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PRINCIPAL INVESTIGATOR: Rajeshwari R. Mehta, Ph.D.

CONTRACTING ORGANIZATION: University of Illinois, Chicago  
Chicago, Illinois 60612-7227

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13. ABSTRACT (Maximum 200 words) Recently we have shown that $1\alpha(\text{OH})\text{D}_5$ , a novel vitamin D analog has both cell differentiating and growth inhibitory effects in selected human breast carcinoma cell lines. We observed enhanced expression of intracytoplasmic casein and lipid droplets, nm23 and ICAM-1 proteins following 7 days <i>in vitro</i> treatment with $1\alpha(\text{OH})\text{D}_5$ . In the proposed study we further evaluated effect of this novel compounds on other biomarkers associated with breast cancer cell differentiation. We studied <i>in vivo</i> and <i>in vitro</i> effects of $1\alpha(\text{OH})\text{D}_5$ on 4 different cell lines. $1\alpha(\text{OH})\text{D}_5$ showed pronounced growth inhibitory effect in all ER+ breast carcinoma cells. It enhanced expression of alpha2 integrin, beta-1 integrin, and CK-8 proteins in breast cancer cells irrespective of their ER status. Changes in expression of later proteins was only observed in VDR+ cells. <i>In vivo</i> , $1\alpha(\text{OH})\text{D}_5$ supplemented in diet inhibited growth of ZR-75-1 and UISO-BCA-4 cells. Our ultimate goal is to conjugate and link this vitamin D analog to Her-2/neu antibody in order to specifically target breast cancer cells in women. We selected ZR-75-1 cells (known to have overexpression of Her-2 receptor) for further <i>in vivo</i> work. <i>In vivo</i> growth of ZR-75-1 cells was significantly reduced by Her-2 antibody treatment.					
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*R. R. Mehta*

PI - Signature

8/25/98

Date

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## Introduction

In recent years, several natural and synthetic agents, especially those with antiproliferative and differentiating properties, have been the primary focus of therapeutic and chemopreventive research. A synthetic analog of vitamin A, N-[4-hydroxyphenyl] retinamide (HPR), is recognized as a chemopreventive agent for breast carcinoma in experimental animals. In addition to vitamin A, Vitamin D has also shown promising results. Vitamin D is classified as a hormone within a steroid hormone family.<sup>5</sup> It is a secosteroid that is biologically inert until hydroxylated on the carbon 25 position in the liver to form 25-hydroxyvitamin D, which is further metabolized to 1 $\alpha$ ,25 dihydroxy vitamin D<sub>3</sub> (1 $\alpha$ D<sub>3</sub>). When it is no longer needed, the hormone gets metabolized to an inactive form (24-hydroxyvitamin D) and excreted from the body.<sup>6</sup> In addition to its function in maintaining blood calcium level and mobilizing calcium from bone, 1 $\alpha$ (OH) D<sub>3</sub> has growth-suppressing and cell-differentiating actions in many malignant cell types.<sup>7,8</sup>

One major factor limiting successful use of vitamin D or 1 $\alpha$ D<sub>3</sub> in cancer prevention or therapy is its calcemic activity. The concentration needed to cause reduced growth of neoplastic cells would cause hypercalcemia and death. Therefore, in recent years, attention has been directed to developing analogs that preserve vitamin D's growth suppressive activity but reduce its calcemic activity.<sup>9</sup> In experimental systems, addition of vitamin D analogs to adriamycin or tamoxifen treatment has shown enhanced growth inhibitory action of drugs.<sup>10,11</sup> We recently evaluated a novel vitamin D analog, 1 $\alpha$ (OH)D<sub>5</sub>, as a potential antiproliferative or cell-differentiating agent for breast cancer cells. This analog was synthesized by Dr. Robert M. Moriarty, Professor, Department of Chemistry, University of Illinois at Chicago. Table 1 summarizes the results obtained previously in our laboratory. 1 $\alpha$ (OH)D<sub>5</sub> is nontoxic in athymic mice.

**Table 1. Previous findings**

Evaluation	Animal model used	Optimal Effective dose	Results
Chemopreventive effect	Mouse mammary gland organ culture <sup>12</sup> system, effect on DMBA-induced preneoplastic lesions (in vitro).	1 $\mu$ M	1 $\alpha$ (OH)D <sub>5</sub> inhibited DMBA-induced premalignant lesion formation in vitro. <sup>12</sup>
Growth inhibitory effect	Human breast cancer cell lines, *UISO-BCA-1, *BCA-2, *BCA-4, T47D, MCF-7 (in vitro).	1 $\mu$ M (10 days exposure)	1 $\alpha$ (OH)D <sub>5</sub> had a dose-related growth inhibitory effect on BCA-4, MCF-7, and T47 D cell lines, irrespective of their estrogen and progesterone receptor status. <sup>13</sup>
Cell-differentiating effect	Human breast cancer cell lines, UISO-BCA-1, BCA-4 and MCF-7 (in vitro).	1 $\mu$ M (7 days exposure)	It induced expression of various proteins associated with cell differentiation, namely nm23, ICAM-1, and casein. <sup>13</sup>

Other effects on various other biomarkers	Human breast cancer cell lines, UIISO-BCA-4 (in vitro)	1 $\mu$ M (7 days exposure)	Histological changes, induction of TGFB1, and VDR. <sup>13</sup> Downregulation of UPA, UPAR, TGFB3, EGFR, and BCL-2
Effect on in vivo tumorigenicity	Human breast carcinoma cell line BCA-4 (in athymic mice)	8 ng/animal 3 times weekly for 2 months, s.c. injection	Complete inhibition of tumor growth; originally injected cells appeared differentiated histologically
Toxicity	in mice (in vivo)	at 400 ng/animal, 3 times weekly, 21 days treatment	No apparent toxicity, (non-calcemic activity)

\*These cell lines were established by the PI and are characterized in detail.<sup>14,15</sup>

