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13. ABSTRACT *(Maximum 200*

We continue to pursue our *in vitro* and *in vivo* studies of novel vitamin D_3 analogs used alone and in combination against breast cancer. We have altered our 19-nor analogs in an attempt to minimize hypercalcemic effects while retaining potency. We have synthesized a series of novel 20-cyclopropyl-cholecalciferol vitamin D_3 analogs that were found to be potent inhibitors of clonal growth of MCF-7 breast cancer cells. We examined a new class of Vitamin D analogs that have a novel 5,6-trans motif. We examined the use of vitamin D analogs in combination with All-trans-retinoic acid in human breast tumors in BNX mice and found an additive effect which decreased tumor mass nearly 3-fold with minimal toxicity. Due to the dearth of in vivo studies examining the long term effects of Vitamin D analogs, we administered unique analogs for approximately one year to Balb/C mice and performed extensive toxicity analyses. We have found that Vitamin D analogs modulate BRCA1 expression in breast cancer. We have initiated studies using the organic arsenical Melarsoprol (Mel-B)and All-trans-retinoic acid (ATRA) in human breast cancer cell lines and in human breast tumors in BNX mice and found significant apoptosis and a synergistic anti-cancer effect. We have recently initiated a clinical trial to examine the PPARγ synthetic ligand Troglitazone and All-*trans*-retinoic acid in patients with metastatic breast cancer. We have continued to search for genetic alterations of breast cancer. We have recloned SNAP 43 and thymosin β4 two genes important for breast cancer. Our studies include examination of a mutation of the $p16^{INK4A}$ binding domain of the CDK4 gene, evaluation of the novel tumor suppressor gene DPC4/SMAD4, and studies of $p21^{WAF1}$, $p27^{KIP1}$, $p15^{INK4B}$, $p16^{INK4}$, $p18^{INK4C}$ and 19^{INK4} alterations in various human malignancies.

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INTRODUCTION

The following provides descriptions of our Vitamin D, Retinoid, Melarsoprol and Troglitazone studies, as well as our molecular biology work.

VITAMIN D3 ANALOGS IN BREAST CANCER

Previously, we have studied the in vitro biologic activities of 19-nor-hexafluoride analogs of vitamin D_3 on proliferation and differentiation of breast cancer, prostate cancer and HL-60 leukemic blast cells. These compounds, which have six fluorines on their side chain, demonstrated strong inhibition of clonal growth and induction of differentiation of cancer cells. Especially potent was compound LH (also known as Ro25-6760). The work on compound LH was submitted in last years annual report and has since been published. (M Koike, E Elstner, MJ Campbell, H Asou, M Uskokovic, N Tsuruoka and HP Koeffler. 19-nor-hexafluoride analogue of vitamin D_3: a Novel class of potent inhibitors of proliferation of human breast cell lines. **Cancer Research** (57), 4545-4550, 1997).

However, compound LH induced prominent hypercalcemia in mice. Therefore, we synthesized a new class of analogs that has a novel 19-nor motif by replacing the six fluorines with a bishomo modification as well as retaining many structural elements previously shown to increase potency (Fig.1). This was done in an attempt to maintain efficacy while simultaneously decreasing the hypercalcemic effects.

These analogs were examined for their effects on a human breast cancer cell line (MCF-7), prostate cell lines (PC-3, LNCaP, and DU-145), and an acute myeloid leukemia cell line HL-60 (Fig.2).

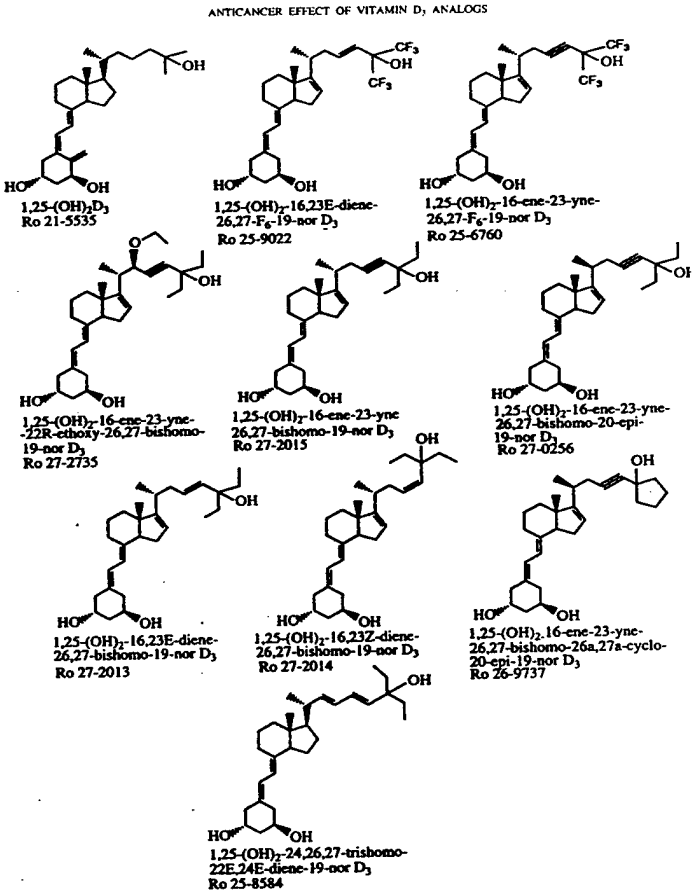


Fig. 1. Chemical structures of vitamin D_3 analogs used in this study.

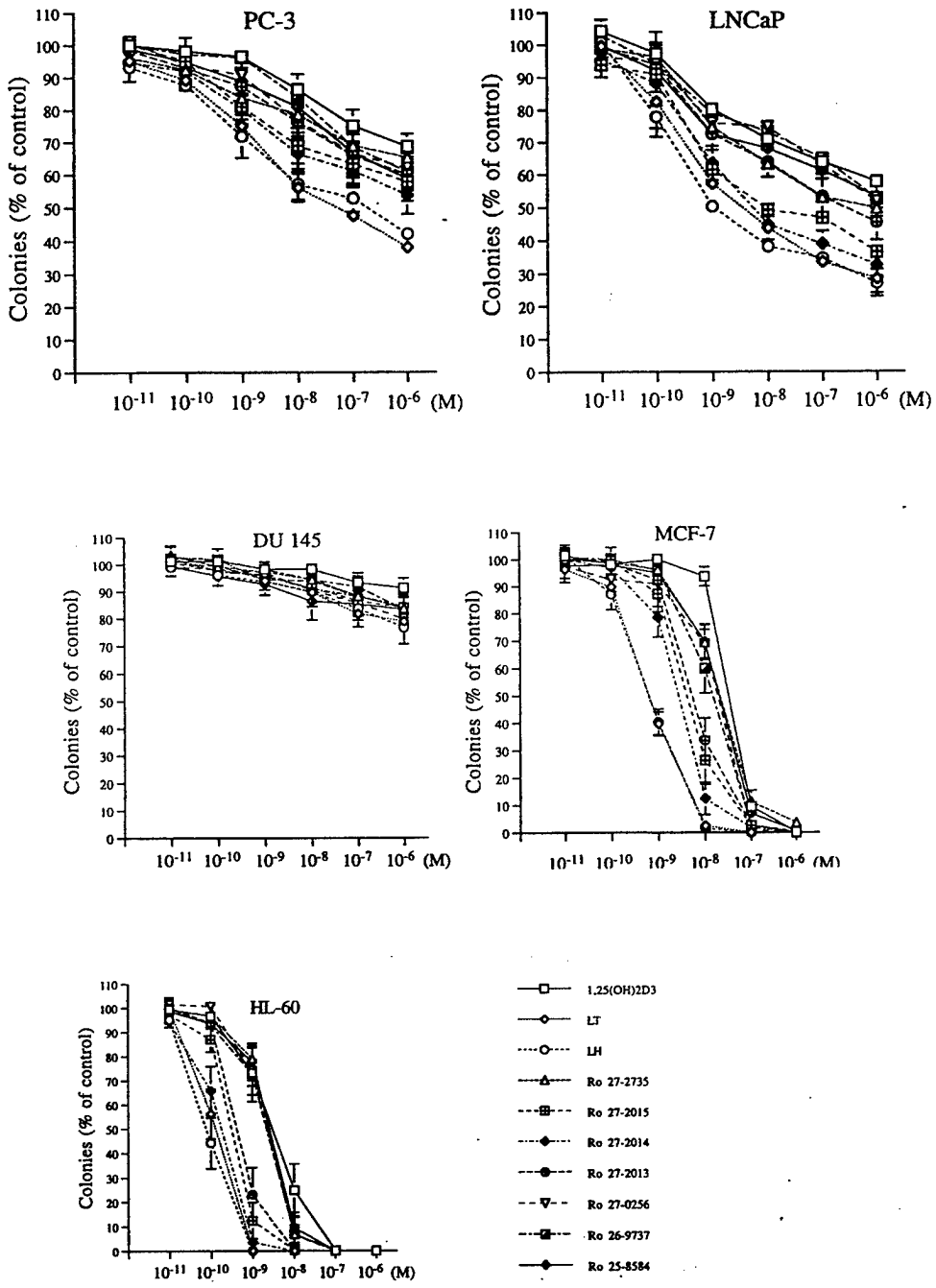


Fig. 2. Dose-response effects of vitamin D_3 compounds on clonal proliferation of PC-3, LNCaP, DU 145, MCF-7, and HL-60 cancer cells. Results are expressed as a mean percentage of control plates containing no vitamin D_3 compounds. Each *data point* represents a mean of at least three independent experiments with triplicate dishes. *Bars*, SD.

