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ABSTRACTS OF THE SECOND INTERNATIONAL SYMPOSIUM ON VITAMIN D ANALOGS IN CANCER PREVENTION AND THERAPY

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1
ENVIRONMENTAL SENSING CAPACITY BY VDR AND RELATED NUCLEAR RECEPTORS

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VDR and related nuclear receptors sense a range of micro- and macronutrients to regulate genes that govern proliferation, differentiation and xenobiotic metabolism. We investigated these actions in the bladder carcinoma cell lines RT4, RT112, HT1376 and EJ28, by examining antiproliferative responses to a range of nuclear receptor ligands. The high-grade cell lines (HT1376 and EJ28) proliferated at the fastest rate and invaded through Matrigel. The cells also demonstrated a spectrum of responsiveness to the ligands including $1\alpha25(OH)_2D_3$ and 9 *cis* retinoic acid (VDR and RAR/RXR), the bile acids chenodeoxycholic acid and lithocholic acid (VDR and FXR), 27-hydroxycholesterol and (LXR α, β) and the omega 6 fatty acid 5,8,11,14-eicosatetraenoic acid (PPAR γ). RT-4 was inhibited by 1 $\alpha25(OH)_2D_3$, 5,8,11,14-eicosatetraenoic acid and chenodeoycholic acid with ED_{50} of 80 nM, 6 μM and 11 μM, respectively, whereas EJ28 was insensitive to these treatments.

We comprehensively profiled, by Q-RT-PCR, expression levels of these nuclear receptors, co-repressors (*NCoR1, NCoR2/SMRT, TRIP15/Alien*), histone deacytelases (*HDAC1, HDAC2, HDAC4, HDAC5, HDAC7, hSIRT1*), histone methyltyransferases (*SUV39H1, SUV39H2*) and histone acetyltransferases (*P300, CBP, PCAF*) in the cell lines and a cohort of primary bladder tumors and normal bladder. These data suggest that epigenetic mechanisms corrupt environmental sensing in bladder cancer, with cellular responsiveness to ligand reflecting an altered ratio of receptor to co-repressor levels. Thus EJ28 displayed reduced *PPARγ* and *FXR* levels (0.03 and 0.0007 fold reduction) compared to RT4, with elevated levels of NCoR1. The cellular sensitivity in these cells towards ligands was restored by combination treatment with SAHA (an HDAC inhibitor), particularly with PPAR, LXR and FXR ligands.

These studies demonstrate that dietary components are sensed locally by tissues and, in malignancy, this capacity is corrupted by an epigenetic mechanism. Potentially the actions of dietary sensing nuclear receptors are chemoprevention/chemotherapy targets.

2
DESIGN AND SYNTHESIS OF 19-NORVITAMIN D_3 ANALOGS AS PROSTATE CANCER CHEMO-PREVENTION AGENTS: LESS CALCEMIC AND PROHORMONE-LIKE ACTIVITY

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1α,25-dihydroxyvitamin D_3 (**1**) is known to inhibit the proliferation and invasiveness of prostate cancer cells. However, **1** can cause hypercalcemia; therefore, its analogs, that are less calcemic but exhibit potent antiproliferative activity, would be attractive as therapeutic agents. It has been shown that prostate cells possess 1α-OHase, and can convert 25-hydroxyvitamin D_3 to **1** intracellularly to inhibit their proliferation. It is also known that the 19-demethylenated analog of **1**, 1α,25-dihydroxy-19-norvitamin D_3 (**2**) and 1α,25-dihydroxy-19-norvitamin D_2 (**3**) possess similar pro-differentiation and antiproliferative activities as **1**, and are less calcemic. Since 25-hydroxyvitamin D_3 and **2** are known to be less calcemic than **1** when administered systemically, we investigated whether 25-hydroxy-19-norvitamin D_3 (**4**) and its 2-substituted analogs exert antiproliferative activity toward prostate cells which possess 1α-OHase activity, and therefore could be used as chemopreventive agents without causing hypercalcemic side-effects. Synthesis of **4** was achieved through our new coupling method utilizing Julia-type olefination between the C5 and C6 positions. Using an immortalized normal prostate cell line, PZ-HPV-7, which has high 1α-OHase activity, we demonstrated that **4** had potent antiproliferative activity, although the activity was slightly less than that of **1**. Furthermore, we demonstrated that **4** can be hydroxylated at the 1α position to form **2** by 1α-OHase in a cell-free system, suggesting that **4** itself was probably hydroxylated to **2** in PZ-HPV-7 cells, which was then responsible for the antiproliferative activity observed in these prostate cells. Our data suggest that compound **4** and its 2-substituted analogs may be useful as chemopreventive agents for prostate cancer.

3
VITAMIN D INHIBITS THE EXPRESSIONS OF MATRIX METALLOPROTEINASES (MMPS) IN SQUAMOUS CELL CARCINOMA (SCC) CELLS AND NON-TUMORIGENIC KERATINOCYTES

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Matrix metalloproteinases (MMPs) are responsible for the degradation of most extracellular matrix proteins during normal tissue turnover, and under pathological conditions. In particular, gelatinases, MMP-2 and MMP-9 have been implicated in cancer progression and metastasis in animal models and patients, including those with head and neck carcinomas. It is probable that the level of MMPs in the tumor microenvironment is the decisive factor, irrespectively of whether they are expressed by the tumor cells or by surrounding uninvolved cells. We examined the notion that the *in vivo* anticancer activity of vitamin D may be associated with the regulation of MMP activity in the tumor milieu. The head and neck SCC cell line, UM-SCC-14C, and HaCaT cells were employed to represent tumor and surrounding uninvolved stromal cells, respectively. MMP activity was identified and quantified in culture media by gelatin zymography and MMP mRNA levels were quantified by real-time quantitative PCR. The SCC cells constitutively expressed and secreted MMP-2, this expression increasing upon treatment with the tumor promoter, PMA. Incubation with calcitriol markedly inhibited both constitutive and stimulated MMP-2 expression. HaCaT cells express low levels of MMP-9, that increase upon exposure to ionizing radiation (the most common therapeutic modality of SCC) or to the immune cytokine TNF (present during immune attack and partaking in the inflammatory response to irradiation). MMP-9 expression was markedly inhibited by calcitriol. The EGF receptor (EGFR) tyrosine kinase in HaCaT cells is activated by autocrine ligands and is further stimulated following exposure to TNF. Addition of the EGFR tyrosine kinase inhibitor, AG1478, inhibited the induction of MMP-9 mRNA by TNF. Furthermore, exposure of HaCaT cells to EGF induced MMP-9 and this activity was also inhibited by calcitriol, indicating that the hormone acts downstream of the transactivation of EGFR by TNF. Our results suggest that vitamin D can blunt the pro-metastatic potential of MMPs during tumor growth, antitumor action of the host immune system and radiotherapy.

4

VITAMIN D ANALOGS AS SELECTIVE VDR MODULATORS FOR CANCER

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Vitamin D, its natural metabolites and especially the hormone 1,25-$(OH)_2D_3$ meet several proteins during their life cycle: chylomicrons, DBP, intracellular vitamin D binding proteins, a nuclear receptor, a (putative) membrane receptor and vitamin D metabolizing enzymes, especially belonging to the CYP family. These proteins determine the metabolic activation and inactivation, extra- and intracellular transport and, finally, the access to the binding pocket of the vitamin D receptor (VDR). Several thousands of analogs of vitamin D have been synthesized with modifications of the fexible side chain, the A ring and the more rigid central CD-region of 1,25-$(OH)_2D_3$. Among the long list of analogs, several compounds with superagonistic activity on a wide variety of end-points (cell proliferation/differentiation and gene expression) have been identified (up to ≥100-fold more potent than 1,25-$(OH)_2D_3$). Some of these have low calcemic effects *in vivo*. Tissue and gene selectivity have been demonstrated for a number of compounds with either (1) bone anabolic properties; or (2) enhanced antiproliferative; or (3) immunomodulatory effects in *in vivo* animal models. The molecular basis for the superagonist's low calcium activity is probably a mixture of modified bioavailability, metabolism and gene regulation at a postreceptor level. The early, mid and late events responsible for vitamin D's action on cell proliferation are only partially understood. The involvement of cyclins, cyclin-dependent kinase inhibitors and retinoblastoma pocket proteins is repeatedly mentioned. At a late stage, E2F transcription factors (TF) are certainly involved as they translate 1,25-$(OH)_2D_3$'s effect to down-regulate a very large number of cell cycle-regulating genes and genes interfering with DNA replication, DNA repair, chromosome modifications and mitosis. Better insights into the molecular mechanism of action of 1,25-$(OH)_2D_3$ and its analogs will certainly contribute to the development of a second generation of selective vitamin D receptor modulators with possible applications as chemopreventive agents or as chemotherapeutic drugs when associated with classcal anticancer drugs.