## Hypothesis

Vitamin D and its analogs have growth-suppressing and cell-differentiating actions in various neoplastic cell types. However, their use as therapeutic or cancer preventive agents is hindered due to their high calcemic activity. We recently studied the effects of a new synthetic vitamin D analog  $1\alpha(\text{OH})\text{D}_3$  in breast carcinoma cells. This newly synthesized analog appears to have no significant calcemic activity. In experimental systems,  $1\alpha(\text{OH})\text{D}_3$  inhibited the development of premalignant lesions in DMBA-exposed mammary gland explants. In malignant cells, it induced expression of various markers associated with breast cell differentiation, namely ICAM, casein, and nm23. Also, altered phenotypic changes were associated with induction of vitamin D receptor (VDR) and TGF $\beta$ <sub>1</sub> protein. In women, tumors showing overexpression of nm23, ICAM, and e-cadherin are generally noninvasive. In the present study, we hypothesize that  $1\alpha(\text{OH})\text{D}_3$  treatment could induce breast cancer cell differentiation, render them non-aggressive, and alter their tumorigenicity and metastatic potential. If vitamin D analog proves to induce functional and biological differentiation in breast carcinoma cells, it will be of great value as a chemopreventive agent, particularly in women with premalignant lesions and at high risk of developing aggressive tumor.  $1\alpha(\text{OH})\text{D}_3$  could be easily given as a dietary supplement. Alternatively, it could be administered at low concentrations as an immunoconjugate with c-erbB2 antibody and specifically targeted for breast carcinoma cells, without any effect on normal cells.

## Technical Objectives

$1\alpha(\text{OH})\text{D}_3$  inhibited the development of premalignant lesions in DMBA-exposed mammary gland explants. In malignant cells,  $1\alpha(\text{OH})\text{D}_3$  induced expression of various biomarkers (namely ICAM-1, casein, and nm23) associated with breast cell differentiation. Generally, in women, in-situ ductal carcinomas showing overexpression of ICAM-1, nm23, and e cadherine are noninvasive<sup>16-20</sup>. Thus, it is likely that induction of various differentiation markers observed following vitamin D<sub>3</sub> treatment may alter functional characteristics of malignant cells, render them nonaggressive, and alter their tumorigenic and invasive potential. In the present study, we aim to evaluate the potential therapeutic and antimetastatic properties of  $1\alpha(\text{OH})\text{D}_3$ .

- 1) Determine the effects of  $1\alpha(\text{OH})\text{D}_3$ , a synthetic vitamin D analog, on morphological or phenotypic, functional, and biological characteristics of malignant cells.
- 2) Evaluate therapeutic efficacy of  $1\alpha(\text{OH})\text{D}_3$  immunoconjugated with c-erbB2 antibody.
- 3) Study the effects of dietary supplementation of  $1\alpha(\text{OH})\text{D}_3$  on growth and metastasis of human breast carcinomas in experimental animals.

Successful completion of the proposed study will identify a new safe, nontoxic chemopreventive and/or therapeutic agent for breast cancer.

### **Statement of Work for 1997-98**

**Task 1. 0-6 months:** Initiate specific aim 1a. Study the effects of vitamin D analog on various differentiation markers in three different human breast carcinoma cell lines by immunohistochemistry and by Western blot analysis. Markers to be studied are cytokeratins, integrins, vimentin, catenins, e-cadherins, casein, and nm23. The cells will be exposed to vitamin D analog for 10 days, after which we will study changes in biomarkers.

**Task-2. 7-9 months:** Study specific aim 1b. Immunoconjugate  $1\alpha(\text{OH})\text{D}_3$  with c-erbB2 antibody. Purify the conjugate. Study the effect of  $1\alpha(\text{OH})\text{D}_3$  on cell aggregation and cellular morphology.

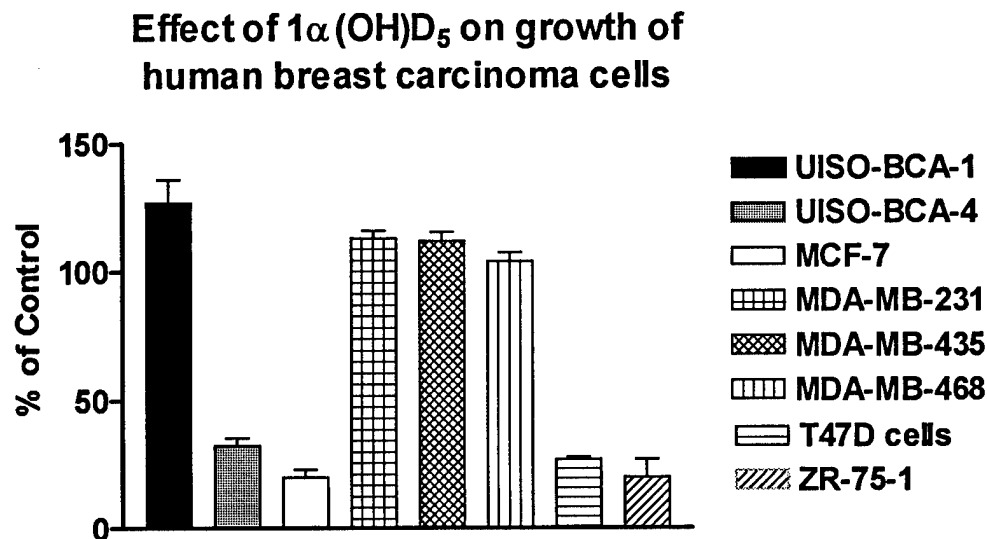
**Task-3. 10-15 months:** Study the effect of dietary supplementation of  $1\alpha(\text{OH})\text{D}_3$  in athymic mice. We will study three different cell lines. Each will be injected into 20 animals; 10 animals will receive regular diet, and 10 animals will receive  $1\alpha(\text{OH})\text{D}_3$ -supplemented diet. A total of 60 animals will be used. Experiments will take 60 days.

### **Experimental Methods and Results**

**Specific Aim 1:** Determine whether  $1\alpha(\text{OH})\text{D}_3$  will alter morphological, biological, and functional characteristics of malignant breast cells and differentiate them to normal condition.

We first determined response to  $1\alpha(\text{OH})\text{D}_3$  in various established human breast carcinoma cell lines. Cells (approximately 10,000-15,000/well) were plated in 24-well tissue culture plates. After 24 hours, media were changed. Control cells received MEM-E containing 5% charcoal stripped serum alone; experimental cells received  $10^{-6}\text{M}$   $1\alpha(\text{OH})\text{D}_3$  in MEM-E containing 5% charcoal-stripped serum. Medium was changed on days 4 and 7 of initiating treatment. On day 10, the number of cells was counted using a coulter counter. The number of cells in the control group was considered 100%. Data represent mean + SE of control value. Each group contained quadruplet observations.