Dose-response studies showed that each of the analogs had more potent antiproliferative activity against cancer cells than 1,25 $[OH]_2$ D_3, and that 1,25-$(OH)_2$-16,23Z-diene-26,27-bishomo-19nor-D_3 (Ro27-2014) was the most potent analog (10-fold increased activity compared to 1,25 $[OH]_2$ D_3). Further studies were done using Ro27-2014.

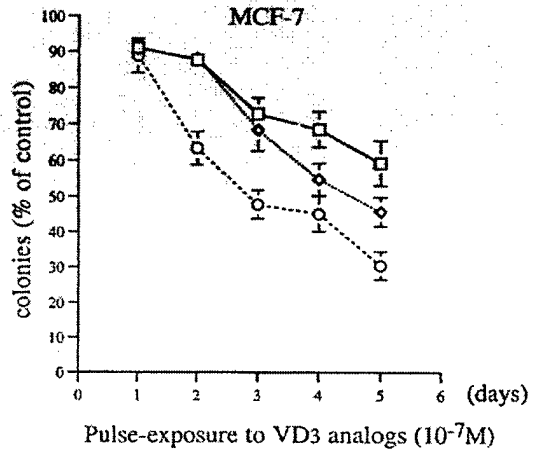


Fig. 3. Effect of pulse-exposure of vitamin D_3 compounds on the clonal growth of MCF-7 breast cancer cells. MCF-7 cells were exposed to 10^{-7} M of either 1,25$(OH)_2D_3$ (□), compound Ro 27-2014 (◇), or compound LH (Ro 25-6760; ○) for the indicated periods of time, thoroughly washed, and plated in soft agar; colonies were enumerated after 14 days of culture. The results are expressed as a mean percentage of colonies in

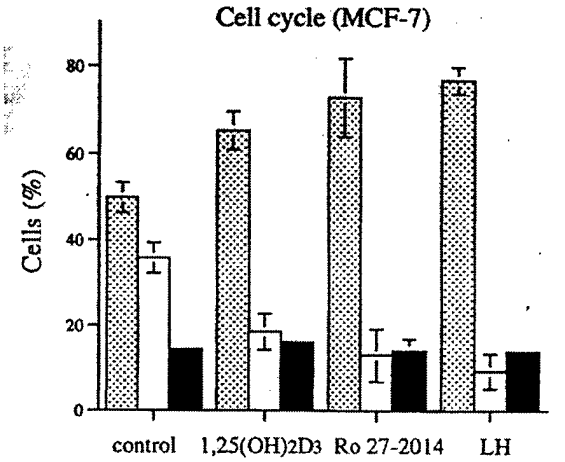


Fig. 4. Cell cycle analysis of MCF-7 breast cancer cells. MCF-7 cells were cultured with either 1,25$(OH)_2D_3$, compound Ro 27-2014, or compound LH (Ro 25-6760; 10^{-7} M) for 4 days. Control cells represent those not exposed to vitamin D_3 compounds. *Columns*, mean of three independent experiments; *bars*, SD. ▨, G_1; □, S; ■, G_2-M.

Pulse exposure studies (Fig. 3) showed that a 5-day pulse-exposure to Ro27-2014 (10^{-7M}) in liquid culture was adequate to achieve 50% inhibition of MCF-7 clonal growth in soft agar in the absence of the analog, suggesting that the growth inhibition mediated by the analog was irreversible. Cell cycle analysis using MCF-7 cells (Fig. 4) showed that Ro27-2014 (10^{-7M} for 4 days) induced a significant increase in the number of cells in G_0 and G_1 (72.8 ± 8.9 versus 49.9 ± 3.5% in control cells), with a concomitant decrease in the percent of cells in S phase (13.1 ± 6.2% versus 35.8% ± 3.5 in control cells.)

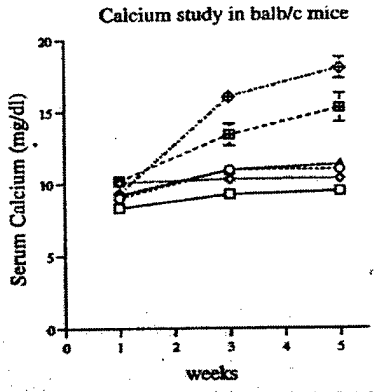


Fig. 5. The effect of vitamin D_3 analogs on serum calcium in mice. Each *data point* represents the mean ± SD of serum calcium levels of five mice. The mice received i.p. injections of vitamin D_3 compound three days a week (Monday, Wednesday, and Friday). If the SD was less than 0.6 mg/dl, it does not appear on the graph. □, PBS; ○, 1,25-$(OH)_2D_3$ (0.05 μg); ◇, Ro 27-2014 (0.025 μg); △, Ro 27-2014 (0.05 μg); ▨, Ro 27-2014 (0.1 μg); ◆, LH (0.05 μg).

The chief toxicity of vitamin D_3 analogs is hypercalcemia. Therefore, we examined calcemic activity of Ro27-2014 in mice and found it not to induce hypercalcemia at doses of .05μg i.p. three times per week (Fig. 5). The serum calcium levels of the mice receiving Ro27- 2014 were almost identical to those of mice receiving 1,25 $[OH]_2$ D_3. In contrast, the same dose of 19-nor vitamin D_3 compound with 6 fluorines on the side chain (1,25-$(OH)_2$-16,ene-23-yne-26,27-F_6-19nor-D_3), although also having potent anti-cancer activity in the mice, caused severe hypercalcemia (18mg/dl).

In summary, 19-nor vitamin D_3 compounds with desaturation and lengthening of their side chains results in a series of compounds with a good therapeutic index, having anti-cancer activity and low toxicity. Further testing using animal models in the setting of low tumor burden may result in a new therapeutic approach.

This work was recently published. T Kubota, K Koshizuka, M Koike, M Uskokovic, I Miyoshi, HP Koeffler. 19-nor-26,27-bishomo-vitamin D_3 analogs: a unique class of potent inhibitors of proliferation of prostate, breast and hematopoietic cancer cells. **Cancer Research** 58,3370-3375, 1998.

2. In addition, we have synthesized and studied the ability of a series of nine novel 1,25 dihydroxyvitamin D_3 (1,25 $[OH]_2$ D_3) analogs with a cyclopropyl moiety at carbon 20, to inhibit clonal growth of breast (MCF-7) and prostate (LNCaP, PC-3 and DU-145) cancer cells and of myeloid leukemic cells (HL-60). To the cyclopropyl backbone, we also incorporated previously identified active structural motifs that are known to enhance activity, including desaturation at carbon C-23, addition of six fluorines at C-26 and -27 and removal of C-19 (Fig.1).

The three prostate cell lines were initially relatively resistant to the 9 compounds (Cmpd) with these alterations; however, removal of C-19 (E, 1,25 dihydroxy-23E-ene-26,27-hexafluoro-19-nor-20-cyclopropyl-cholecalciferol) resulted in an analog that was inhibitory against the MCF-7 breast cancer cell line, all three prostate cell lines, and the HL-60 cell line (Table 1).

Further analysis showed that pulse exposure (3 days, 10^{-7}), washing, plating in soft agar, and enumerating colony formation 14 days after plating resulted in inhibition of clonal growth of PC-3 cells by 50%. These results suggest that Cmpd E (E, 1,25 dihydroxy-23E-ene-26,27-hexafluoro-19-nor-20-cyclopropyl-cholecalciferol) inhibited growth of the cancer cells by a mechanism other than one that is merely cytostatic.