5

1α,25-DIHYDROXYVITAMIN D_3 AND INTERLEUKIN-6: POSSIBLE INTERSECTIONS OF SIGNALING PATHWAYS ON CONTROL OF HUMAN COLON CARCINOMA CELL GROWTH

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The usefulness of the steroid hormone 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) as an anti-mitogen may be curtailed by its potential to induce the release of

interleukin-6 (IL-6) from tumor-infiltrating lymphocytes and possibly also from carcinoma cells. IL-6 has also been implicated in autocrine/paracrine growth stimulation of various solid tumors. However, with respect to colorectal cancer, no relationship between IL-6 expression in the cancer lesions and tumor growth has ever been established.

Using RT-PCR, we studied the mRNA expression of IL-6 and of the IL-6 receptor complex in human colon cancers of different grades. In the human colon carcinoma primary cell clones COGA-1A and COGA-13, and in the Caco-2 cell line, we evaluated 1,25-$(OH)_2D_3$-induced IL-6 production, as well as cellular growth in response to rhIL-6 (0.01-100 ng/ml).

IL-6 expression in human colorectal cancer tissue rises with tumor progression and is thus abundant in moderately- to poorly-differentiated carcinomas. 1,25-$(OH)_2D_3$ (10^{-10}-10^{-7} M) stimulated the release of IL-6 only up to 2-fold in COGA-1A cultures, and had no effect on COGA-13 and Caco-2 cells. Although both the cell clones studied expressed the IL-6R complex, only in the differentiated Caco-2 cells did exogenous rhIL-6 induce a significant increase in cell proliferation (at and above 1.0 ng/ml). 1,25-$(OH)_2D_3$ (10^{-8} M) significantly counteracted IL-6-induced proliferation and stimulated cellular differentiation regardless of the proliferative effect of the cytokine. Inhibition of the MAPK and PI3K pathways strongly suggest involvement of p38 in IL-6-mediated proliferation, but indicate no interaction with 1,25-$(OH)_2D_3$.

IL-6-mediated proliferation was observed selectively in differentiated cancer cells, a cell population which is also sensitive to 1,25-$(OH)_2D_3$ action. Thus, we conclude from our results that the anti-mitogenic effect of the steroid hormone can still override the proliferative action of IL-6. On the other hand, 1,25-$(OH)_2D_3$ effected only week IL-6 release from the colon carcinoma cells derived from rather undifferentiated cancer lesions in our study. These results underscore the relevance of the steroid hormone in colorectal cancer prevention.

6
FUNCTIONAL VDREs OF THE HUMAN GENES CYP24, PPAR, CYCLIN C AND P21 IN CHROMATIN CONTEXT

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The nuclear vitamin D receptor (VDR) controls gene expression by binding discrete DNA sequences in promoter regions, referred to as response elements (REs). Although the sequences of these elements are well characterized, the mechanistic details of REs *in vivo* are still largely unknown. To resolve this issue, a variety of known and new VDREs in MCF-7 human breast cancer cells were examined by the chromatin immunoprecipitation (ChIP) method. First, four different breast and prostate cancer cell lines were screened by quantitative real-time PCR analysis to verify that the human genes CYP24, PPARα, cyclin C and p21 were primary 1α,25$(OH)_2D_3$ responding genes. This was followed by ChIP assays using antibodies against acetylated histone H4 (to assess global chromatin status) and various other components of VDR-dependent gene activation. These included VDR, RXRα and coregulator molecules, such as p160 coactivator family members (SRC-1, TIF2 and RAC3), histone acetyltransferases (HATs), histone deacetylases (HDACs) and corepressors (NCoR and Alien). For the three examined genes, up to four vitamin D-responsive regions (VDRRs) were found, which show elevated, time-dependent, histone H4 acetylation upon the addition of 1α,25$(OH)_2D_3$. The level of increase in acetylated histone H4 also depends on the level of transcriptional activity before the addition of ligand. Examination of the VDRR sequences found motifs resembling known VDREs. Additionally, many of the VDRRs examined are associated with VDR in the absence of ligand and these also associate with corepressor molecules and HDACs. After ligand treatment, the VDR exchanges corepressors for coactivators and HATs. This is consistent with the observed increases in histone H4 acetylation status. However, the particular coregulator proteins found at any particular VDRR appeared to be different. In conclusion, we found that VDR-RXR heterodimers associate with coregulators in an idiosyncratic fashion and this suggests that complexes formed at VDRRs are heterogeneous in their composition and are dependent on coregulator availability, chromatin context and RE sub-type. These results will have an impact on the development of therapeutic regimes for diseases, such as cancer, that use 1α,25$(OH)_2D_3$ and its analogs.

7
AUTOCRINE REGULATION OF VITAMIN D METABOLISM IN PROSTATE CANCER PREVENTION AND TREATMENT

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Although 1α,25-dihydroxyvitamin D [1α,25-(OH)2D] promotes the differentiation and inhibits the proliferation, invasiveness and metastasis of prostate cells, this hormone is not suitable as a chemopreventive agent because its administration can cause hypercalcemia. The expression of vitamin D-25-hydroxylase (25-OHase), 25-hydroxyvitamin D-1α-hydroxylase (1α-OHase) and 25-hydroxyvitamin D-24R-hydroxylase (24R-OHase) in prostate cells demonstrates that these cells have the ability to synthesize

1α,25-$(OH)_2$D from vitamin D_3 and that 1α,25-$(OH)_2$D can be degraded locally without acting on bone and intestine to cause hypercalcemic side-effects. Furthermore, the lack of regulation by PTH and calcium suggests that the administration of vitamin D or 25(OH)D will probably cause an increased synthesis of 1α,25-$(OH)_2$D within prostate cells as a result of increased serum and tissue levels of 25(OH)D. It is well established that epidermal growth factor (EGF) is a primary regulator of prostate cell proliferation. The demonstration that 1α-OHase was differentially expressed between noncancerous (PZ-HPV-7) and cancerous (LNCaP) prostate cells, and that the enzyme was up-regulated by EGF in PZ-HPV-7 cells, but not in LNCaP cells, suggests that a defect in the up-regulation of 1α-OHase activity and/or expression by EGF in cancer cells may lead to insufficient synthesis of 1α,25-$(OH)_2D_3$. This, in turn, may contribute to uncontrollable growth of prostate cancer cells. Thus, the data suggest that dysregulation of 1α-OHase may play an important role in the natural history of prostate cancer, and that vitamin D or 25(OH)D may be useful as chemopreventive agents for protstate cancer because these two compounds have longer half-lives and are less toxic than those of 1α,25-$(OH)_2D_3$.

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VITAMIN D STATUS AND BREAST CANCER RISK

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Vitamin D appears to exert a protective effect against breast cancer, which may be mediated *via* localized activation of vitamin D. The balance between local generation of 1,25-D from circulating 25-hydroxyvitamin D (25OH-D) and its catabolism *via* 24-hydroxylation within breast tissue is likely to be important in determining the responsiveness of cells. In addition, genetic differences in the vitamin D receptor (VDR) could contribute to an individual's response to vitamin D compounds. In this regard, a number of polymorphisms in the VDR gene have been identified, resulting in differing genotypes that may alter susceptibility to certain cancers including carcinoma of the breast.

To address the association of vitamin D status and VDR genotype with breast cancer risk, we determined plasma 25OH-D concentrations and *Bsm* I VDR genotype in 179 breast cancer patients and matched controls. Analysis showed that the odds ratio (OR) for breast cancer risk for women with insufficient levels of 25OH-D (<50nM) was 3.54 (CI 1.89-6.61 $p<0.001$) compared to those with levels >50nM. The risk for breast cancer was further increased for women with both insufficient 25OH-D levels and the *bb* VDR genotype (OR 6.82, CI 2.57-17.1, $p<0.001$).

To assess the potential for 1,25-D activation and catabolism in normal and malignant breast tissue, quantitative RT-PCR analysis of mRNA expression was carried out for the 1a- and 24-hydroxylases in 41 paired tumor and normal breast tissues. The results showed significantly higher expression of 24 hydroxylase (mean 4-fold) and VDR (mean 7-fold) in tumors compared to normal tissue. Surprisingly, the mRNA levels of 1a-hydroxylase were also increased in malignant tissue (mean 27-fold).