Figure 1



Cells were incubated in the presence/absence of  $1\alpha(\text{OH})\text{D}_5$  ( $10^{-6}\text{M}$  concentration) for 10 days. Data represent % of growth in control (cells treated in the basal medium).

As shown in Figure 1,  $1\alpha(\text{OH})\text{D}_5$  significantly inhibited growth of MCF-7, ZR-75-1, UIISO-BCA-4, and T47D cells. No growth inhibitory effect was observed in other cell lines studied.

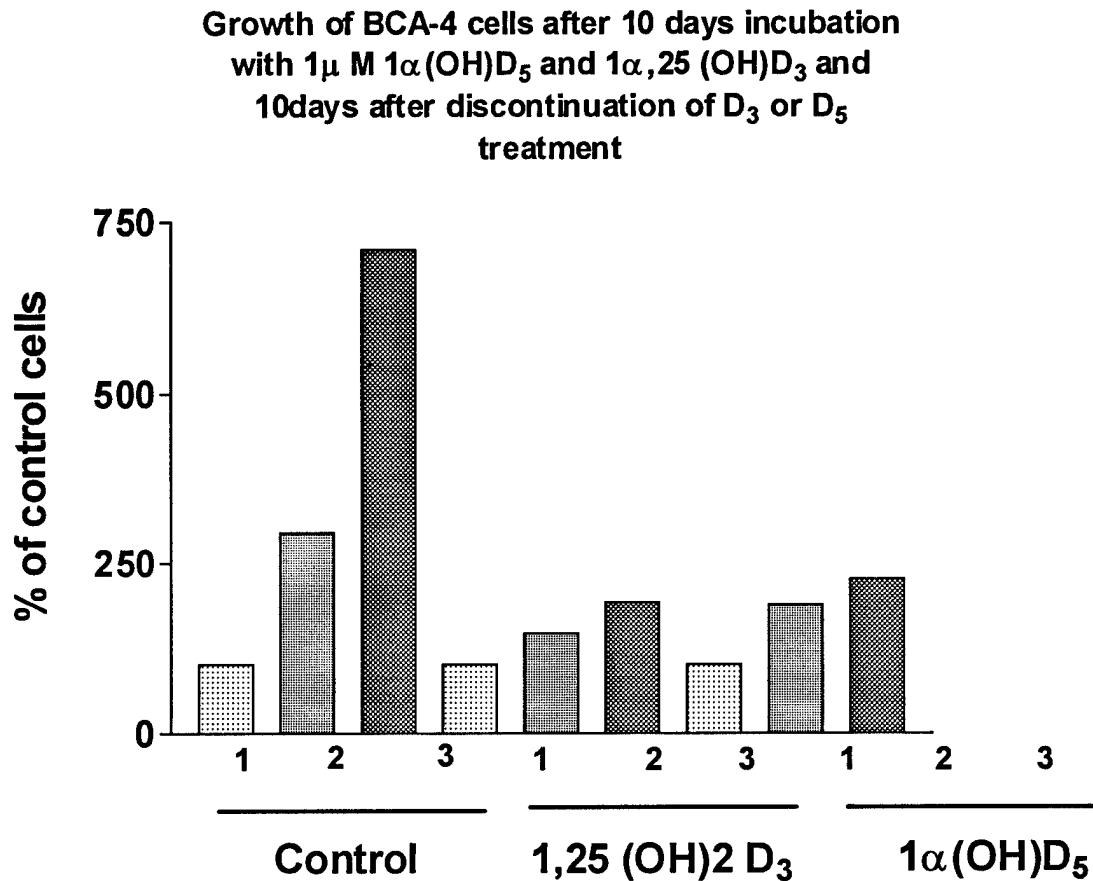
We further examined whether the effect of  $1\alpha(\text{OH})\text{D}_5$  is transient. UIISO-BCA-4 cells were first treated with/without  $1\alpha(\text{OH})\text{D}_5$  for 10 days as mentioned above. After 10 days, all cells were fed with regular MEM-E medium containing 15% FBS and were further allowed to grow for 10 days. At the end of 10 days, the number of cells was counted in each treatment and control group. Data represent % of control growth. Lane 1 represents the initial number of cells plated (100%); lane 2 represents the number of cells following 10 days of  $1\alpha(\text{OH})\text{D}_5/\text{D}_3$  treatment; lane 3 represents % of cells allowed to recover for 10 days following 10 days  $1\alpha(\text{OH})\text{D}_5$  or  $\text{D}_3$  treatment. Results are shown in Fig. 2.

**The effect of  $1\alpha(\text{OH})\text{D}_5$  on differentiation markers:** ICAM-1, nm23, e-cadherin, catenins ( $\alpha$ ,  $\beta$ ), casein, cytokeratins (CK5, CK-8, CK14, CK-18), vimentin, and integrins ( $\alpha 2\beta 1$ ,  $\alpha 5\beta 1$ ,  $\alpha 6\beta 1$ ) are known markers for breast cell differentiation.<sup>22-29</sup> In the present study, we aim to assay all these markers in vitamin D analog-treated cells and control cells by immunohistochemical method, flow cytometric analysis, and Western blot analysis.

We studied the expression of various differentiation markers in 4 different human breast carcinoma cell lines: UIISO-BCA-4, MCF-7, MDA-MB-231, and ZR-75-1. For immunohistochemical studies, cells were plated on coverslips (Nunc Corp., Naperville, Illinois), then incubated for 7 days in the culture medium containing  $1\alpha(\text{OH})\text{D}_5$  ( $10^{-7}\text{M}$ ,  $10^{-6}\text{M}$ ) or  $1\alpha(\text{OH})_2 \text{D}_3$  for 7 days. These compounds were added to medium containing charcoal-stripped serum. At the end of incubation, coverslips were rinsed in PBS, and either processed for immunohistochemical staining or fixed in 10% buffered formalin, 70% ice cold methanol, and acetone, then processed for immunohistochemical staining.



Figure 2.



