemopreventive agent suppresses the latency of the tumour occurrence. However, in the present study, there was no statistically significant increase in the median latency times observed in any of the groups. These results clearly support developing 1α (OH)D5 for further studies and clinical trials.

Conflict of Interest

None.

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