Taken together, these results suggest that breast cancer development may be associated not only with genetic variation and expression of VDR, but also with local generation of 1,25-D determined by both 25OH-D availability and relative expression of the vitamin D hydroxylases.

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MODULATION OF MAPK ERK1 AND ERK2 INDUCED BY 1α-25-DIHYDROXYVITAMIN D_3 IN VITAMIN D-RECEPTOR-POSITIVE AND -NEGATIVE BREAST CANCER CELL LINES

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1α-25-Dihydroxyvitamin D_3 (Calcitriol), the biologically active metabolite of vitamin D, is known to regulate calcium and phosphate levels in bone metabolism. It is also known to regulate proliferation and differentiation in carcinoma cells. These effects are mediated by the vitamin D receptor (VDR). The antiproliferative effects of calcitriol are believed to be mediated by the nuclear pathway *via* binding of the activated receptor to vitamin D-response elements and induction of vitamin D-responsive genes, or by the rapid response pathway through the MAP-kinase cascade. The interaction of calcitriol and the MAP-kinase cascade was evaluated on VDR-positive MCF-7 cells and VDR-negative MDA-MB-231 breast cancer cells. The cells were incubated with calcitriol solution at 10^{-7} M and 10^{-9} M and with ethanol as controls for up to 48 hours. The effects of calcitriol were measured by semi-quantitative Western blotting. Calcitriol stimulated the MAP-kinases ERK1 and ERK2. A biphasic activation was found for calcitriol in VDR-positive cells at an incubation of 5 to 20 minutes and from 2 to 24 hours. However, an early activation of ERK1 and ERK2 was also demonstrated in VDR-negative cells. It was shown that the MAPK-cascade was also induced by ethanol after 5 to 20 minutes. Calcitriol-induced activation

was found on incubation from 2 to 24 hours. In summary, it seems that the early induction of the MAPK-cascade was independent of the VDR. A calcitriol-induced MAPK activation was shown after 4 hours. This may be caused by an activation of the nuclear receptor pathway.

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GENOMIC ANALYSIS TO UNRAVEL THE MOLECULAR MECHANISMS OF VITAMIN D DURING ONCOGENESIS

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We have initiated studies where we employ genomic approaches to unveil the molecular actions of vitamin D during oncogenesis. In our studies, we made use of four independently-derived vitamin D-resistant MCF-7 cell lines and a variety of genomics platforms, including expression arrays, 10K SNP chips and high-resolution array CGH. The four cell lines with acquired resistance to 1,25-dihyroxyvitamin D_3 were developed in independent laboratories and are labelled MCF-7/VD^R, MCF-7 DR, DRA and D_3RE. We and others have shown that studying drug resistance may point directly towards pivotal genes underlying cancer progression. For expression and CGH analysis, RNA and DNA were isolated from all four resistant cell lines and the respective parental cell line. To monitor gene expression alterations, the RNA was hybridized to human oligonucleotide (60-mer) arrays with 30,000 genes. To monitor chromosomal copy number changes as a consequence of the acquired resistance, high resolution oligo array CGH was performed. In addition to measuring the chromosome copy number changes, DNA was used for genome-wide SNP analysis. For this purpose, the Affymetrix 10K SNP chip was used initially for the MCF-7/VD^R cell line, and will be confirmed in the other three cell lines. Array CGH experiments pointed to areas in chromosomes 1q and 20q as possible candidates for regions of DNA copy number changes. The expression analysis revealed a set of genes that were consistently altered in all four vitamin D-resistant cell lines when compared to the respective non-resistant parental cell line. Some of these genes will be reported and discussed during the meeting. For example, preliminary results identified CYP24 (located at chromosome 20q) as a candidate gene for conferring vitamin D resistance. The findings from array CGH and the expression arrays are in line with our previous findings that identified CYP24 as a candidate oncogene in breast cancer. Knock-in and knock-out experiments are underway to confirm this hypothesis, in addition to the production of a series of new vitamin D-resistant cell lines for breast and colon.

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REGULATION OF VITAMIN D HYDROXYLASE EXPRESSION FOR PREVENTION OF COLON AND PROSTATE TUMOR PROGRESSION

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Both colorectal and prostate tumor incidence show a positive correlation with low serum levels of 25-OH-D_3, but not of the active vitamin D hormone, 1,25-$(OH)_2$-D_3. However, it is 1,25-$(OH)_2$-D_3 that has antimitotic, prodifferentiating and proapoptotic activity *in vitro*, though only at supraphysiological nanomolar levels, which would cause hypercalcemia if used in patient therapy. Human colon and prostate cell lines have a high capacity for conversion of 25-OH-D_3 to 1,25-$(OH)_2$-D_3 or to 24,25-$(OH)_2$-D_3. While our data, derived from human colon tumor tissue, suggest up-regulated colonic synthesis of 1,25-$(OH)_2$-D_3 early during tumorigenesis as an endogenous defense against tumor progression, it became evident that in higher grade tumors 24-hydroxylation, *i.e.* catabolism of 1,25-$(OH)_2$-D_3, potentially synthesized in the colon, is prevalent. In contrast to the colon, this enhanced catabolism apparently exists very early in the prostate, while CYP27B1 expression is already reduced during the transition from normal to hyperplastic tissue. In prostate cancer cells, high levels of CYP24 are expressed. Therefore, in order to maintain an adequate concentration and activity of extrarenal tissue-localized 1,25-$(OH)_2$-D_3, approaches to increase CYP27B1 and to decrease CYP24 expression were explored. Several dietary substances, such as calcium, phytoestrogens and folate, showed promising results. The mechanisms of action are discussed in cell lines, in a mouse model and in a human pilot study.

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FUNCTIONALITY OF CYP27B1 SPLICE VARIANTS METABOLISM OF CALCIDIOL AND EFFECT OF CALCITRIOL IN HUMAN GLIOBLASTOMA MULTIFORME

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A better understanding of vitamin D_3 metabolism is required to evaluate its potential therapeutic value for cancers. We found amplification of the gene encoding the P450 cytochrome 25-hydroxyvitamin D_3-1alpha-hydroxylase (CYP27B1) in 16% of human *glioblastoma multiforme* (GBM). At least 16 alternatively spliced transcripts of CYP27B1 are expressed in a variety of normal tissues, including kidney, brain and skin, and in malignant tissues, including GBM, melanoma, basal cell carcinoma and cervix carcinoma cells. Western blot analysis revealed a different expression pattern of CYP27B1 variants in GBM and in normal tissues. GBM tumor biopsies with gene amplification of CYP27B1 showed increased CYP27B1 mRNA expression in comparison to normal brain. GBM cell lines showed an elevated mRNA expression of GBM, not only in comparison to normal brain, but also in comparison to the corresponding tumor. Western blot analysis confirmed the elevated expression in GBM cell cultures. High-performance thin-layer chromatography showed preliminary evidence for enzymatic activity of endogenous CYP27B1 in all the GBM cell cultures tested. Measurements of the production of calcitriol in transfected COS-1 cells provided no evidence for the functionality of the splice variants. By adding calcitriol to GBM cell cultures, we found a proliferative effect in some cell lines depending on the dose of calcitriol. The administration of calcitriol led to an elevated expression of CYP27B1 and 1,25-dihydroxyvitamin D_3-24-hydroxylase (CYP24), but left the expression of the vitamin D_3 receptor unaltered. Our findings show that GBM cell lines metabolize calcidiol. We provide evidence for proliferative effects mediated by calcitriol.

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VITAMIN D METABOLISM IN BREAST CANCER AND OVARIAN CANCER

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1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is the biologically active metabolite of vitamin D and has been shown to regulate the growth of various cell types. There are two principal enzymes involved in the formation of circulating $1,25(OH)_2D_3$ from vitamin D; vitamin D 25-hydroxylase (25-OHase) and 1α-hydroxylase (1α-OHase). Recently, extrarenal activity of 1α-OHase has been reported in various cell types. The aim of this study was to analyze the expression of vitamin D receptor (VDR) and the main enzymes involved in the synthesis and metabolism of calcitriol in gynecological malignancies and corresponding normal tissue. The expressions of VDR, 25-OHase, 1α-OHase and 24-OHase were analyzed in breast carcinomas (BC), ovarian cancer (OC), cervical carcinomas (CC) and normal corresponding tissues, using real-time PCR and specific hybridization probes, as well as using immuno- histochemistry. RNA for VDR, 1α-OHase, 24-OHase and 25-OHase was up-regulated in breast, cervical and ovarian carcinomas as compared to normal tissues. VDR immunoreactivity was increased in breast and ovarian cancer and in cervix carcinomas as compared to corresponding normal tissues. Our findings indicate that cervical carcinomas, breast cancer and ovarian cancer may be considered as potential targets for prevention or therapy with the new vitamin D analogs that exert little or no calcemic side-effects, or by pharmacological modulation of $1,25(OH)_2D_3$ synthesis and metabolism in these tumor cells.