Effect of  $1\alpha(\text{OH})\text{D}_5$  on growth of UIISO BCA-4 cells: lane 1, (100%) cells at initial plating, lane 2, after 10 days incubation in charcoal stripped serum containing medium alone (control) or with  $1,25(\text{OH})_2\text{D}_3/1\alpha(\text{OH})\text{D}_5$ ; lane 3, after treatment as in lane 2, medium was changed to a regular MEM-E containing 15% FBS and cell growth was maintained for additional 10 days. Data represent % (of initial plating) of control cell growth.

For FACS analysis, cells growing in the culture flasks were incubated with  $1\alpha(\text{OH})\text{D}_5$  or  $1\alpha(\text{OH})_2\text{D}_3$  for 7 days. At the end of incubation, cells were harvested, rinsed with PBS, and incubated with appropriate primary antibody or IgG as control for 1 hour. Cells were rinsed with PBS and incubated with fluorescent-labeled secondary antibody. Cells after thorough washing were fixed in 0.5% buffered formalin and then subjected to FACS analysis. Table 1 summarizes the original (without treatment) status of various biomarkers in cell lines examined by FACS analysis.

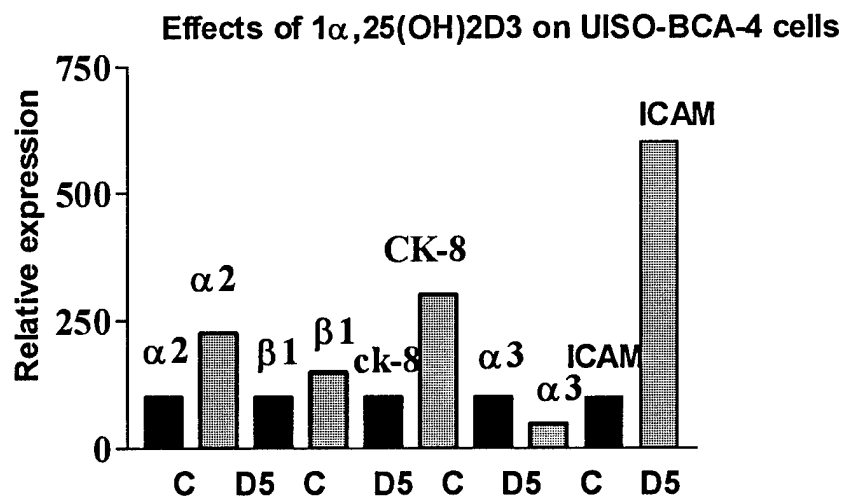
Biomarker	UIISO-BCA-4	MCF-7	MDA-MB-231	ZR-75-1
Alpha 2 integrin	+	+	+	+
Alpha3 integrin	+	+	Neg/low	+
Alpha5 integrin	+	+	+	Low/neg
Beta 1 integrin	+	+	+	+
Beta 4 integrin	-	+	low	-
Alpha6 integrin	+	+	+	+
Cytokeratin 8	+	-	-	+/low
Cytokeratin 18	-	-	+	-
Cytokeratin 19	+	+	+	+
ICAM-1	+	+	+	Low/neg
EGFR	-	-	-	-
Insulin-like Growth Factor I	+	+	+	Nd

These results were further confirmed by immunohistochemical studies.

#### The effect of $1\alpha(\text{OH})\text{D}_5$ and $1\alpha(\text{OH})2\text{D}_3$ on UIISO-BCA-4 cells

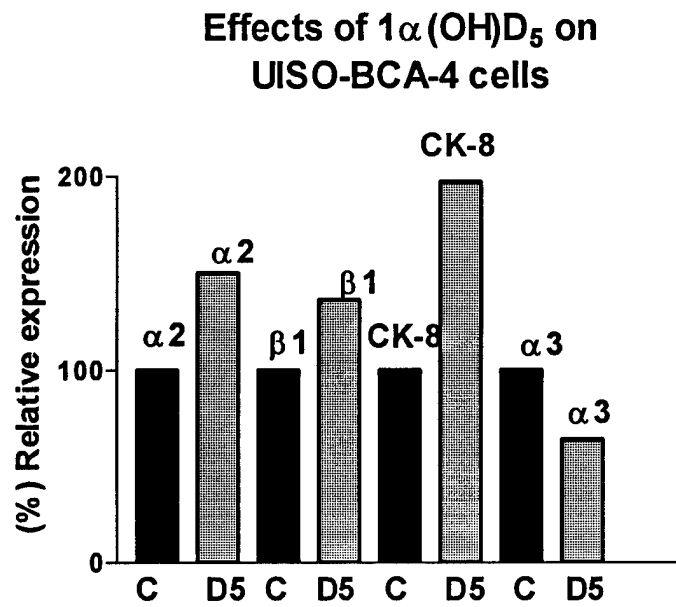
We evaluated the effect of  $1\alpha(\text{OH})2\text{D}_3$  (used as a standard vitamin D metabolite) in UIISO-BCA-4, ZR-75-1, MCF-7 and MDA-MB-231 cells. As mentioned earlier, results were evaluated by both immunocytochemistry and FACS analysis. Following treatment with  $1\alpha(\text{OH})2\text{D}_3$ , we observed enhanced expression of alpha2 integrin, beta 1 integrin, Ck-8 and ICAM-1 proteins. Expression of alpha3 integrin was reduced following  $1\alpha(\text{OH})2\text{D}_3$  treatment. Results in UIISO-BCA-4 cells are shown in Fig. 3.