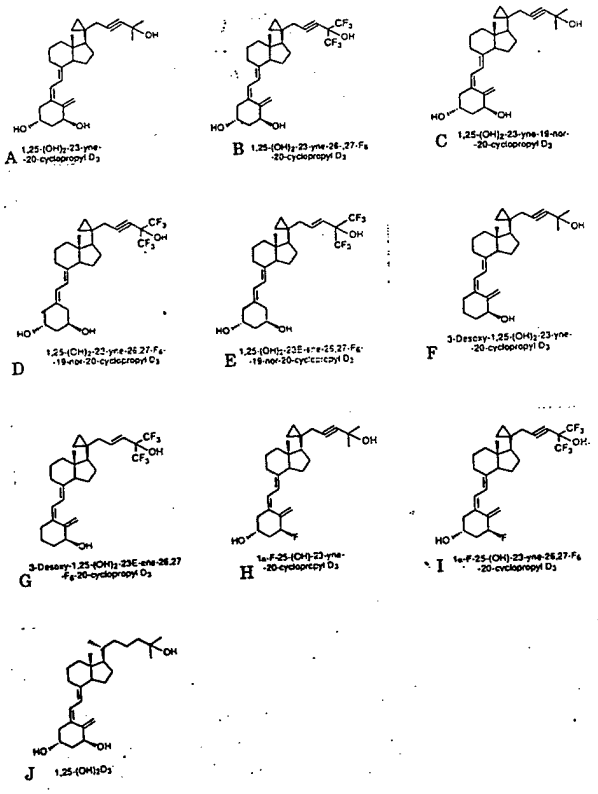


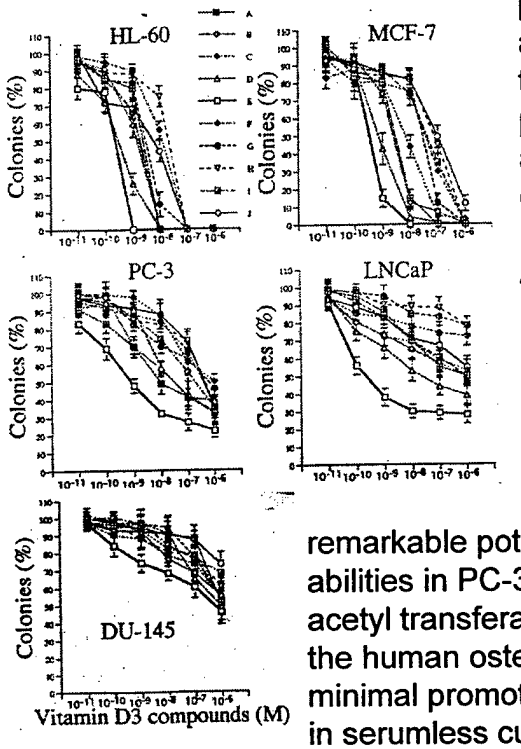
Fig.1

Table 1. Inhibition of clonal proliferation of cancer cell lines by vitamin D_3 analogs

	Inhibition of clonal proliferation ED_{50} (M)*				
Vitamin D_3 analogues	HL-60	MCF-7	PC-3	LNCaP	DU-145
A	5×10^{-9}	5×10^{-9}	1×10^{-8}	1×10^{-8}	N.R.*
B	2×10^{-8}	4×10^{-8}	3×10^{-8}	N.R.	N.R.
C	5×10^{-8}	8×10^{-8}	1×10^{-7}	N.R.	N.R.
D	5×10^{-10}	8×10^{-10}	8×10^{-9}	3×10^{-8}	1×10^{-7}
E	2×10^{-10}	4×10^{-10}	9×10^{-10}	3×10^{-10}	8×10^{-9}
F	2×10^{-8}	6×10^{-8}	8×10^{-8}	N.R.	N.R.
G	5×10^{-8}	5×10^{-8}	9×10^{-7}	N.R.	N.R.
H	3×10^{-8}	8×10^{-8}	5×10^{-7}	N.R.	N.R.
I	6×10^{-8}	8×10^{-8}	2×10^{-7}	N.R.	N.R.
J (1,25(OH)$_2$D$_3$)	8×10^{-9}	1×10^{-7}	8×10^{-7}	N.R.	N.R.

* Dose-response curves (Fig.2) were used to calculate the concentration of the analogues achieving a 50% inhibition (ED_{50}) of clonal growth. N.R., the ED_{50} was not reached at $\leq10^{-6}$M of the 1,25 D_3 analog.

Fig. 2 Dose response curves



1,25 $[OH]_2 D_3$ mediates its biologic activities through specific binding to the vitamin D_3 receptor (VDR) and subsequent association with vitamin D_3 response elements (VDRE) in genes modulated by 1,25 $[OH]_2 D_3$. Cmpd E increased the number of PC-3 cells in G_1 and decreased the number in S phase. Prior studies have found that several 1,25 $[OH]_2 D_3$ analogs caused a G_1 to S-phase block in parallel with an upregulation of $p27^{KIP1}$ in HL-60 cells, and breast and prostate cancer cell lines.

We have found that the levels of $p21^{WAF1}$ and $p27^{KIP1}$ in LNCaP cell line increased by several fold after exposure to Cmpd E (3 days, 10^{-7}) supporting the hypothesis that the levels of $p21^{WAF}$ and $p27^{KIP}$ protein may be mediators of the antiproliferative activity of the vitamin D_3 Cmpds by blocking entry of these cancer cells into the S-phase.

Analogs may also extend the half-life of the activated VDR or they may induce novel VDR conformation which may then allow either more efficient interaction with vitamin D_3 response elements and /or alter the array of VDREs that can be activated. To clarify the mechanism by which nine of these vitamin D_3 analogs mediate their remarkable potent biological activities, we have investigated their abilities in PC-3 prostate cells to transactivate a chloramphenical acetyl transferase (CAT) reporter gene containing a VDRE from the human osteocalcin gene attached to a thymadine kinase minimal promoter. Dose-response studies of Cmpd E showed that in serumless culture conditions, transactivation of the VDRE-CAT was stronger than Cmpd J (1,25 $[OH]_2 D_3$). At 10^{-7} mol/L, Cmpd E increased CAT reporter gene activity nearly 12.5-fold compared with similar cells exposed to 10^{-10} mol/L of the same analog.

We also investigated the effects of Cmpd E in mice. Our in vitro data showed that the growth inhibitory action of Cmpd E in the prostate cancer cell line (PC-3) was statistically superior (as measured by tumor size and weight) to the non-treatment group without inducing hypercalcemia (Fig. 3 & 4).

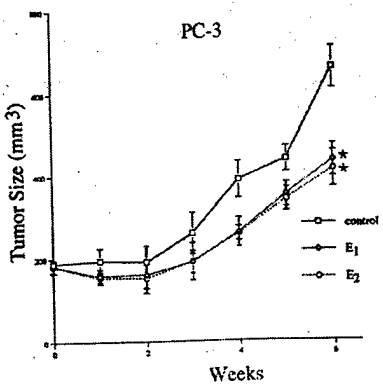


Fig. 3 Tumor Size PC-3 Cells

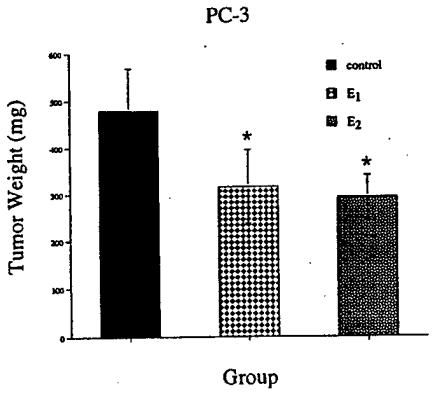


Fig. 4 Tumor Weight PC-3 Cells

Previously we found that Cmpd J at given at a dose of .0125 μg /mouse given in a similar time schedule caused mild hypercalcemia. It is not totally clear why Cmpd E was more potent that Cmpd J in its antiproliferative effects without the attendant hypercalcemia.

In summary, this is the first report of a potent series of 20-cyclopropyl-cholecalciferol vitamin D_3 analog with the ability to inhibit proliferation of LNCaP, PC-3, DU-145, MCF-7 and HL-60 cell lines. These compounds may mediate their potent anti-proliferative activity through a cell cycle arrest pathway.

A manuscript documenting this work has been submitted for publication. M Koine, K Koshizuka, H Cebidae, R Yang, H Taub, H Said, M Uskokovic, N Tsuruoka, H P Koeffler. 20-cyclopropyl-cholecalciferol Vitamin D_3 analogs; A unique class of potent inhibitors of proliferation of human prostate, breast and myeloid leukemia cell lines.

3. Another new class of analogs of 1,25 $[OH]_2 D_3$ has been synthesized that has a novel 5,6-trans motif. Seven of these analogs were examined for their antiproliferative effects on breast (MCF-7), prostate (LNCaP) and acute myeloid leukemic (HL-60) cell lines. Two analogs had more potent antiproliferative effects than 1,25 $[OH]_2 D_3$. The most potent analog of this group was 1,25 $(OH)_2$-16-ene-5,6-trans-D_3 (Ro 25-4020), inhibiting 50% clonal growth of HL-60 clonal proliferation at 1.65 $x10^{-9}$M, and significantly increasing the proportion of MCF-7 cells in the G_0 and G_1 phase and decreasing those in the S phase at $x10^{-7}$M. In MCF-7 cells, Ro 25-4020 (10^{-7}M x 3 days) induced about 3-fold increased expression of the cyclin dependent kinase inhibitors, $p21^{WAF1}$ and $p27^{KIP1}$. Furthermore, Ro 25-4020 induced expression of the novel tyrosine phosphatase TEN/MMAC1, a candidate tumor suppressor gene. To examine the effect of Ro25-4020 on telomerase activity in HL-60 cells, we performed telomeric repeat expression of human telomerase reverse transcriptase (hTRT), using RT-PCR and Northern blot analysis. Ro25-4020 (10^{-8}M x 4 days) inhibited telomerase activity more than did equal molar amounts of 1,25 $[OH]_2 D_3$. Taken together, these results suggest that this new class of analogs is worthy of an *in vivo* study for possible clinical trials.

Abstract submission to ASH 1998. Accepted for presentation. J Hisatake, T Kubota, Muskokovic, HP Koeffller. 5,6-trans-vitamin D_3 analogs: a unique class of potent inhibitors of proliferation of myeloid leukemic cells as well as prostate and breast cancer cells.