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EXPRESSION OF VITAMIN D-METABOLIZING ENZYMES IN THE HUMAN BREAST CANCER CELL LINE MCF-7

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The three main enzymes of vitamin D metabolism, vitamin D_3-25-hydroxylase (25-OHase), 25-hydroxyvitamin D_3-1α-hydroxylase (1α-OHase) and 25-hydroxyvitamin D_3-24-hydroxylase (24-OHase), have been described in malignant breast tissue. This *in vitro* study aimed at obtaining more information about the regulation of these enzymes in the human breast cancer cell line MCF-7. Cells were cultured in cell culture flasks, then sown in Petri dishes and stimulated with the vitamin D metabolites vitamin D_3 (calciferol), 25-calciferol and calcitriol for 24, 48 and 72 hours, in physiological (10^{-9} M) and supraphysiological (10^{-7} M) concentrations. Before stimulation, the abundance of the vitamin D-receptor was proven by conventional PCR. After stimulation, RNA was extracted and the expression of the enzymes was assessed by real-time PCR. The results were normalized to the "house-keeping gene" HPRT as an internal standard for each sample. The expression of 25-OHase was not influenced by the substrates. The expression of 1α-OHase was slightly induced after stimulation with 10^{-7} M calcitriol

after 72 hours. However, after stimulation with supra-physiological concentrations of calcitriol, the expression of 24-OHase was increased 144-fold after 24 hours, 32-fold after 48 hours and still 10-fold after 72 hours. These results were not evident with physiological calcitriol doses. Our results demonstrate that MCF-7 cells not only possess 24-OHase, but also are able to regulate the expression of this calcitriol-inactivating enzyme. This might be a mechanism for these tumor cells to protect themselves against the antiproliferative and apoptosis- inducing effects of calcitriol.

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ENHANCED VITAMIN D RECEPTOR – COACTIVATOR INTERACTION UNDERLIES THE SUPERAGONISTIC ACTION OF 14-EPI-VITAMIN D ANALOGS

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Two 14-epi-analogs of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], 19-nor-14-epi-23-yne-1,25-$(OH)_2D_3$ (TX522) and 19-nor-14,20-bisepi-23-yne-1,25-$(OH)_2D_3$ (TX527), show enhanced antiproliferative (at least 10-fold) and markedly lower calcemic effects both *in vitro* and *in vivo*, when compared with 1,25-$(OH)_2D_3$. In this study, their superagonistic action was evaluated at the level of the interaction between the vitamin D receptor (VDR) and coactivators. Two-hybrid assays with VP16-fused VDR and GAL4-DNA-Binding-Domain-fused SRC-1, TIF2 or DRIP205 showed the 14-epi-analogs to be more potent inducers of VDR – coactivator interactions than 1,25-$(OH)_2D_3$ (up to 16- and 20-fold stronger induction of VDR-SRC-1 interaction for TX522 and TX527 at 10^{-10} M). Comparable assays with a selective inhibitor of the 1,25-$(OH)_2D_3$-metabolizing enzyme CYP24 showed that the enhanced potency of these analogs in establishing VDR – coactivator interactions can only partially be accounted for by their increased resistance to metabolic degradation. Crystallization of TX522 complexed to the Ligand Binding Domain (LBD) of the human VDR demonstrated that the epi-conformation of C14 caused the CD ring of the ligand to shift by 0.5A, thereby bringing the C12 atom into closer contact with Val300. Moreover, the C22 of TX522 made an additional contact with the CD1 atom of Ile268 due to the rigidity of the triple bond-containing side chain. The position and conformation of the activation helix H12 of VDR was found to be strictly maintained. In conclusion, this study provides deeper insight into the docking of TX522 in the ligand-binding pocket of the LBD and shows that stronger VDR – coactivator interactions underlie the superagonistic activity of these two analogs.

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NOVEL 2,2-FUNCTIONALIZED ANALOGS OF 1α,25-DIHYDROXYVITAMIN D_3: SYNTHESIS, BIOLOGICAL EVALUATION AND THEIR POSSIBLE BINDING MODES TO VITAMIN D RECEPTOR

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The hormonally-active metabolite of vitamin D, 1α,25-dihydroxyvitamin D_3 (**1**), has a wide variety of biological activities, which makes it a promising therapeutic agent for the treatment of cancer, psoriasis and osteoporosis. An insight into the structure-activity relationships of the A-ring of (**1**) is needed to assist the development of more potent and selective analogs, as well as to define the molecular mode of action. We synthesized all eight possible stereoisomers of 2-methyl-1,25-dihydroxyvitamin D_3, demonstrating that the introduction of a simple methyl group into the A-ring of (**1**) yields analogs with unique activity profiles. In particular, 2α-methyl-1α,25-dihydroxy- vitamin D_3 (**2**) was a 4-fold better binder to bovine thymus vitamin D receptor (VDR) with a 2-fold higher cell differentiation-inducing activity in comparison with (**1**). The X-ray crystal structure of VDR complexed with (**1**) indicated an extra space in the vicinity of the A-ring, suggesting that substituents of synthetic A-ring analogs could occupy this additional space. In view of these important results, we have now designed 2,2-functionalized analogs having substituents projecting in both directions in the cavity. All possible stereoisomers of 2,2-ethano-, 2,2-propano-, 2,2-butano- and 2,2-pentano-1,25-dihydroxyvitamin D_3 were synthesized as novel spiro-ring analogs having cyclopropane, cyclobutane, cyclopentane and cyclohexane fused at the C2-position, respectively, in addition to the 2,2-dimethyl analogs. Biological evaluation of the compounds, in terms of the VDR binding affinity as well as the transcriptional potency using ROS 17/2.8 cells, revealed the importance of the ring size at position 2.

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EPIGENETIC DISRUPTION OF VDR SIGNALING IN SOLID TUMORS

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The ligand-mediated switch from binding co-repressor to co-activator complexes is central to the transcriptional actions of vitamin D receptor (VDR). We examined, in prostate, breast and bladder cancer models, whether these interactions are disrupted resulting in loss of VDR sensitivity. Both primary tissue models and cancer cell lines displayed a spectrum of suppressed responsiveness towards $1\alpha,25(OH)_2D_3$, which correlated with elevated co-repressor content. Specifically, elevated NCoR2/SMRT was detected in prostate cancer cell lines and primary tumor cultures, and elevated NCoR1 in breast and bladder cancer cell lines and matched breast cancer tumor and normal biopsies. Interestingly, whilst the cancer cell lines frequently displayed reduced VDR content, compared to their non-malignant counterparts, primary tumor material retained and/or elevated VDR mRNA, correlating with co-repressor content. Functional approaches towards NCoR2/SMRT (siRNA) in prostate cancer cells or NCoR1 (over-expression) in non-malignant breast epithelial cells confirmed a role in suppressing VDR transcriptional and cellular actions. Targeted co-treatments of $1\alpha,25(OH)_2D_3$ plus HDAC inhibitors (*e.g.* NaB or TSA) resulted in re-expression of antiproliferative target genes (*e.g.* GADD45α $p21^{(waf1/cip1)}$ and VDUP-1) and synergistic inhibition of proliferation. This was further potentiated by using potent analogs of $1\alpha,25(OH)_2D_3$. Suppression of GADD45α induction by VDR was found in primary prostate tumor cultures with elevated NCoR2/SMRT, compared to matched peripheral zone (normal) cultures from the same donor. We are currently investigating global and promoter-specific acetylation events associated with VDR activation. These data suggest that VDR actions in solid tumors are retained, but skewed by epigenetic mechanisms to selectively suppress antiproliferative target gene promoter responses. This molecular lesion in a range of solid tumors provides a novel chemoptherapy target utilizing acceptable doses of $1\alpha,25(OH)_2D_3$ plus HDAC inhibitors.