Figure 3



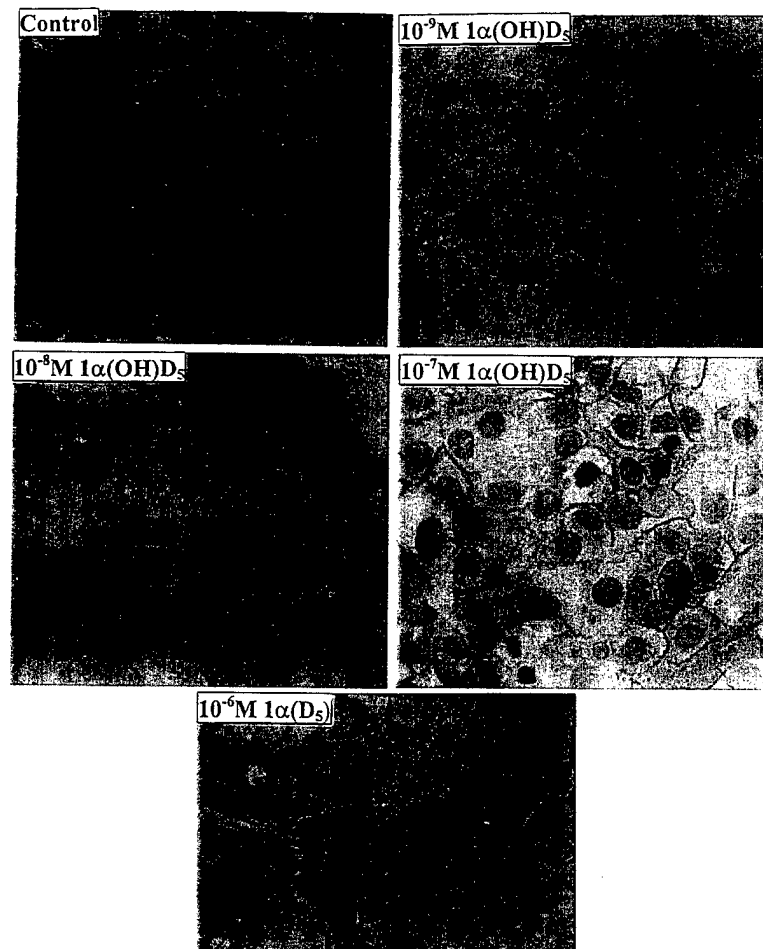
Effect of  $1\alpha(\text{OH})\text{D}_5$  was similar to that of  $1\alpha(\text{OH})2\text{D}_3$ . We observed enhanced expression of alpha2, beta 1, and ck-8 proteins (fig. 4). Results on ICAM-1 expression need to be reconfirmed by FACS analysis.

Figure 4



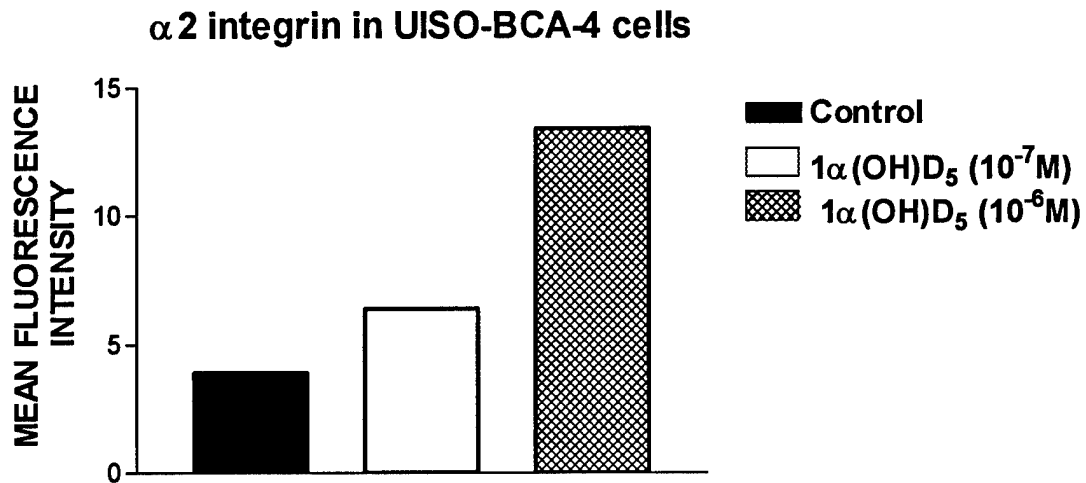
Changes in the expression of  $\alpha 2$  integrin following  $1\alpha(\text{OH})\text{D}_3$  treatment was dose-dependent. Fig. 5 shows the immunohistochemical staining for  $\alpha 2$  integrin following treatment with  $10^{-9}$ - $10^{-6}\text{M}$   $1\alpha(\text{OH})\text{D}_3$ .

Figure 5.



Dose-dependent changes observed by immunohistochemistry were further confirmed by FACS analysis (Fig. 6).

**Figure 6.**



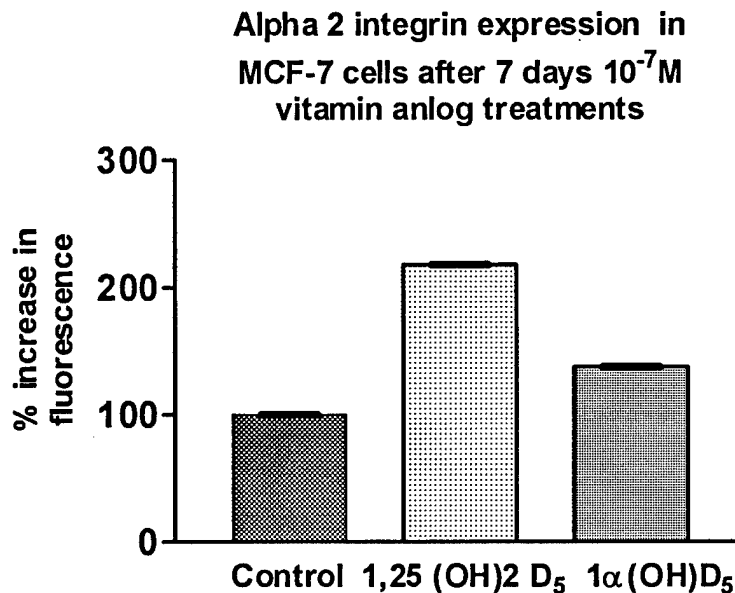
UIO-BCA-4 cells were incubated in the presence or absence of  $1\alpha(\text{OH})\text{D}_5$  for 7 days. Expression of  $\alpha 2$  integrin was determined by flow cytometry.

#### Effect of $1\alpha(\text{OH})\text{D}_5$ in MCF-7 cells

We evaluated the effects of  $1\alpha(\text{OH})\text{D}_5$  and  $1\alpha(\text{OH})2\text{D}_3$  on MCF-7 cells. Our flow cytometry and immunohistochemical data suggest that both these vitamin D analogs induced ICAM-1, CK-8,  $\alpha 2$ , and  $\beta 1$  integrins in these cells. The effects on other markers need to be confirmed.