4. Vitamin D_3 analogs and all-trans-retinoic acid (ATRA) are able to inhibit growth of a variety of malignant cells. We examined the ability of three vitamin D_3 analogs to inhibit growth of a human mammary cancer (MCF-7) in Beige Nude xid (BNX) mice either alone or in combination with ATRA. Vitamin D_3 analogs were 1,25 $[OH]_2$ D_3 (code name compound C), 1,25-$(OH)_2$-16,ene-23-yne-26,27-F_6-19nor-D_3 (code name compound LH) and 24A,26A,27A,-trihomo-22,24-diene-1,25 $(OH)_2$ D_3 (code name EB1089).

At the doses chosen, the antitumor effect of ATRA alone was greater than that of either vitamin D_3 analogs alone, and an additive effect was observed when a vitamin D_3 analog and ATRA were administered together. EB 1089 was the most potent vitamin D_3 analog; and EB 1089 plus ATRA was the most potent combination. This was most impressively shown when examining tumor weights at the end of the study (Fig. 1).

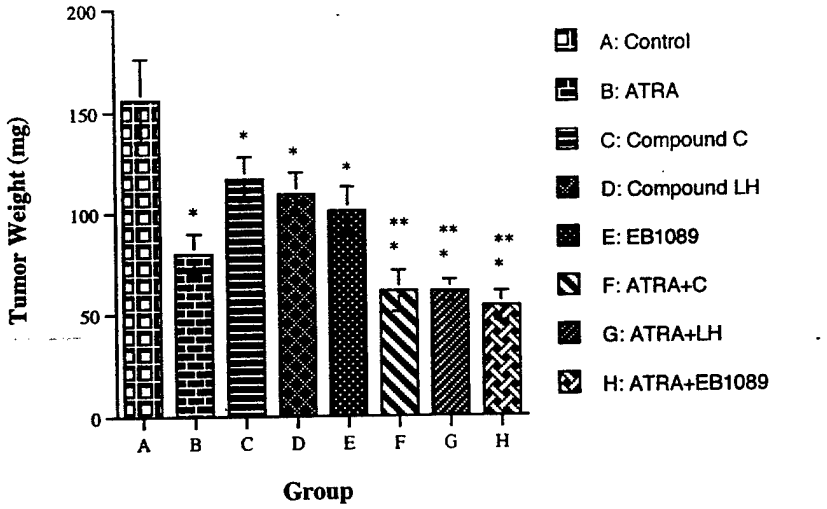


Fig. 1 Effect of a vitamin D_3 analog either alone or in combiantion on the weight of MCF-7 human breast tumors in BNX Mice

The tumor weights of the mice that received the combination of ATRA and EB 1089 were approximatelly 70% less thatn those in the control group. This combination decreased tumor mass nearly three-fold compared to tumors of diluent control mice. In addition tumors treated with ATRA and vitamin D_3 analogs revealed necrosis and fibrosis involving approximately 30% of the tumor mass (Fig. 2).

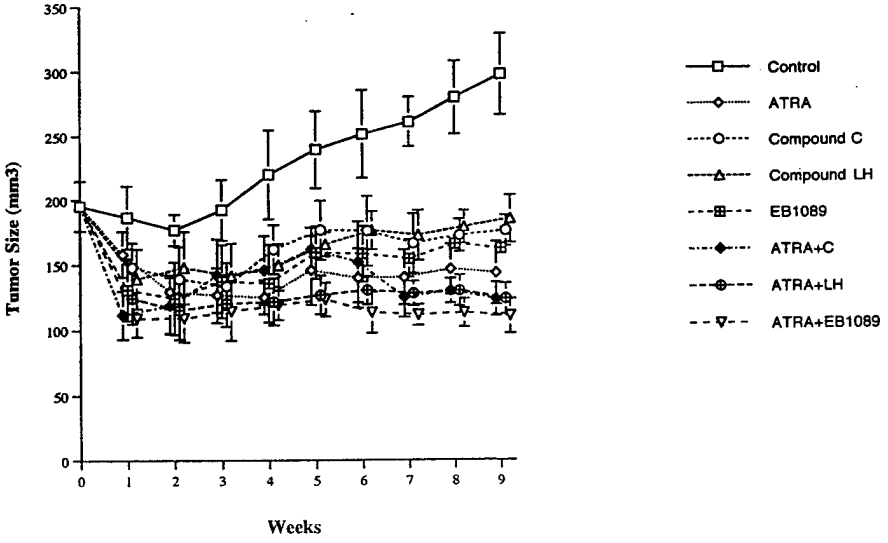


Fig. 2 Size of MCF-7 human breast tumors in mice receiving a vitamin D_3 analog and ATRA.

None of the animals became hypercalcemic. Their complete blood counts, serum electrolytes analysis as well as their liver and renal functions were all fairly similar and within the normal range.

We believe that this is the first report showing that one of several active vitamin D_3 analogs combined with ATRA is efficacious in inhibiting the growth of human MCF-7 breast tumors in BNX nude mice. This is important because these compounds mediate their antiproliferative effects via different receptors and have non-overlapping toxicities.

This work was recently submitted for publication. K Koshizuka, T Kubota, J Said, M Koike, L Binderup, M Uskokovic, HP Koeffler. Combination therapy of a vitamin D_3 analog and All-trans-retinoic acid: effect on human breast cancer in nude mice.

5. The breast cancer susceptibility protein BRCA1 was shown to be mutated in families with a high incidence of inherited breast cancer. Its exact function remains unclear, having little homology with other cloned genes, although it has subsequently been shown to play a role in regulating DNA repair and apoptosis. Retroviral transfection of MCF-7 cells has previously been shown to control cell growth *in vitro* and *in vivo*. Previously we have shown that BRCA1 protein is expressed in MCF-7 breast cancer cells after treatment with a 1,25 $[OH]_2$-20-epi-D_3. We have therefore attempted to investigate the mechanism of induction of this protein and determine the extent to which it is a target for mediating the antiproliferative effects in all breast and prostate cancer cell lines.

We examined the effects of 1,25 $[OH]_2$-D_3 on BRCA1 expression in a panel of breast

and prostate cancer cell lines. We have demonstrated increased RNA accumulation in the presence of cyclohexamide, thus suggesting that 1,25 $[OH]_2$-D_3 when bound to vitamin D response elements (VDRE) induces transcription of BRCA1. This would necessitate the presence of a VDRE in the promoter region of BRCA1 which as yet remains to be identified. To explore the VDR mediated effects on sensitivity to 1,25 $[OH]_2$-D_3 and BRCA1 modulation we reduced VDR expression by stable transfection with antisense VDR and isolated a number of clones for further study. The antiproliferative effects of 1,25 $[OH]_2$-D_3 were reduced in a dose dependent fashion in the two clones chosen for further study, that is the clone (MCF-7-AS#4) with the greater reduction in expression was the one with the greatest reduction in sensitivity (Table 1), and interestingly also did not modulate BRCA1 to any significant extent in the presence of 1,25 $[OH]_2$-D_3.

Table 1

	C		LH		IE	
	Clonal inhibiton (ED_{50})[1]	Fold Induction BRCA1	Clonal inhibiton (ED_{50})	Fold Induction BRCA1	Clonal inhibiton (ED_{50})	Fold Induction BRCA1
LNCaP	20	2.1	6	2.2	0.05	2.7
PC-3	100	1.9	4.5	2.7	>1000	2
MCF-7	20	1.4	0.8	1.8	0.05	2.4
MCF-7-neo	30	1.5	nd	nd	0.02	nd
MCF-7-AS#4	>100	1.0	nd	nd	20	nd

[1]Clonal growth in the presence or absence of vitamin D_3 analogues was examined using a two-layer soft-agar colony assay (Materials and Methods). Each experiment utilised triplicate dishes and was repeated three times. After 14 days, colonies were enumerated and expressed as a percentage of colonies in untreated control plates. These data were used to construct dose-response curves and the dose required to inhibit clonal growth by 50% [ED_{50} (nM)] was interpolated from the curve.

Taken together, our data suggest that one of the antiproliferative effects of 1,25 $[OH]_2$-D_3 in MCF-7 cells is to upregulate BRCA1 which in turn regulates proliferation via its effects on control on DNA replication and apoptosis.

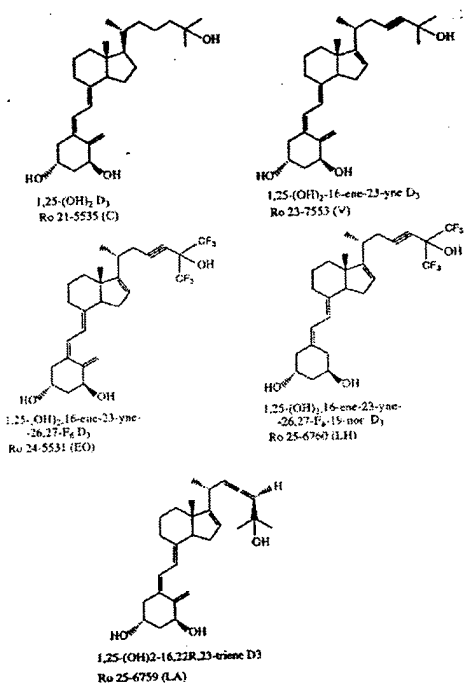
We then set out to determine in what other cell line BRCA1 was modulated. Of the panel of breast and prostate cancer cell lines chosen for study only MCF-7, LNCaP and PC-3 modulated BRCA1. In MCF-7 and LNCaP it s interesting to note that the extent to which the protein was modulated correlated directly with the potency of the analog.