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THE ROLE OF UVB AND VITAMIN D IN REDUCING THE RISK OF CANCER

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The primary objective of this work was to determine the role of solar UVB irradiance in the U.S. as a risk reduction factor for cancer. The secondary objective was to link the geographic variations of cancer mortality rates to various risk factors. An epidemiological study was performed for over 20 types of cancer using state-averaged mortality rates for Caucasian Americans from 1970-94 and 1950-69, along with data on alcohol consumption, Hispanic heritage, latitude, lung cancer (smoking), poverty status, UVB irradiance and degree of urbanization, in Poisson multiple linear regression analyses. Sixteen types of cancer were inversely correlated to July UVB irradiance, adding gall bladder, Hodgkin's lymphoma, laryngeal, oral, pancreatic and vulvar cancer to the list of cancers inversely correlated with UVB irradiance. The association with UVB irradiance was generally stronger than that of any other single factor. Most cancers had correlations with alcohol, smoking and Hispanic heritage in very good agreement with the literature. These results provide additional support for the hypothesis that solar UVB irradiance, through the production of vitamin D, is a significant risk reduction factor for up to 18 types of cancer. These results also largely confirm previous results for alcohol, Hispanic heritage and smoking, as risk factors for various cancers. These results should be studied further using other approaches.

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VITAMIN D AND ITS ANALOGS FOR THE PREVENTION AND TREATMENT OF COMMON CANCERS. EVALUATION OF A NOVEL CLASS OF GEMINI ANALOGS

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To evaluate the role of vitamin D deficiency in colon tumor growth, adult male Balb/c mice were fed either a vitamin D sufficient or vitamin D deficient diet for 10 weeks. Groups of six mice received subcutaneously 10,000 MC-26 cells in the posterior trunk. The tumor size was documented daily. By day 9, there was a significant difference in tumor volume between the vitamin D-sufficient and vitamin D-deficient

mice. By day 18, the vitamin D-deficient animals had a 108% larger tumor size compared to the animals that were vitamin D-sufficient. To determine whether treatment with active vitamin D analogs could further decrease colon tumor growth in a vitamin D sufficient state, groups of mice were treated with novel 19-Nor-Gemini 1,25-dihydroxyvitamin D_3 analogs. The mice were fed a low calcium diet. They recieved 10,000 MC-26 cells in the dorsal back subcutaneously. Twenty-four hours after implantation, they received one of the active vitamin D analogs or placebo vehicle 3x/week. The group that received $1,25(OH)_2$D-19-Nor-Gemini-D_3(S) showed a dose-dependent decrease in tumor volume; at 0.02 μg the tumor volume was reduced by 41% by the end of the study, day 19, compared to the control group. The hexa-deuterated derivative of $1,25(OH)_2$D-19-Nor-Gemini-D_3 (S-D_6) at 0.002 μg showed a 52% reduction in tumor volume ($p<0.05$) compared to the control group at day 19. Animals that received $1,25(OH)_2$D at 0.002 μg and 0.02 μg showed a trend to reducing tumor volume at the highest dose, but it was not statistically significant. Serum calcium levels revealed that the calcium was normal in all groups. These studies demonstrate that vitamin D deficiency may exacerbate metastatic colorectal cancer and that using novel Gemini analogs of $1,25(OH)_2D_3$ may be an effective new approach for mitigating colon cancer tumor growth and metastasis.

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EXPRESSION OF 1,25-HYDROXYVITAMIN-D_3-RECEPTOR, VITAMIN-D25-HYDROXYLASE, VITAMIN-D24-HYDROXYLASE AND VITAMIN D-1ALPHA-HYDROXYLASE IN ENDOMETRIOSIS

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Endometriosis is a common benign gynecologic disease, which affects about 10% of all women in the reproductive phase of life. It has several similarities to malignant diseases, with continuous and destructive growth into surrounding tissue, recurrence and the need for surgical intervention. In addition, endometriosis patients have a doubly increased risk of developing ovarian cancer in comparison to healthy women. If endometriosis patients additionally suffer from long-term infertility, the incidence rate for ovarian cancer is increased more than four times. *Materials and Methods*: Total RNA was extracted from 17 ovarian endometrioma, 13 ovarian cancer samples and 7 benign ovaries without signs of endometriosis. Real-time PCR was performed for 1,25-hydroxyvitamin-D_3-receptor, vitamin-D25-hydroxylase, vitamin-D24-hydroxylase and vitamin D-1alpha-hydroxylase. Immunohistochemistry with monoclonal antibodies against 1,25-hydroxyvitamin-D_3-receptor was perfomed on formalin-fixed tissue sections of ovarian endometriosis and endometrium. *Results*: The 1,25-hydroxyvitamin-D_3-receptor was expressed in ovarian endometrioma, but the expression in ovarian cancer was four times higher. The expression of vitamin-D25-hydroxylase was similar to ovarian cancer samples, whereas the expression of vitamin-D24-hydroxylase and vitamin D-1alpha-hydroxylase in endometriosis was markedly lower compared to ovarian cancer samples. The 1,25-hydroxyvitamin-D_3-receptor was mainly detected in the glandular epithelium of ovarian endometrioma and endometrium, whereas the surrounding stroma showed weaker staining intensity. *Conclusion*: Our findings indicate that endometriosis, in the same way as cancer, might be considered as a potential target for prevention or therapy with new vitamin D analogs. Further studies are needed to prove this concept.

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THE ANTITUMOR EFFICACY OF CALCITRIOL: PRECLINICAL STUDIES

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Studies in our laboratory demonstrate that vitamin D (1,25 dihydroxycholecalciferol or calcitriol) has significant antitumor activity *in vitro* and *in vivo* in murine and human squamous cell, prostate, lung, pancreatic and myeloma model systems. Calcitriol induces G_0/G_1 arrest, modulates p27 and p21, the cyclin dependent kinase (cdk) inhibitors implicated in G_1 arrest, and induces cleavage of caspase 3, PARP and the mitogen-activated protein kinase (MEK) in a caspase-dependent manner. Calcitriol also decreases phospho-Erk (P-Erk) and phospho-Akt (P-Akt), kinases that regulate cell survival pathways and up-regulates the pro-apoptotic signaling molecule, MEKK-1. Calcitriol significantly enhances the *in vitro* and *in vivo* antitumor efficacy of the platinum analogs and taxanes. Enhancement of drug-mediated apoptosis is associated with an increase in PARP-, MEK- and caspase 3-cleavage, the expression of p73 and MEKK-1 and a decrease in P-Erk and P-Akt. Glucocorticoids enhance calcitriol-mediated activities pre-clinically *in vitro* and *in vivo*. We have demonstrated that dexamethasone (dex) significantly potentiates the antitumor effect of calcitriol and decreases calcitriol-induced hypercalcemia. Both *in vitro* and *in vivo*, dex increases vitamin D receptor (VDR) ligand binding in the tumor, while decreasing binding in intestinal mucosa, the site of calcium absorption. P-Erk and P-Akt are also decreased with calcitriol/dex, as compared to either agent alone. Induction of CYP24, the enzyme primarily responsible for calcitriol

catabolism, may be a factor in bioavailability and calcitriol exposure; therefore, studies were initiated to investigate the effect of ketoconazole, a CYP24 inhibitor, on the *in vitro* and *in vivo* antitumor effects of calcitriol/dex in the PC-3 prostate tumor model. Ketoconazole enhanced the antitumor activity of calcitriol/dex and decreased CYP24 activity. These studies demonstrate that calcitriol has significant antiproliferative activity in a number of pre-clinical model systems and form the groundwork for ongoing clinical studies investigating calcitriol as an anticancer agent. (Supported by NCI grants CA67267, CA85142 and CA95045).

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HEPATIC ACTIVATION AND INACTIVATION OF CLINICALLY-RELEVANT VITAMIN D ANALOGS AND PRODRUGS

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Like most pharmaceutical agents, vitamin D analogs are subject to hepatic metabolism by a variety of cytochrome P450 (CYP)-based systems. Metabolism can involve activation as well as inactivation of the vitamin D analog and one of the more successful families includes the 1α-hydroxyvitamin D prodrugs (1α-OH-D_2,1α-OH-D_3,1α-OH-D_4,1α-OH-D_5), that all require a step of activation. Some of these prodrugs are in use or clinical trials because they have a therapeutic advantage over calcitriol. However, the nature of the activation of these molecules is poorly understood, particularly with regard to the CYP isoform involved. We have used various transfected CYPs and hepatic cell lines combined with tandem LC-MS analysis to investigate the metabolism of a spectrum of vitamin D analogs, including 1α-OH-Ds and the topical analog, calcipotriol. In the case of the 1α-OH-Ds, evidence of multiple sites of side chain hydroxylation consistent with the generation of more than one active form was found. We also showed the potential involvement of CYP27A and other putative 25-hydroxylases in 1α-OH-D activation, as well as the potential for CYP24 activation and inactivation. In the case of calcipotriol, the respective roles of non-vitamin D-related CYPs and CYP24 in the catabolism of this anti-psoriatic drug were elucidated using cell lines with or without CYP24 expression, allowing us to demonstrate the potential contribution of CYP24 to "vitamin D resistance". The implications of hepatic metabolism in the context of other facets thought to play a role in the mechanism of action of anticancer and antiproliferative vitamin D analogs are discussed.