Studies on the effect of these compounds on expression of catenins, vimentin, and e-cadherin are currently in progress. We are also evaluating the effects of  $1\alpha(\text{OH})\text{D}_5$  on differentiation of ZR-75-1 cells.

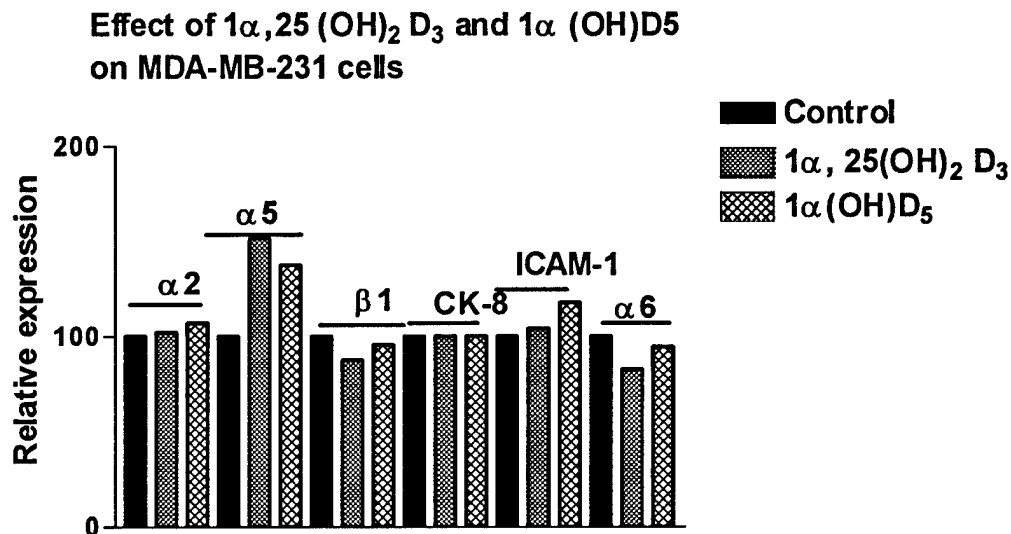
**Figure 7**



### The effect of $1\alpha(\text{OH})\text{D}_5$ in MDA-MB-231 cells.

We examined the effects of  $1\alpha(\text{OH})\text{D}_5$  and  $1\alpha(\text{OH})_2\text{D}_3$  in VDR-ER- MDA-MB-231 cells on  $\alpha 2$ ,  $\alpha 5$ ,  $\beta 1$ ,  $\text{ck-8}$ ,  $\text{ck-18}$ ,  $\text{ck-19}$ , and  $\text{ICAM-1}$ ,  $\alpha 6$  expression.. We failed to observe significant changes in the expression of any of these biomarkers following  $1\alpha(\text{OH})\text{D}_5$  treatment (10-7M). Fig. 7 shows expression of various biomarkers in control and  $1\alpha(\text{OH})\text{D}_5$ -treated MDA-MB-231 cells.

**Figure 8**



**Specific Aim 2:** Evaluate the therapeutic efficacy of  $1\alpha(\text{OH})\text{D}_5$  conjugated with c-erbB2. Whether conjugated  $1\alpha(\text{OH})\text{D}_5$  is more effective than c-erbB2 antibody or  $1\alpha(\text{OH})\text{D}_5$  treatment alone.

Recently, tumor cell-targeted antibody therapy has been considered a promising approach for the treatment of cancer.<sup>33</sup> The antitumor effect of monoclonal antibodies may be achieved directly through its effector function. Alternatively, antibody specific for the cancer cell could be used to deliver toxins or chemotherapeutic drugs to cancer cells.<sup>34</sup> We aim to evaluate the therapeutic efficacy of  $1\alpha(\text{OH})\text{D}_5$  conjugated with c-erbB2 antibody. C-erbB2 is a membrane receptor belonging to the EGFR receptor family.<sup>35</sup> Overexpression of c-erbB2 has been reported in about 33%-40% of human breast tumors. Also, an association between overexpression of this protein and poor prognosis is established.<sup>36,37</sup> Generally, DCIS overexpressing c-erbB2 are at high risk of developing invasive cancer.<sup>38</sup> Recently, c-erbB2 antibody treatment has shown both cell-differentiating and growth-inhibiting actions in breast carcinoma cells.<sup>39,40</sup> Treatment with  $1\alpha(\text{OH})\text{D}_5$  conjugated to c-erbB2 antibody will generate a high concentration of vitamin D analog exclusively in breast cancer cells and will have enhanced cell-differentiating action compared to either compound administered alone. We aim to approach this specific aim in 5 different phases: a) prepare  $1\alpha(\text{OH})\text{D}_5$ -erbB2 conjugate; b) evaluate whether the conjugation process preserved the breast carcinoma cell-binding property of c-erbB2 antibody; c) evaluate whether the cell differentiating action of  $1\alpha(\text{OH})\text{D}_5$  is maintained following conjugation with c-erbB2 antibody; d) determine the  $1\alpha(\text{OH})\text{D}_5$  concentration in tumor and normal tissues following conjugate administration in experimental animals; and e) determine the *in vivo* efficacy of conjugate as antitumorigenic and antimetastatic therapeutic drug.

Currently, we have obtained sufficient  $1\alpha(\text{OH})\text{D}_3$  to use the compound for conjugation. All information on procedures available for conjugation of various compounds to specific antibody has been obtained.

During the course of study, we found the following *in vivo* experiments necessary.

- 1) Identify cell lines that express high levels of c-erbB2 protein.
- 2) Determine whether c-erbB2 alone is effective in inhibiting growth of breast cancer cells.
- 3) Determine whether combination of c-erbB2 +  $1\alpha(\text{OH})\text{D}_3$  treatment will be more effective than either compound alone.