In summary, we have demonstrated that a 1,25 $[OH]_2$-D_3 modulates BRCA1 expression in a panel of breast and prostate cancer cell lines and that the extent to which either 1,25 $[OH]_2$-D_3 or its analogs modulate BRCA1 expression is often proportional to their ability to be clonally inhibited. Reducing VDR content in one cell line (MCF-7) reduces the clonal sensitivity and ability to induce BRCA1. Taken together, these data suggest that BRCA1 protein expression is an important pathway for controlling cell proliferation in breast and prostate cancer cells. Signaling via 1,25 $[OH]_2$-D_3 has been shown to play an important role in maintenance of the normal prostate gland and therefore, we speculate that some aspect of 1,25 $[OH]_2$-D_3 signaling, such as co-activator is lost in prostate and breast cancer cells that selectively reduced transactivation of genes that are critical to controlling cell proliferation.

This work has been submitted for publication. J Moray, A Gombart, S Park, HP Koeffler. Modulation of BRCA1 expression is associated with the antiproliferative action of 1α, 25 dihydroxyvitamin D_3 and two of its analogs in prostate and breast cancer cells.

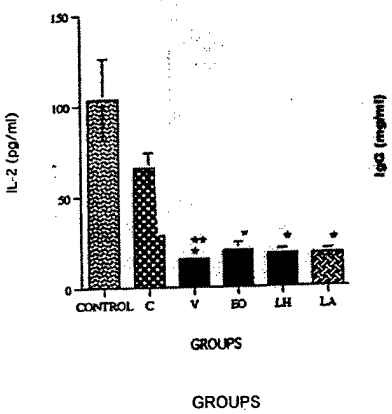
6. Although a plethora of vitamin D_3 analogs have been tested *in vitro* over the past several years, their long term effects *in vivo* remain largely unknown. We administered 1,25 $[OH]_2$-D_3 (code mane compound C) and four unique vitamin D_3 analogs (code names, compounds V, EO, LH, LA) intraperitoneally for 55 weeks to Balb/C mice (Fig.1). Each analog had previously shown to potent *in vitro* activities.

Fig. 1



At one year, these mice had a profound decrease of their levels of IL-2 (Fig. 2). Likewise, several analogs depressed sera IgG concentrations (Fig. 3) (compounds LH an LA), but levels of lymphocyte subsets (CD3, CD4, CD8 and CD19) were not significantly depressed. The percent of committed meloid hematopoietic stem cells were 4-5 fold elevated in the bone marrow of the mice that received analogs LH and V (Fig. 4), but their peripheral blood white and red blood cell counts and platelets were not significantly different in any of the groups.

Weight gains were significantly decreased in all experimental mice compared to control mice. The mice that received the physiologically active 1,25 $[OH]_2$-D_3 had a decrease in bone quantity and quality with a decrease in cross-sectional area and cortical thickness, and a 50% reduction in both stiffness and failure load compared to control group. In contrast, the cohort that received fluorinated analog (compound EO) developed bones with significantly larger cross-sectional area and cortical thickness, as well as stronger mechanical properties compared to the control group. Extensive gross and microscopic analysis of all mice at conclusion of the study were normal including their kidneys (Table 1, 2A, 2B, 3).



GROUPS

Fig. 2 IL-2 levels after one year

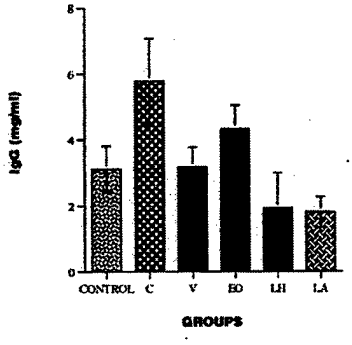


Fig. 3 IgG Serum levels at one year

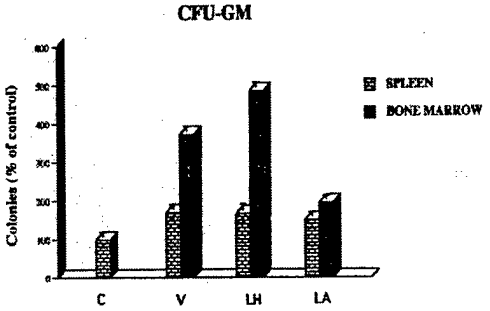


Fig. 4 Numbers of CFU-GM in Murine Bone Marrow and Spleens

Table 1

B- and T-lymphocyte populations in spleens of mice after 55 weeks adminstration of vitamin D_3 analogs

	Control	Comp C	Comp V	Comp EO	Comp LH	Comp LA
CD4 (%)	55.5±3.8	60.0±1.9	58.1±2.1	61.7± .0	61.0±6.8	61.3±3.5
CD8 (%)	31.4±2.9	29.5±0.5	28.3±3.0	27.0±0.9	28.1±3.9	26.1±3.2
CD3 (%)	29.5±3.5	26.1±0.4	27.2±5.0	28.4±2.7	32.0±4.8	33.7±10.1
CD19 (%)	59.8±4.7	65.6±1.5	62.8±3.9	59.3±6.6	54.9±0.5	55.5±10.8
CD4/CD8	1.764	2.036	2.049	2.379	2.172	2.351
CD3/CD19	0.492	0.398	0.433	0.479	0.584	0.607

CD3, T lymphocytes; CD, helper T lymphocytes; CD8, cytotoxic T lymphocytes; CD19, B lymphocytes.

Analysis of T- and B-lymphocyte subsets in the spleen at the end of the study. Data are expressed as the mean ± SD for three mice for each cohort

Table 2A

Hematopoietic blood values of mice after 55 weeks injections of vitamin D_3 compounds

	Control	C	V	EO	LH	LA
WBC (10^3/mm^3)	5.88±1.94	6.26±1.14	4.24±1.76	5.78±0.83	5.90±1.47	4.90±1.34
RBC (10^6/mm^3)	9.72±1.02	11.4±1.46	9.49±0.96	11.1±0.55	9.39±0.66	9.42±0.60
Hgb (g/dl)	15.1±1.50	16.7±1.47	14.6±1.29	16.6±0.97	14.6±0.91	14.4±0.72
Hct (%)	45.7±4.76	50.9±5.39	43.8±4.15	51.1±3.05	43.6±3.05	43.3±2.18
MCV	47.0±0.13	44.9±1.14	47.2±2.43	46.0±0.69	46.5±0.65	46.0±0.86
MCH	15.6±0.11	14.7±0.69	15.4±0.43	14.9±0.15	15.6±0.30	15.3±0.57
MCHC	33.1±0.22	23.8±0.82	33.3±0.25	32.5±0.25	33.6±0.36	33.3±0.68
Plt (10^3/mm^3)	1127±98.9	1405±92.9	1132±253	1090±78.3	1147±193	1188±76.1

The effect of intraperitoneal administration of vitamin D_3 analogs on hematopoietic parameters (Table 2A) and blood chemistries (Table 2B). All the vitamin D3 analogs were administered intraperitoneally three times a week (M,W,F). Data are expressed as the mean ± SD for five mice.

Abbreviations: WBC, white blood cell counts; RBC, red blood cell; Hgb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular henoglobin; MCHC, mean corpuscular hemoglobin concentration; Plt, platelet counts.

Table 2B

Serum chemistry data of mice after 55 weeks of injections of Vitamin D_3 compounds

	Control	C	V	EO	LH	LA
ALP	142.0±9.49	184.4±26.4	150.8±15.1	160.0±11.4	113.6±5.86	123.2±14.1
GOT	151.4±31.5	106.3±16.3	154.8±25.7	160.3±32.6	111.6±24.5	163.8±35.2
GPT	178.2±94.4	ND	113.4±60.1	127.8±68.3	68.8±45.4	181.8±75.2
BUN	19.1±1.35	20.9±3.06	24.1±0.86	25.7±2.41	19.0±1.36	17.4±0.87
Cr	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Glu	131.0±14.3	93.4±11.7	145.0±8.80	94.8±7.95	145.2±23.0	116.6±20.2
Chol	150.6±13.1	146.4±5.41	136.4±11.9	152.6±14.3	140.2±21.9	118.6±9.7
TG	113.0±12.2	97.2±21.7	107.8±18.5	112.6±35.4	93.8±34.2	80.4±13.0
UA	2.44±0.22	4.12±1.00	5.84±1.53	6.12±0.59	5.58±0.72	4.13±0.15
Bil	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
T-pro	5.92±0.49	5.88±0.45	5.32±0.69	5.54±0.38	5.52±0.33	5.16±0.46

Data are expressed as the mean ± SD for five mice. Abbreviations: ALP, alkaline phosphatase; GOT, glutamic oxalacetic transaminase; GPT, glutamic pyruvic transaminase; T-PRO, total protein; BUN, blood urea nitrogen; Cr, creatinine; Bil, bilirubin; UA, uric acid; Glu, glucose; Chol, cholesterol.