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EFFICIENT SYNTHESIS OF 2-ALKYLATED 1α,25-DIHYDROXY-19-NORVITAMIN D_3 WITH JULIA OLEFINATION AND ITS BIOLOGICAL ACTIVITY

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Previously, we reported that several analogs of 2-alkylated and 2-(α-hydroxyalkoxy)lated 1α,25-dihydroxyvitamin D_3 exhibit stronger VDR binding affinity than that of the natural hormone, 1α,25-dihydroxyvitamin D_3 (**1**), which contribute to their unique biological activities in HL-60 leukemia cells. On the other hand, the 19-demethylenated analog of (**1**), 1α,25-dihydroxy-19-norvitamin D_3 (**2**), possesses a selective active profile that is non-calcemic and maintains the cell pro-differentiation and antiproliferative activities. We were interested in incorporating both "2-alkylation" and "19-demethylenation" into the molecule of natural hormone (**1**). Therefore, six novel 2-alkylated analogs of (**2**) were synthesized, using a radical alkylation reaction to introduce a three carbon unit into the A-ring and a Julia-type olefination to connect the modified A-ring (a cyclohexanone derivative) with a C2-elongated CD-ring part (a sulfone derivative). This C5-C6 connective approach was found to be very efficient in constructing the diene between the A-ring, bearing a rather long alkyl chain at the C2-position, and the CD-ring. The coupling yields, including a deprotection step, were 47-62%. Stereochemistry at the C2-position (2α or 2β) was determined by ^{1}H NMR experiments. The synthesized 2α-(3-hydroxypropyl)-1α,25-dihydroxy-19-norvitamin D_3 (3) showed similar potency in binding to the bovine thymus VDR as **1** and was about 36-fold more potent than **1** in the induction of HL-60 cell differentiation. Interestingly, although 2α-(3-hydroxy- propyl)-1α,25-dihydroxy-19-norvitamin D_3 (4) had lower receptor binding affinity than **1**, it still showed a 6.7-fold higher potency in the cell differentiation activity than that of **1**. Our data suggest that 2-alkylated analogs of 1α,25-dihydroxy-19-norvitamin D_3 may be excellent candidates for human clinical trials in myeloid malignancies, as well as other types of cancers including prostate cancer.

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VITAMIN D COMPOUNDS AND HEMATOPOIETIC MALIGNANCIES

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Studies that showed human and murine acute myeloid leukemia cells can differentiate towards macrophages in the presence $1,25(OH)_2D_3$, popularized the concept of differentiation therapy. Because of concerns about hypercalcemia, investigators have turned their attention to the synthesis of vitamin D analogs that can induce differentiation and inhibit proliferation of cancer cells without causing hypercalcemia. We found that the FDA-approved 19-nor-$1,25(OH)_2D_2$ (Paricalcitol, Zemplar) had prominent antiproliferative/pro-differentiation activities against leukemia cells *in vitro* with little hypercalcemia in mice. A small MDS clinical trial, initiated by us, found 1/12 individuals had a response to Paricalcitol, with a rise of platelet count from 40,000 to 120,000/μl blood. Oral Paricalcitol doses ranged between 4 and 54 micrograms/day with no hypercalcemia or any other toxicity. Thus, the compound was well tolerated, but had only modest effects. Further studies *in vitro* showed a synergy between As_2O_3 and vitamin D analogs, particularly in acute promyelocytic leukemia. We also found that Retinovir, a protease inhibitor which inhibits cyp24 [a cytochrome p450 involved in $1,25(OH)_2D_3$ metabolism], markedly increased the activity in $1,25(OH)_2D_3$. Notably, we have additionally found that $1,25(OH)_2D_3$ could greatly enhance the expression of anti-microbial compounds such as cathelicidin antimicrobial peptide (CAMP) in normal and leukemic myeloid cells. This finding may lay the ground work for a novel form of anti-microbial therapy. Finally, we found, by microarray expression studies, that the transcription factor C/EBP-α was markedly induced in LNCaP prostate cancer cells cultured with $1,25(OH)_2D_3$; and the antiproliferative effects of $1,25(OH)_2D_3$ on these cells were attenuated by siRNA to C/EBP-α. These studies suggest that the growth inhibitory activity of vitamin D_3 may be mediated, in part, by induction of expression of C/EBP transcription factors.

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UV-RADIATION AND CANCER PREVENTION - WHAT IS THE EVIDENCE?

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Using special key words, a total of 281 articles for "Prostate Cancer and Vit-D", 244 for "Breast Cancer and Vit-D", and 119 for "Colon Cancer and Vit-D" were reviewed. *Results*: Focusing on the key words: natural sunlight, UV-radiation, and environment-latitude, only 13, 9 and 7 publications were found, respectively. The spectrum of the papers included reviews, epidemiological observations, clinical studies and laboratory experiments. *Discussion*: For all three types of cancer, a higher exposure to sunshine (UV-radiation) was inversely correlated with the incidence of cancer and mortality. Environmental risk factors were low UV-exposure in urban areas (like rickets in childhood) or living at high latitudes. Additionally at risk are colored people living north of tropical latitudes. Furthermore, there is a correlation with different genotypes and VDR polymorphisms. The observation that the overall mortality is higher during wintertime at high latitudes could be discussed in relation to UVB exposure, Vit D- induced infection, myocardial infarction or stroke. *Conclusion*: Compensation for the lack of UVB during wintertime at high latitudes seems to be an important goal in preventive health strategies.

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LACK OF UV AND VITAMIN D – A FATAL RISK IN RENAL PATIENTS?

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Aim: In the general population, there is a higher prevalence of cardio-vascular episodes and an increasing incidence of mortality during wintertime. The aim of the investigation was to evaluate whether there is a seasonal risk of death for end-stage kidney renal patients. *Methods and Results*: Using the data of the German RRT Registry from 1995 (start of registration) up to 2003, statistically significant differences between winter and summer were found: the highest rates were found in February/March and the lowest in August/September for starting dialysis ($\Delta + 2\% = p<0.05$) as well as for mortality ($\Delta + 0.7\% = p<0.005$). *Discussion and Conclusion*: UV radiation in Germany shows a 6.5-fold difference between the lowest in January/December and the highest in June/July. Therefore, the seasonal gap of UV radiation due to the disease-related disturbance of vitamin D metabolism in renal patients can lead to the final loss of kidney function and fatal detoriation.

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KINETICS OF VITAMIN D METABOLITES DURING SERIAL UV RADIATION

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Aim: For the cutaneous synthesis of vitamin D, only a small

amount of UV radiation from natural sunlight and the exposure of only small parts of the body, *e.g.* hands, forearms and/or face, are necessary. The aim of this study was to evaluate the correlation between Vitamin D-weighted UV dosage (H_{vd}) *versus* the increase of circulating $25(OH)D_3$ and $1,25(OH)_2D_3$. *Patients and Methods*: Twenty-two dialysis patients were partial-body (frontal part of the legs, approx. 15% of body surface) irradiated over a period of 14 weeks using an artificial UV-source (UVB 3.5%); blood samples were taken every two weeks. *Results*: The peak value of $25(OH)D_3$ was found after 8 weeks (increase Δ + 13μg/l = +33%, median) and the peak of $1,25(OH)_2D_3$ followed 6 weeks later (increase Δ + 9ng/l = +90%). Therefore, the following algorithm can be calculated: $25(OH)_2D_3 = (H_{vd}^2 \times 10^5)+25$, as a nonlinear correlation ($r^2=0.992$); following a linear correlation (r=0.32) between $25(OH)D_3$ and $1,25\ (OH)_2D_3$. *Conclusion*: A sufficient pool of circulating $25(OH)D_3$ is necessary for conversion to $1,25(OH)_2D_3$. In renal patients, the threshold level of $25(OH)D_3$ seems to be ≥35 μg/l.