Three-to-four-week-old female Balb/c athymic mice were obtained from the Frederick Cancer Research Facility, Bethesda, MD. Breast carcinoma cells ( $2 \times 10^6$  cells/animal) suspended in Hank's Balanced Salt Solution and matrigel (1:1 vol.) were injected S.C. into the dorsal region of the mice. Animals receiving ZR-75-1 cells also received estradiol pellet (0.72 mg, 60 days release). Animals were divided into two groups: 1) Control group, receiving regular diet; 2) experimental group receiving diet supplemented with  $1\alpha(\text{OH})\text{D}_3$  (12.5  $\mu\text{g}/\text{kg}$  diet). Each group consisted of five animals. Experiments were repeated at least 3 times. All animals were observed weekly for any sign of toxicity. Animals were palpated at the site of cell injection or other cutaneous sites for the development of palpable tumors. Tumor volume was determined using vernier calipers. Animals were sacrificed 60 days postinoculation or if they became moribund or tumor became necrotic or reached  $2.0\text{cm}^3$  in volume.  $1\alpha(\text{OH})\text{D}_3$  was effective in inhibiting growth of UISO-BCA-4 and ZR-75-1 cells. No effect on the growth of MDA-MB-231 cells was observed. The *in vivo* effect on MCF-7 cells needs to be re-evaluated because, due to severe sickness of animals in both control and experimental groups, the experiment was terminated prematurely.

Figure 9

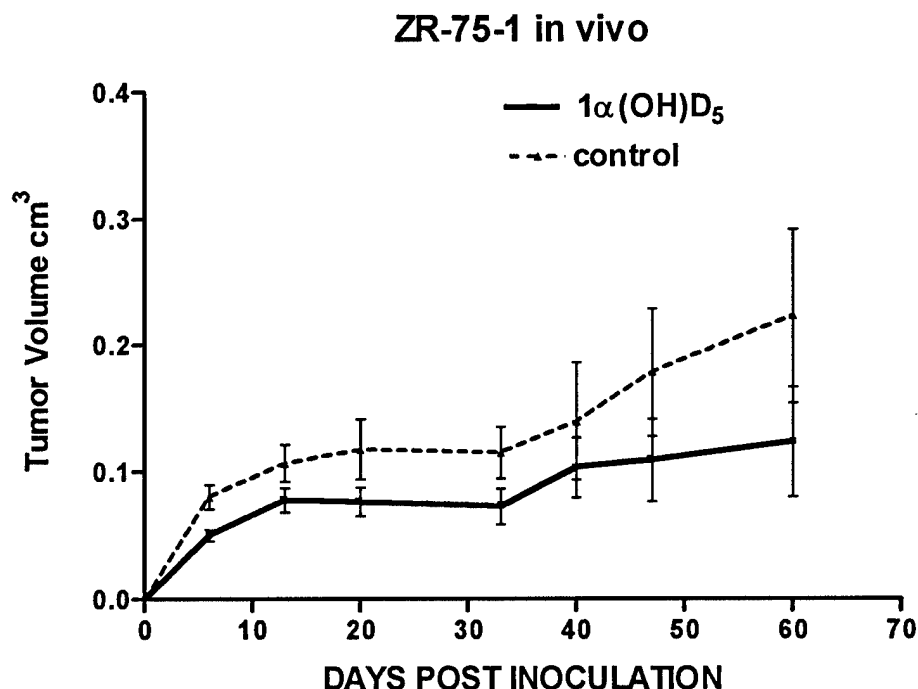
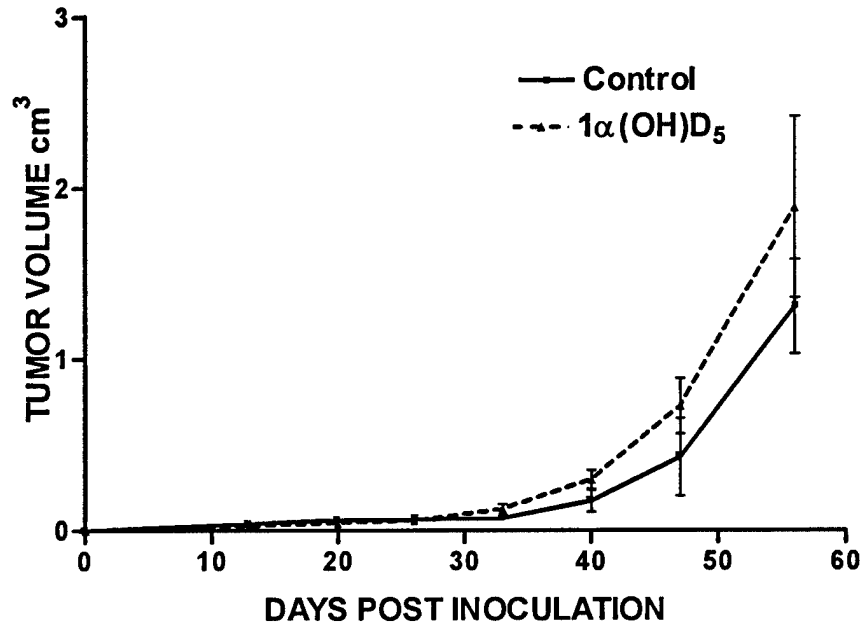