Table 3

Vitamin D_3 Analogs: Effects on mechanical and geometric properties of bone

Treatment	C	Control	EO	LA	LH	V
Yield Load (N)	16.4±3.9	30.2±9.9	37.7±11.4	34.4±8.5	31.3±6.6	30.1±8.3
Failure Load (N)	18.6±2.6	37.4±9.4	48.0±14.3	40.3±7.8	39.2±8.8	37.4±8.6
Stiffness (N/mm)	136.2±37.0*	275.3±72.2	394.5±66.0*	321.7±67.6	281.7±58.1	291.4±68.9
CSA (mm^2)	0.917±0.035	1.042±0.112	1.257±0.087+	1.017±0.111	1.103±0.083	0.922±0.078
CT (mm)	0.232±0.007+	0.287±0.020	0.336±0.026*	0.281±0.018	0.309±0.039	0.254±0.013
Ixx (mm^4)	0.300±0.035	0.280±0.054	0.332±0.033	0.268±0.063	0.308±0.039	0.236±0.045
Iyy(mm^4)	0.161±0.017	0.137±0.025	0.120±0.016+	0.139±0.030	0.153±0.016	0.126±0.019
J (mm^4)	0.46±0.051	0.417±0.079	0.532±0.048	0.4-7±0.091	0.460±0.054	0.363±0.063

*p<0.05 vs. Control
+p<0.01vs. Control

Mean results represent the bones studied from 5 mice per cohort after 55 weeks of administration of vitamin D_3 compounds.
CSA, cross-sectional area; CT, cortical thickess; Ixx,Iyy, and J, moments of inertia.

In conclusion, the vitamin D_3 analogs were fairly well tolerated; they are of potential interest as suppressors of immunity after organ transplantation and for autoimmune diseases. Also, they enhance both mechanical and geometric properties of bone, especially those analogs containing fluorines. These analogs have been shown to prevent development and growth of a variety of tumors *in vitro* and *in vivo,* and this study shows that long-term clinical trials are feasible.

7. We have also embarked on a study to investigate the anti-tumor effects of the organic arsenical (Melarsoprol) and All-trans-retinoic acid (ATRA) in breast (MCF-7) human breast cancer cells as well as PC-3 and DU-145 prostate cancer cell lines *in vitro* and *in vivo.* Melarsoprol (Mel-B) is used to treat African trypanosomiasis. Recently Mel-B was shown to be effective in treatment of acute promyelocytic leukemia.

Clonogenic assays showed that these cancer cell lines were sensitive to the inhibitory effect of Mel-B (effective dose that inhibited 50% clonal growth (ED_{50}) 7 x 10^{-9} M (MCF-7), 2 x 10^{-7} M (PC-3), 3 x 10^{-7} M (DU-145) (Fig. 1). Remarkably, the combination of Mel-B and ATRA had an additive antiproliferative activity against all three cell lines. Furthermore, the combination of Mel-B and ATRA induced a high level of apoptotic cells in all three cell lines and levels of bcl-2 protein decreased 7-fold in MCF-7 cells. Treatment of PC-3 and MCF-7 bearing triple immunodeficient mice with Mel-B and ATRA either alone or in combination markedly decreased tumor size and weight of PC-3 and MCF-7 tumors with major side effects. A synergistic anti-cancer effect occurred after treatment of MCF-7 tumors (Fig. 5,6) with a combination of both drugs;

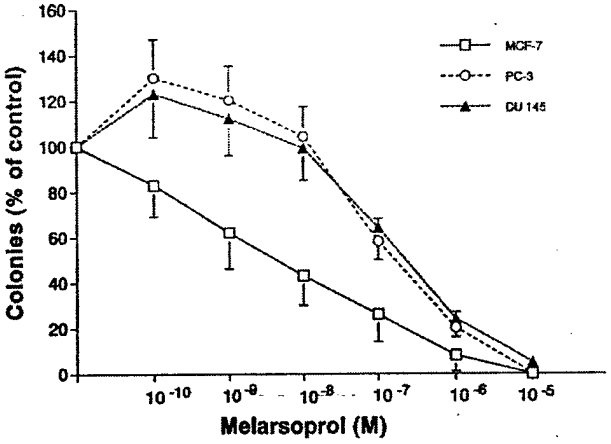


Fig.1 Dose response studies

Fig. 2 Clonal Proliferation

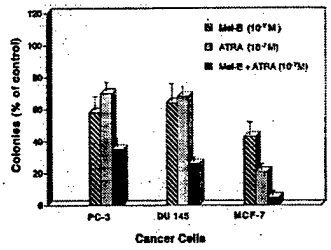
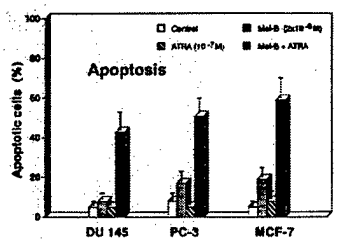


Fig. 3 Apoptosis of cancer cells



sections from mouse tumors treated with Mel-B and ATRA revealed extensive necrosis, apoptosis and fibrosis involving approximately 30-60% of the tumor area. The mechanism of activity remains

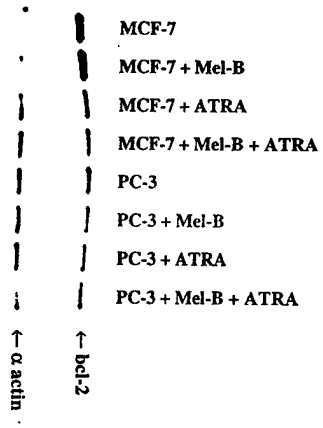


Fig. 4 Western blot analysis

unclear, but it is associated with significant apoptosis.

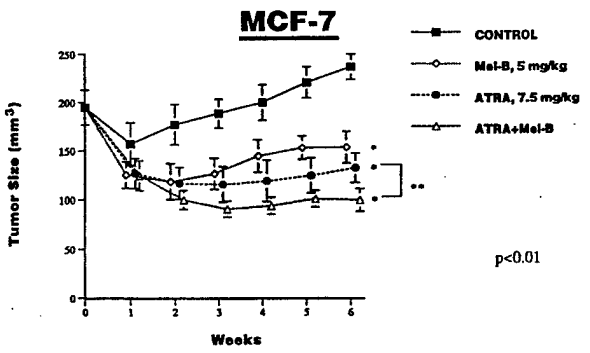


Fig. 5 Tumor size

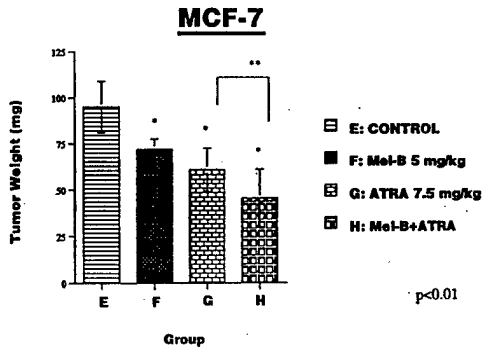


Fig. 6 Tumor Weight

In summary, our results suggest that the combination of Mel-B and ATRA warrant further study and may prove useful as novel therapy for breast or prostate cancer patients with minimal disease burden after definitive curative attempt with surgery and/or radiation. This work was recently submitted for publication.

8. In last years annual report we submitted information regarding the use of the peroxisome proliferator/activated receptor gamma (PPARγ), a member of the nuclear hormone receptor superfamily that is activated through a synthetic ligand, the anti-diabetic drug Troglitazone. Troglitazone, when used in combination with All-*trans*-retinoic acid (a ligand for retinoic acid receptor) decreased proliferation and induced differentiation and apoptosis *in vitro* and *in vivo* in human breast cancer cells. This effect may be realted to the striking down-regulation of bcl-2, because forced over-expression of bcl-2 in MCF-7 cells cultured with Troglitazone and ATRA blocked cell death. This combination significantly inhibits MCF-7 tumor growth in triple immunodeficient mice, causing prominant apoptosis and fibrosis of these tumors without toxic effects on the mice.

This work was recently published. E Elstner, C Muller, K Koshizukz, EA Williamson, D Park, H Asou, P Shintaku, JW Said, D Heber, HP Koeffler. Ligands for peroxisome proliferator-activated receptorgamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. **Proceedings of the National Academy of Sciences of the United States of America.** 95(15):8806-8811,1998 Jul21.

These results prompted the initiation of a clinical trial using Troglitazone and ATRA in histologically confirmed metastatic breast cancer (Stage IV [M1]) either at diagnosis or at recurrence. All patients must have measurable disease that has failed first and second line therapies (hormonal manipulation and/or chemotherapy), either as adjuvant therapy or for metastatic disease. We have just opened this study and currently have 2 patients enrolled.

PROTOCOL SYNOPSIS

Trial of All-trans-Retinoic Acid and Troglitazone in the Treatment of Metastatic Breast Cancer

Objective: To evaluate the maximum tolerated dose (MTD), safety and efficacy of All-trans-Retinoic Acid and Troglitazone in the treatment of patients with Metastatic Breast Cancer.

Population: Patients with Metastatic (Stage IV {M1}) Breast Cancer

Sample Size: 30

Dosage: Patients will receive a daily oral dose of Troglitazone continuously for the duration of the study treatment period. All-trans-retinoic acid will be given in a divided, daily oral dose for two consecutive weeks out of each four week cycle. The first five patients will receive ATRA 50 mg/m^2 and Troglitazone 600 mg. If no grade 3 or 4 major organ or hematologic toxicities are obtained, the next five patients will be treated with ATRA 75 mg/m^2 and Troglitazone 600 mg. If no grade 3 or 4 major organ or hematologic toxicities are obtained, the next five patients will be treated with ATRA 100 mg/m^2 and Troglitazone 600 mg. If two or more patients experience a grade 3 or 4 major organ or hematologic toxicity, the maximum tolerated dose (MTD) will be defined as that dose below which 2 patients experienced a grade 3 or 4 major organ or hematological toxicity. The remaining 15 patients will begin treatment at the MTD.