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EFFECT OF UVB RADIATION EMITTED FROM THE NARROWBAND TL-01 LAMP (311 NM) ON CALCITRIOL SYNTHESIS IN ORGANOTYPIC CULTURES OF KERATINOCYTES

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The skin is the only tissue known in which the complete UVB-induced pathway from 7-dehydrocholesterol (7-DHC) to hormonally-active calcitriol (1α,25-dihydroxyvitamin D_3) occurs under physiological conditions. It is well known that both calcitriol and UVB radiation exert potent antipsoriatic effects. We speculate that the therapeutic effect of UVB radiation can be attributed to UVB-triggered cutaneous synthesis of calcitriol, for which the optimum wavelength was 300 ± 3 nm *in vitro* and *in vivo*. On the other hand, the narrowband Philips TL-01 lamp, which is commonly used as a UVB source for the treatment of psoriasis, has a maximum spectral irradiance at around 311 nm. The aim of this study was to investigate the calcitriol-inducing potential of the TL-01 lamp in organotypic cultures of keratinocytes supplemented with 25 μM 7-DHC at different radiant exposures (125-1000 mJ/cm^2). We found that the maximum calcitriol-generating capacity of the TL-01 lamp at 500 mJ/cm^2 (corresponding to 2.1 SED [Standard Erythema Dose]) and 16 hours after irradiation still amounted to approximately 45% of that of monochromatic radiation at 300 nm and 30 mJ/cm^2. We conclude that irradiation with the narrowband TL-01 lamp in a therapeutic dose range can affect calcitriol synthesis in epidermal keratinocytes. Thus, the antipsoriatic effect observed after TL-01 lamp exposures may be, at least partially, explained by the known action of newly-synthesized calcitriol on epidermal cell proliferation and differentiation.

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PATHWAYS MEDIATING THE GROWTH INHIBITORY ACTIONS OF VITAMIN D IN PROSTATE CANCER

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Vitamin D is an important hormone that affects the incidence and progression of many malignancies including prostate cancer (PCa). 1,25-dihydroxyvitamin D (calcitriol), the active form of vitamin D, inhibits the growth and stimulates the differentiation of PCa cells. We studied established human PCa cell lines as well as primary cultures of normal or cancer-derived prostatic epithelial cells to elucidate the molecular pathways of the action of calcitriol. These pathways are varied and some appear to be cell-specific. We used cDNA microarray analysis to ascertain additional genes regulated by calcitriol, in order to identify novel therapeutic targets for the treatment of PCa. Several potentially useful target genes have emerged from these studies including two new target genes, both involved in prostaglandin (PG) metabolism.

Accumulating evidence has implicated PGs in stimulating the development of many types of cancer including PCa. PGs have been associated with the progression of PCa, tumor invasiveness and tumor grade. Prostatic PGs are formed by the action of the cyclooxygenase enzyme COX-2. The first step in PG inactivation is mediated by 15-hydroxyprostaglandin dehydrogenase (PGDH). We found that calcitriol down-regulates the expression of COX-2 and up-regulates PGDH. There is much current interest in the use of second-generation COX-2 inhibitors or non-selective nonsteroidal anti-inflammatory drugs (NSAIDs), to prevent and/or treat PCa, due to their ability to inhibit growth and induce apoptosis. Moreover, PGDH has recently been proposed as a tumor suppressor. The actions of calcitriol to induce PGDH and inhibit COX-2 constitute a pathway to reduce and/or remove active PGs, thereby diminishing PCa proliferation. Combination therapy of LNCaP cells with calcitriol and NSAIDs revealed synergistic growth inhibition. In combination, calcitriol and NSAIDs allowed the use of

reduced doses of both drugs that still resulted in enhanced antiproliferative activity. These findings suggest that therapy combining calcitriol and NSAIDs will increase efficacy while decreasing side-effects. We propose that this combination of already approved drugs can be brought to clinical trial swiftly, particularly in patients with early recurrent PCa that demonstrate rising PSA after primary therapy. In conclusion, our research is directed at understanding the mechanisms of vitamin D action in prostate cells with the goal of developing treatment strategies to improve PCa therapy. The ability of calcitriol to inhibit PG synthesis and stimulate PG destruction appears to be an additional pathway by which calcitriol can enhance PCa therapy.

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BIOLOGICAL EFFECTS OF 1α,25-DIHYDROXYVITAMIN D_3 ON HUMAN KERATINOCYTES AFTER IONIZING RADIATION

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Exposure of human skin to ionizing radiation results in various early and late effects such as an inflammatory reaction, keratosis, fibrosis, radiation vasculitis and cancer. 1α,25-Dihydroxyvitamin D_3, the biologically active metabolite of vitamin D, has been shown to exert pleiotropic effects in the skin. We evaluated whether the radiation reaction of human keratinocytes (HaCaT cells) can be modulated by 1α,25-dihydroxyvitamin D_3. The cell growth of keratinocytes after ionizing radiation was significantly increased in the presence of 1α,25-dihydroxyvitamin D_3 as compared to the untreated control. Moreover, 1α,25-dihydroxyvitamin D_3 also exerted a positive influence on the cell survival of irradiated keratinocytes, as shown by clonogenic assay. As the cutaneous radiation reaction is determined by various inflammatory parameters, including adhesive interactions mediated by cellular adhesion molecules, we analyzed the cell surface expression of intercellular adhesion molecule-1 (ICAM-1) and β1-integrin in keratinocytes and the effect of 1α,25-dihydroxyvitamin D_3 using flow cytometry and immuno- histochemistry. The results revealed that ionizing radiation causes an up-regulation of both ICAM-1 and β1-integrin in keratinocytes, which was inhibited by pretreatment of the cells with 1α,25-dihydroxyvitamin D_3. Taken together, our data suggest that 1α,25-dihydroxyvitamin D_3 might be a promising agent to modify the radiation reaction, offering new options in radiotherapy and oncology.

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SNAIL REPRESSES VITAMIN D RECEPTOR EXPRESSION AND BLOCKS THE EFFECTS OF 1α,25-DIHYDROXYVITAMIN D_3 ON HUMAN COLON CANCER CELLS *IN VITRO* AND *IN VIVO*

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We have previously reported that 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and several non-hypercalcemic analogs (EB1089, MC903 and KH1060) inhibit proliferation and promote differentiation of human SW480-ADH colon cancer cells. They induce the expression of E-cadherin and the translocation of β-catenin from the nucleus to the plasma membrane. The Wnt/β-catenin signaling pathway is deregulated in most colon cancers as a result of mutation of APC or β-catenin (*CTNNB1*) genes. In several human colon cancer cell lines analyzed, $1\alpha,25(OH)_2D_3$ repressed β-catenin/TCF-4 transcriptional activity and thus inhibited the expression of β-catenin/TCF-4-responsive genes. Using oligonucleotide microarrays, the genetic profile induced by $1\alpha,25(OH)_2D_3$ in human colon cancer cells was identified. $1\alpha,25(OH)_2D_3$ changed the expression levels of numerous previously unreported genes, including many involved in transcription, cell adhesion, DNA synthesis, apoptosis and intracellular signaling. Vitamin D receptor (VDR) is expressed in normal colon epithelium and during the early stages of colon cancer, but is lost at later stages of tumor progression. High VDR expression has been associated with good prognosis. We found that the SNAIL transcription factor represses human *VDR* gene expression in colon cancer cells and blocks the antitumor action of EB1089 in xenografted mice. In human colon cancer, elevated SNAIL expression correlates with the down-regulation of *VDR* and E-cadherin. Our data predict that colon cancer patients with high levels of SNAIL are likely to be poor responders to therapy with $1\alpha,25(OH)_2D_3$ analogs.

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HIGH-DOSE PULSE CALCITRIOL IN PROSTATE CANCER

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In pre-clinical models of prostate cancer, calcitriol, the principal active metabolite of vitamin D, displayed significant antineoplastic activity alone and in combination with cytotoxic drugs, but only at substantially supraphysiologic concentrations. The reported mechanisms of activity include inhibition of proliferation and cell cycle

arrest, induction of apoptosis, and reduction of invasiveness and angiogenesis. Intermittent administration of calcitriol has allowed significant dose escalation. A weekly dose of 0.5 μg/kg weekly was safe and further dose escalation did not result in additional gains in peak blood calcitriol concentrations or total drug exposure. Assessed by immunohistochemistry in 33 patients who underwent radical prostatectomy, vitamin D receptor (VDR) was expressed in all 33 tumors. VDR was expressed in 83 to 100% (mean 98%) of cancer cells in 17 untreated tumors and its expression was modestly reduced with 4 weeks of weekly calcitriol therapy. Administered to 22 patients with a rising PSA without hormonal therapy, weekly calcitriol treatment resulted in a slower rise in serum PSA, but no confirmed 50% reductions in serum PSA. Phase II studies in combination with chemotherapy for the treatment of androgen-independent prostate cancer yielded mixed results. One out of 17 patients (6%, 95%CI 0% - 28%) treated with oral calcitriol 0.5 μg/kg on day 1 and intravenous carboplatin AUC 7 (AUC 6 in patients with prior radiation) on day 2, repeated every 4 weeks, had a confirmed PSA reduction in excess of 50%. Thirty out of 37 patients (81%, 95%CI 68% - 94%) achieved this degree of PSA reduction in response to treatment with oral calcitriol 0.5 μg/kg on day 1 followed by docetaxel 36 mg/m^2 on day 2, repeated weekly for 6 weeks of an 8-week cycle. An international placebo-controlled randomized trial, currently under way, will provide more robust information about the safety and efficacy of calcitriol and docetaxel.