Figure 10

## MDA-MB-231

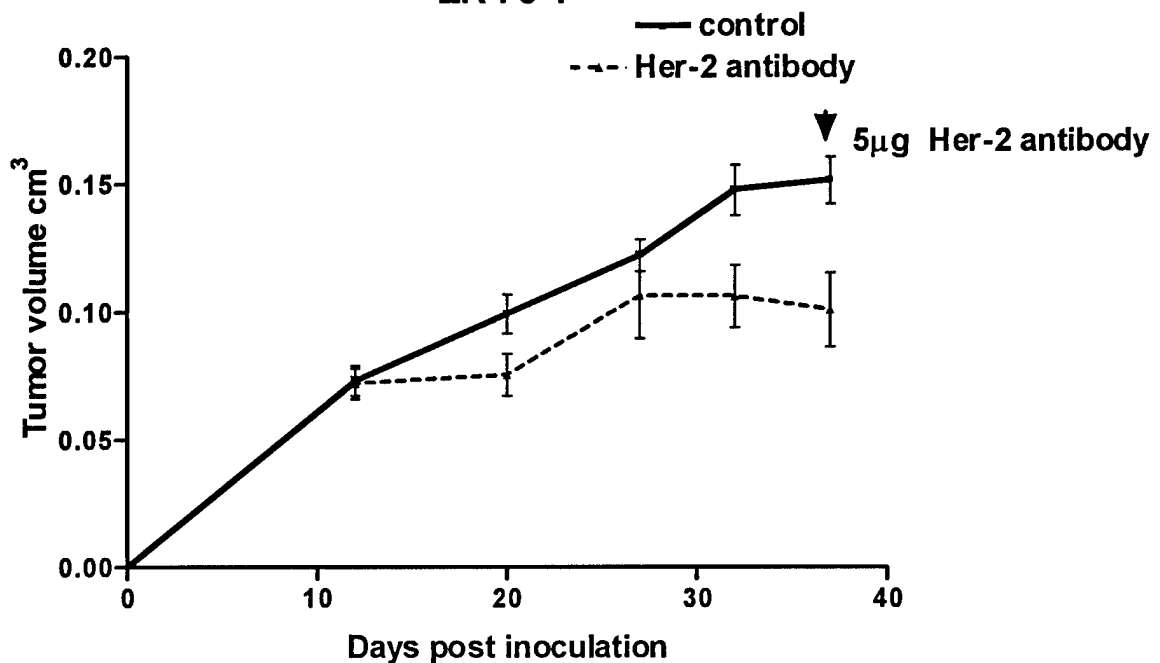


Among all the cell lines included in the study, ZR-75-1 and UIISO-BCA-4 showed overexpression of Her-2 /neu protein. MCF-7 and MDA-MB-231 have relatively low expression of HER-2/neu. In vivo, ZR-75-1 cells are highly tumorigenic and also respond to 1α(OH)D<sub>5</sub> treatment (see Fig. 11). Response to Her-2 antibody was evaluated using this cell line. ZR-75-1 cells mixed with Matrigel were transplanted S.C. into 3- to 4-week-old female athymic mice. All animals received estradiol pellets as mentioned above. Animals were injected with saline only (control group) or Her-2 antibody (2 μg/100 ml saline) three times per week. Animals were monitored for the growth of tumor.

After 30 days, we increased the dose of Her-2 antibody to 5μg in order to determine the maximum effect of antibody. As shown in Fig. 11, animals receiving Her-2 antibody had significantly smaller tumors than those receiving saline only. This experiment is currently in progress.

Figure 11

## ZR-75-1



## Conclusion

We evaluated the effects of  $1\alpha(\text{OH})\text{D}_3$ , a novel synthetic analog of vitamin D, on various established human breast carcinoma cell lines (MCF-7, ZR-75-1, UIISO-BCA-4, MDA-MB-231). MCF-7 and ZR-75-1 cells are ER+ and also contain VDR. UIISO-BCA-4 cells are ER-VDR+. MDA-MB-231 cells are ER-VDR-.  $1\alpha(\text{OH})\text{D}_3$  had significant growth inhibitory action on ER+VDR+ human breast carcinoma cell lines. Growth inhibition achieved by these agents was permanent. Cells pre-exposed to  $1\alpha(\text{OH})\text{D}_3$  ( $10^{-6}\text{M}$ ) or  $10^{-7}\text{M}$   $1\alpha,25(\text{OH})_2\text{D}_3$  failed to achieve normal growth pattern following treatment withdrawal.  $1\alpha(\text{OH})\text{D}_3$  had cell-differentiating action on selected breast carcinoma cell lines. Following 7 days treatment with  $1\alpha(\text{OH})\text{D}_3$ , we observed enhanced expression of ICAM-1, nm23, casein, intracellular lipids,  $\alpha_2$  integrin,  $\beta_1$  integrin, and CK-8. These markers are known to be associated with breast cell differentiation. The effect of  $1\alpha(\text{OH})\text{D}_3$  was evaluated on growth of ZR-75-1, UIISO-BCA-4, and MDA-MB-231 cells transplanted into athymic mice.  $1\alpha(\text{OH})\text{D}_3$  ( $12.5 \mu\text{g/kg}$  diet) was supplemented in the diet.  $1\alpha(\text{OH})\text{D}_3$  had significant growth inhibitory effect on ZR-75-1 cells. In animals receiving  $1\alpha(\text{OH})\text{D}_3$ , tumor size at the site of injection was significantly reduced compared to in those animals receiving regular diet. In slow-growing UIISO-BCA-4 cells,  $1\alpha(\text{OH})\text{D}_3$  had cell-differentiating action. No effect was observed on in vivo growth of MDA-MB-231 cells. Our ultimate goal is to specifically target breast cancer cells with  $1\alpha(\text{OH})\text{D}_3$ . We aim to conjugate  $1\alpha(\text{OH})\text{D}_3$  to c-erbB2/Her-2 antibody. In the panel of our breast carcinoma cell lines selected, UIISO-BCA-4 and ZR-75-1 cells express high levels of Her-2 protein. Thus, based on the tumorigenic potential and Her-2 expression, further studies were performed with ZR-75-1 cells. Her-2 antibody ( $2-5 \mu\text{g/animal}$ , 3 times weekly) administered S.C. inhibited in vivo growth of ZR-75-1 cells. Further studies will be performed to determine the effect of combined her-2 antibody +  $1\alpha(\text{OH})\text{D}_3$  treatments. Experiments to conjugate  $1\alpha(\text{OH})\text{D}_3$  to Her-2 antibody are in progress.

## Unanticipated problems and issues

Our ultimate goal is to determine whether treatment with  $1\alpha(\text{OH})\text{D}_3$  linked to Her-2 antibody is effective in preventing metastasis of breast cancer cells. From our results, it is evident that VDR is a critical component in mediating the effect of  $1\alpha(\text{OH})\text{D}_3$ . In the cell lines selected in our panel, only MDA-MB-231 cells are known to have metastatic potential when transplanted in vivo in experimental animals. Other cell lines are non-metastatic. However, MDA-MB-231 cells either don't express or show low expression of ER, VDR, and Her-2 proteins. Thus, in order to determine the effect of  $1\alpha(\text{OH})\text{D}_3$  linked to Her-2/neu antibody, we will use MDA-MB-231 cells transfected with 1) ER cDNA, 2) VDR cDNA, and 3) Her-2 cDNA. Alternatively, recently we generated a xenograft line from UIISO-BCA-4 cells, which are highly tumorigenic in mice and show 60%-80% metastasis in animals. UIISO-BCA-4 cells show high expression of Her-2/neu protein and are VDR+. Therefore, the effect of  $1\alpha(\text{OH})\text{D}_3$  + Her-2 antibody will be evaluated also in this xenograft line.

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