If two or more patients experience a grade 3 or 4 major organ or hematologic toxicity at the first dose level, the dose of ATRA will be decreased to 25 mg/m^2 and Troglitazone 400 mg. If dose limiting toxicity is observed at this level, the study will be terminated.

Duration: Treatment will be continued to either a six month endpoint or until disease progression. Treatment may be continued beyond the 6 month endpoint as indicated by response.

Safety: Clinical and laboratory adverse reactions will be closely monitored by periodic physical and laboratory examinations.

Efficacy: Clinical responses will be measured by a 50% or greater reduction of the sum of the products of the perpendicular diameters of all measurable bi-dimensional disease, sustained for a minimum of 6 weeks as determined by radiologic examination or nuclear medicine scans.

MOLECULAR BIOLOGY STUDIES

1. Cancer results from a genetic cellular alteration that produces uncontrolled cell growth. Cyclins form complexes with cyclin dependent kinases (CDKs) that positively regulate the cell cycle. These cyclin/CDK complexes are negatively controlled by cyclin dependent kinase inhibitors (CDKIs). In this manner, CDKIs are regarded as potential tumor suppressor genes.

To better understand the role of CDKI's in cancer we have examined a large number and variety of neoplasms for alterations. Deletions and rearrangements were assessed by Southern blotting. Mutations were detected by single strand conformation polymorphism (SSCP) and defined by direct sequencing. We examined 540 cancer DNA samples from 14 different tumors as shown on Table 1.

Table 1. Frequency of alterations of CDKI's found in various neoplasms

Neoplasm (reference)	Total Samples	CDKI alterations
breast (7)	37	1 deletion of $p15^{INK4B}$
ovarian (6)	22	none
endometrial (6)	15	none
testicular (6)	42	none
cervical (5)	41	none
lung** (8)	34	3 deletions, 1 mutation of $p16^{INK4}$
osteosarcoma (9)	52	2 deletions of $p16^{INK4}$
sarcoma (9)	3	1 mutation of $p16^{INK4}$
myeloid leukemias (10)	46	none
lymphomas (11)	74	2 deletions of $p16^{INK4}$, 1 mutation each of $p21^{WAF}$ and $p27^{KIP1}$
myeloma (12)	7	1 deletion of $p16^{INK4}$
ATL *** (13)	44	8 deletions of $p16^{INK4}$
B-ALL (14)	81	8 deletions $p16^{INK4}$
T-ALL (14)	22	14 deletions $p16^{INK4}$

*CDKI, cyclin dependent kinase inhibitor
** Non-small cell
***ATL, adult T-cell leukemia

All samples were examined for alterations of $p21^{WAF1}$, $p27^{KIP1}$, $p15^{INK4B}$, $p16^{INK4}$, $p18^{INK4AC}$, $p19^{INK4D}$. The frequency of alterations varied considerably between different CDKI and different tumors. Alterations of the $p15^{INK4B}$ and $p16^{INK4}$ were by far the most frequent. Of the 56 alterations affecting the $p15^{INK4B}$ and $p16^{INK4}$, all but two were

homozygous or hemizygous gene deletions; the remaining 2 changes were missense mutations within exon one of the $p16^{INK4}$ gene.

The low rate of point mutations affecting $p16^{INK4}$ coding sequences in these studies contrasts with what is seen for other tumor suppresser genes. The p53 an Rb genes, archetypal tumor suppressor genes are frequently inactivated by mutation with loss of the normal gene. In contrast, T-ALL hemizygous deletion of $p16^{INK4}$ is not accompanied by point mutations of the second allele. Could the nearby $p15^{INK4B}$ or $p19^{ALT}$ genes be the target of these alterations? We have not fund point mutations affecting either $p15^{INK4B}$ or $p19^{ALT}$. Perhaps a closely linked gene is the true target $p16^{INK4}$ deletions. Alternately, inactivation of the remaining $p16^{INK4}$ (or $p15^{INK4B}$ or $p19^{ALT}$) gene by higher order alterations such as methylation may provide the underlying mechanism of these deletions.

In the neoplasms that we have studied, we can not completely exclude a role for other closely linked genes. The range of frequencies of abnormalities in various tumor types may reflect some basic property of tumor phenotype. Characterization of these differences may enhance our understanding of tumor progression and suggest approaches for the treatment of cancer.

This work was recently published. CW Miller, HP Koeffler. Cyclin dependent kinase inhibitors in human neoplasms. **Leukemia**, 11S3:370-371, 1997.

2. A specific mutation of the $p16^{INK4A}$ binding domain of the cyclin dependent kinase (CDK4) gene, R24C, has been reported in some cases of melanoma. This mutation prevented binding of the CDK4 inhibitor $p16^{INK4A}$ to CDK4. To understand the relevance of this mutation, we performed polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis in breast carcinomas and in several diverse types of human cancers. No mobility shifts indicating sequence alterations were observed in 273 tumors and 49 cell lines from this array of cancers. In contrast to melanoma, these results suggest that the mutation of the CDK4 gene is very rare in many types of human cancer. To better understand these findings, we randomly mutagenized the CDK4 gene and used the yeast two-hybrid method to screen for CDK4 mutants that lost the ability to bind to $p16^{INK4A}$. Sequenced analysis and in vitro assays showed that most of the mutation that disrupted interactions with p16 also knocked out the activity of CDK4. This result may explain the rareness of CDK4 mutations in tumors. Our results are summarized on Table 1.

Table 1. Mutations in CDK4 that disrupt binding to p16.

Clone	MCD1	MCD2	MCD5	MCD12	MC5	MC6	T2-1	T1-4	T23	T41	WC 1
Mutations	K142R[1]	E56G T277A	N145S[1]	K142R[1]	S189P	D140G[1]	L104P	L120P	L141P[1] L1281	Y180H[2]	NA
Kinase	NA	NA	NA	NA	NA	-	-	-	NA	NA	-

NOTE: [1]Mutations within the ATP binding site of CDK4. [2]Highly conserved positions of CDK4. NA: not analysed; (-), no kinase activity detected.

The results of this work have recently been submitted for publication. N Mori, R Yang, N Kawamata, C Miller, HP Koeffler. Absence of mutations in the $p16^{INK4A}$ binding domain of the CDK4 gene in diverse types of cancer.

3. Recently, a novel tumor suppresser gene, DPD4/SMAD 4, has been implicated in the development of pancreatic cancers. The DPC 4 gene has a sequence similarity to the Mad (mothers against dpp) genes of Drosophila. Since human homologs of the Mad proteins are involved in the intracellular signaling pathway of the TGFβ superfamily (BMP4/Mad-related protein 1 or TGFβ/Mad related protein 2), DPC4 may also exert its function in this pathway. TGFβ has a growth inhibitory effect on most normal human cells. A disruption of its intracellular signaling pathway may lead to abnormal cell growth.

Allelotyping of solid tumors revealed a loss of heterozygosity (LOH) at locus 18q21 in a variety of human tumors. The location of DPC4 at human chromosome 18q21 prompted us to investigate this gene in a large series of primary tumors located outside the gastrointestinal tract which have been associated with loss of heterozygocity (LOH) at this locus. One hundred and thirty primary solid tumor samples (28 breast, 34 non-small cell lung, 20 prostate cancers, 40 osteosarcomas, and 32 cell lines as well as 162 leukemias and lymphomas were analyzed by Southern blotting and PCR-SSCP for deletions and mutations of the DPC 4 gene. In the breast cancer cell line MDA-MB-468, we found that the gene was homozygously deleted (Fig. 1)

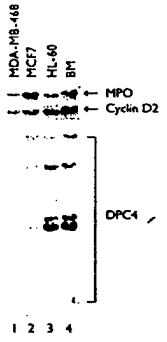


Figure 1. Southern blot of genomic DNA from breast cancer cell lines MDA-MB-468 (lane 1), MCF-7 (lane 2) and promyelocytic leukemia cell line HL-60 (lane 3) as well as normal human bone marrow (lane 4) hybridized to DPC 4 probe. The probe hybridizes to five fragments of EcoRI digested human genomic DNA. The upper part of Fig. 1 demonstrates that all samples contain genomic DNA sequences for myeloperoxidase (MPO) and cyclin D2 (controls). The DPC 4 probe does not hybridise to DNA of breast cancer cell line MDA-MB 468 (lane 1), indicating the deletion of gene.

Figure 1.

This finding has also recently been reported by Schutte et al. The TGFβ responsiveness of MDA-MB-468 transfected with a DPC 4 expression vector may give

interesting insights into the function of this protein. Neither the primary solid tumor samples nor the hematological malignancies had detectable abnormalities (Table 1).

Table 1. Summary of Results

Tumor type	number of samples	DPC4 alterations detected by southern blotting	DPC mutations detected by PCR-SSPC
breast cancer	36	0/28	0/36
breast cancer cell lines	9	1[a]/9	0/9
NSCLC	34	0/34	0/34
osteosarcoma	40	0/40	0/40
prostate cancer	20	0/20	0/20
AML	21	0/20	0/21
AML cell lines	2	0/1	0/2
MDS	24	0/24	0/24
ALL	24	NE	0/24
ALL cell lines	11	NE	0/11
ATL	43	0/40	0/43
ATL cell lines	2	0/2	0/2
NHL	50	0/50	0/50
lymphoma cell lines	8	NE	0/8
primary tumors	292	0/256	0/292
cell lines	32	1/12	0/32
TOTAL	324	1/268	0/324

[a]MD-MB-468 breast cancer cell line (fig. 1); NE, not examined

Our study suggests that alterations of the DPC 4 gene found in pancreatic cancer, are rare in breast, non-small cell lung and prostate cancers, osteosarcomas and hematopoietic malignancies. In most cases of 18q21 LOH, genes other than DPC 4 are involved. These genes may, however, also affect the TGFβ signaling pathway. Interestingly, the Mad-related protein, MADR2, has recently been assigned to chromosome 18q21 and has been found to be altered in 6% of colorectal cancers.

This work was recently published.W Verbeek, K Spirin, Y Hatta, C Miller, N Kawamata, S Takeuchi, M Koike, H Asou, J Simpson and HP Koeffler. DPC4/SMAD 4 in non-pancreatic tumors with frequent LOH 18q21 and in hematological malignancies. **International Journal of Oncology**. 10:257-260, 1997.

4. The $p21^{WAF1}$ (also known as CIPI or SDI1) was the first reported CDKI: it's expression is induced by wild type p53. The $p21^{WAF1}$ is a potent inhibitor of most

cyclin/CDK complexes and also inhibits the ability of the proliferating cell nuclear antigen (PCNA) to activate DNA polymerase-d. Alterations of the cell-cycle can cause cellular transformation. We analyzed 471 primary samples from 15 types of human malignancies and 36 cell lines for structural alterations of the $p21^{WAF1}$ gene (Table 1). No changes were found in the coding regions of $p21^{WAF1}$ gene by polymerase-chain reaction-single-strand conformation polymorphism (PCR-SSCP) analysis. Many of these tumors had a normal p53 gene. Other investigators sowed that $p21^{WAF1}$ knock-out mice did not have an increased incidence of cancer, while p53 knock-out mice did. Taken together, the absence of alterations of $p21^{WAF1}$ in a series of malignancies suggests that $p21^{WAF1}$ may not have a role in either onset or progression of most human malignancies. Furthermore, p53 probably activates additional, critical tumor suppressor genes.

$P21^{WAF1}$ MUTATIONS & HUMAN MALIGNANCIES

TABLE I Analysis for $p21^{WAF1}$ Genetic Alterations in Human Malignancies

Tumor Type	Samples With p53 Mutation (%)	Abnormal SSCP of p21	Exon 2 Heterozygotic Polymorphism/ Total Sample No.	Exon 3 Heterozygotic Polymorphism/ Total Sample No.
AML	0	None	3/17	8/17
MDS	1	None	3/29	3/29
ALL	2	None	11/48	8/48
ATL	10	None	10/45	5/45
Lymphoma	20	None	12/95	9/95
Lung	ND	None	3/36	3/36
Breast	ND	None	3/11	2/11
Renal	9	None	5/23	2/23
Colon	45	None	1/11	0/11
Cervical	8	None	1/12	6/12
Ovarian	ND	None	6/17	4/17
Vulva	24	None	3/17	2/17
Osteosarcoma	27	None	3/18	5/18
Neuroblastoma	1	None	1/29	1/29
Wilms	5	None	4/63	4/63
Cell lines	ND	None	0/36	2/36

Abbreviations: AML, acute myelocytic leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; ATL, adult T-cell leukemia; ND, not done.

This work was recently published. M Shiohara, K Koike, A Komiyama, and HP Koeffler. P $p21^{WAF1}$ mutations and human malignancies. **Leukemia and Lymphoma**, Vol. 26, 35–41, 1997.

5. Differential gene expression is likely to explain the phenotypic difference between normal breast and tumor breast cells. In an attempt to clone genes that are involved in the tumorigenesis of breast cancer, mRNAs from normal breast cell MCF12A and breast carcinoma cell MDA-MB-231 1 were extracted and a PCR-select cDNA subtraction was utilized to construct a subtracted cDNA library. Differential screening of the library isolated five differentially expressed clones: elongation factor-lα, human homologue of murine tumor rejection antigen (Tra1), thymosin β4, SNAP43 and 5A-1. Thymosin ,β4 was highly expressed in nine different breast tumor cell lines but lower expression was observed in the normal breast cells shown by Northern blot. SNAP43, a subunit of the TBP-TAF complex required for transcription of human snRNA genes, was found to have 20 fold higher expression in MDA-MB2-31 than the MCF12A, suggesting

that SNAP43 may be involved in the tumorigenesis of a specific breast cancer. Gene amplification was not observed in this gene by Southern analysis. Expression of both Thymosin β4 and SNAP43 in primary breast tumors will be studied by immunohistochemical staining . Clone 5A-1 did not match the sequences in the GeneBank/EMBL database, indicating that it may be a novel gene. 5A-1 was highly expressed in five breast cancer cell lines compared with the control MCF12A. Further characterization of the gene such as isolation of full length cDNA and protein function assay will be studied.

This work will be submitted for publication. Suppression subtractive hybridization and differential screening identified genes differentially expressed in breast carcinoma cell lines.

6. The role of the p53 tumor suppressor gene in gynecological cancers and heritable cancer syndromes was reviewed. Akashi M, Koeffler HP. Li-Fraumeni Syndrome and the Role of the p53 Tumor Suppressor gene in Cancer Susceptibility. Symposium on Familial Cancer Syndromes, published in **Clinical Obstetrics and Gynecology**, Pitkin RM, Scott JR, (eds.), Lippincott-Raven, 41(1)172-199, 1998.

CONCLUSION

We have identified several novel, potent Vitamin D analogs that decrease proliferation and induce differentiation and apoptosis of breast cancer. We have successfully altered several of these analogs to diminish the hypercalcemic effects while maintaining a good therapeutic index. We examined a new class of Vitamin D analogs that have a novel 5,6-trans motif; the most potent of these analogs affect cell cycle regulatory mechanisms, upregulate expression of cyclin dependent kinase (CDK) inhibitors, inhibit tolomerase activity, and induce expression of a novel candidate tumor suppresser gene. Our studies indicate that Vitamin D analogs upregulate BRCA1 expression, which in turn regulates DNA replication and effects cellular proliferation and apoptosis in breast and prostate cancer cells. We have found that ATRA used in conjunction with EB 1089 has an additive effect against breast cancer *in vitro* and *in vivo* with minimal attendant toxicity. Our extensive analyses of the effects of long term administration of Vitamin D analogs in a murine model, indicate that these analogs are well tolerated and may hold promise as immune modulators after organ transplantation and in autoimmune diseases. The organic arsenical, Melarsoprol, has shown significant activity in breast and prostate cancer and warrants further study as a novel treatment for these tumors. We are enthusiastic about the recent initiation of our clinical trial using Troglitazone and ATRA in metastatic breast cancer. Because these drugs were already approved by the FDA, this novel approach went from "bench-top" to clinic in less than 12 months. We continue to identify molecular alterations associated with breast cancer.

STATEMENT OF WORK:

Specific Aim 1: This year we have continued our study of over 25 novel Vitamin D_3 analogs either alone or in combination with other agents and examined their ability to induce the inhibition of clonal proliferation, apoptosis and differentiation in breast cancer cells.

Specific Aim 2: We have studied Vitamin D analogs in our triple immunodeficient murine models as reported in this submission.

Specific Aim 3: We have begun cloning new genes related to the breast, whose expression is altered in breast cancer. We have also recloned SNAP 43 and thymosin β-4 which are overexpressed in breast cancer.

Specific Aim 4: We continue to examine the effects of TALL-104 cells to inhibit the growth of breast cancer in vitro and in vivo through our collaboration with Dr. Daniela Sanatoli. Dr. Sanatoli has recently started a phase I trial of TALL cells in humans.

REFERENCES

Please see body of grant; references cited after each section.

APPENDIX

The following were submitted for publication at the time of the 1997 annual review and have since been published.

1. M Koike, E Elstner, MJ Campbell, H Asou, M Uskokovic, N Tsuruoka and HP Koeffler. 19-nor-hexafluoride analogue of vitamin D_3: a Novel class of potent inhibitors of proliferation of human breast cell lines. **Cancer Research** (57), 4545-4550, 1997

2. E Elstner, C Muller, K Koshizukz, EA Williamson, D Park, H Asou, P Shintaku, JW Said, D Heber, HP Koeffler. Ligands for peroxisome proliferator-activated receptorgamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. **Proceedings of the National Academy of Sciences of the United States of America**. 95(15):8806-8811,1998 Jul21.

3. K Koshizuka, M Koike, T Kubota, J Said, L Binderup, HP Koeffler. Novel vitamin D_3 analog (CB1093) when combined with paclitaxel and cisplatin inhibit growth of MCF-7 human breast cancer cells *in vivo*. **International Journal of Oncology**, In press, 1998.

LIST OF PERSONNEL SUPPORTED BY THIS GRANT

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