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ANTIPROLIFERATIVE ACTIVITY OF NEW VITAMIN D ANALOGS IN SINGLE OR COMBINED WITH CISPLATIN TREATMENT

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Calcitriol, the seco-steroid hormone, has proven to be a potent antiproliferative agent against various normal and neoplastic cells. Moreover, calcitriol and other vitamin D_3 analogs induce differentiation of many human cancer cells. The biological activity of vitamin D compounds is mediated by the nuclear vitamin D receptor (nVDR). Such properties suggest potential therapeutic applications for these agents, including antitumor therapy. Unfortunately, the strong calcemic activity of calcitriol excludes its use in clinics. Two of the promising, low hypercalcemia-inducing, side-chain modified analogs, PRI-1906 and PRI-2191, were the object of our intensive studies. The results of a study on PRI-2191 or PRI 1906 activity in multidrug antitumor therapy *in vitro* are presented. In particular, applying SRB or MTT cytotoxicity assays, the growth inhibitory effects of the studied compounds in combination with cisplatin were measured against the human cell lines HL-60, CCRF (leukemia), T47D, MCF-7 (mammary gland), and the murine cell line WEHI-3 (leukemia). Treatment of vitamin D-sensitive cancer cells (WEHI-3, HL-60) with the combination of agents significantly decreased the ID_{50} value compared with the cytostatic applied alone, which was not the case for the vitamin D-resistant CCRF cells. In the model of weakly vitamin D-sensitive (MCF-7, T47D) cells, only a weak additive effect of combined with cytostatic therapy was observed. In conclusion, a synergism between PRI 2191 or PRI 1906 and cisplatin against vitamin D-sensitive cancer cells *in vitro* was observed.

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1α,25-DIHYDROXYVITAMIN D_3 INDUCES A DRASTIC CHANGE IN THE PHENOTYPE AND INHIBITS THE EXPRESSION OF MYOEPITHELIAL GENES IN HUMAN BREAST CANCER MDA-MB-453 CELLS

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1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) displays anti-proliferative and pro-apoptotic activities in many cell types, including breast cancer cells. The responsiveness of a panel of human breast cancer cells to $1,25(OH)_2D_3$ was studied. Among them, MDA-MB-453 cells showed the highest activation of a consensus vitamin D response element (VDRE) and so were selected for a detailed study. $1,25(OH)_2D_3$ drastically increased the cell size and induced a change from a rounded to a flat morphology. By phase contrast, laser confocal and electron microscopy, we found that $1,25(OH)_2D_3$ changed the cytoarchitecture of actin filaments and microtubules and augmented the number of filopodia and lamellipodia. No expression of E-cadherin, desmoplakin or connexins was detected, whereas the expression of other epithelial adhesion proteins, such as ZO-1 or occludin, was unchanged. Instead, $1,25(OH)_2D_3$ induced cell to cell and cell to matrix contacts *via* large cytoplasmic extensions and focal adhesion plaques, respectively. Remarkably, Northern and Western analysis revealed that $1,25(OH)_2D_3$ decreased the expression of mesenchymal proteins such as vimentin and N-cadherin. Moreover, $1,25(OH)_2D_3$ also inhibited the expression of myoepithelial marker proteins such as P-cadherin, alpha6-

integrin, beta4-integrin and smooth muscle alpha-actin, as shown by immunofluorescence, Northern and Western analysis and flow cytometry. These findings suggest that, at least in the case of MDA-MB-453 cells, $1,25(OH)_2D_3$ reverts the myoepithelial phenotype, that in human breast cancers is associated with more aggressiveness and poor prognosis. We are currently extending these studies to other human breast cancer cells, analyzing the expression of some target genes in VDR-deficient mice and studying the molecular mechanism of the described effects.

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ROLE OF PROHIBITIN IN THE MECHANISM OF VITAMIN D ACTION IN BREAST CANCER

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It is well established that certain vitamin D analogs protect against the development and progression of breast cancer. Previous studies from our laboratory have shown that 1α-hydroxy-24-ethyl-cholecalciferol (1α(OH)D5) inhibits chemically-induced carcinogenesis in rats, as well as exhibiting antiproliferative properties for VDR-positive breast cancer cells. In ER+ cells, 1α(OH)D5 down-regulates estrogen-inducible genes such as progesterone receptors and pS2, whereas in VDR+, ER− cells it induces cell differentiation. Although it is established that the action of vitamin D is mediated by nuclear VDR, the genomic regulation of the sensitivity to vitamin D is not clear. The microarray analysis showed that transformation of MCF12F cells by MNU resulted in differential expression of 320 genes including prohibitin, which is involved in cell cycle regulation. We therefore hypothesized that prohibitin could play a role in mammary carcinogenesis and in enhancing sensitivity and response to vitamin D. We evaluated overexpression of prohibitin on the 1α(OH)D5-induced cellular effects in breast cancer cells. By using the MCF-7 cells expressing tetracycline-inducible prohibitin-model (Tet-On model), we found that overexpression of prohibitin consistently inhibited cell proliferation and the inhibition was further enhanced by treating the cells with 1α(OH)D5. Co-transfection of the prohibitin expression vector with the CYP24 promoter reporter construct inhibited the CYP24 promoter activity induced by 1α(OH)D5 in both MCF-7 and BT474 cells. RT-PCR analysis demonstrated that 1α(OH)D5 down-regulated prohibitin mRNA expression in ER– cells, which suggested cellular resistance to vitamin D. These results suggest that prohibitin sensitizes the cellular response to vitamin D and may serve as a potential target gene for breast cancer prevention and treatment with efficacious vitamin D analogs. (Supported by the National Cancer Institute USPHS R01-CA82316).

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DYSFUNCTION OF THE VITAMIN D ENDOCRINE SYSTEM AS COMMON CAUSE FOR MULTIPLE MALIGNANT AND OTHER CHRONIC DISEASES

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Extensive research on the *CYP27B1*-encoded 25-(OH)D-1α-hydroxylase has contributed much to our understanding of how locally produced $1,25-(OH)_2D_3$ exerts tissue-specific control of cellular growth, differentiation and function. Because many types of epithelial, mesenchymal and immune cells express the 25-(OH)D-1α-hydroxylase, many organ functions are necessarily affected by changes in the activity of the enzyme. We hypothesize that this is likely to occur under conditions of hypovitaminosis D, *i.e.*, at serum 25-(OH)D levels below 30 nM, because extra-renal 25-(OH)D-1α-hydroxylase activity is critically limited by availability of its substrate. This can provide an explanation, on a molecular and cellular basis, for the many observations that significant associations exist between a compromised vitamin D status and the pathogenesis of frequent chronic diseases. In addition to skeletal disorders, vitamin D insufficiency is a risk factor of malignancies, particularly of colon, breast and prostate gland, of chronic inflammatory and autoimmune diseases (insulin-dependent diabetes mellitus, inflammatory bowel disease, multiple sclerosis *etc.*), as well as of metabolic disorders (metabolic syndrome, hypertension). We hope that a deeper insight into the pathogenic consequences of a compromised vitamin D status will also stimulate discussion on the importance of vitamin D substitution or even supplementation, respectively, for preventive and clinical medicine.

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SKIN CANCER PREVENTION, UV EXPOSURE AND VITAMIN D: HOW MUCH SUNLIGHT DO WE NEED?

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UV exposure is the the main reason for the development of skin cancer. In consequence, strict sun protection recommendations represent a fundamental part of skin cancer prevention programs. However, 90% of all requisite vitamin D is formed within the skin through the action of the sun, creating a real dilemma, since a connection between vitamin D deficiency and various types of cancer (*e.g.* colon, prostate and breast cancer) has been confirmed

in a large number of studies. These cancer protective effects of vitamin D most likely depend on extrarenal, local production of $1\alpha,25(OH)_2D_3$, that has been shown in various tissues. Increasing evidence indicates that lack of exposure to sunlight leads to even more than thinning bones and an increased risk of cancer, since there are added benefits of vitamin D that include control of blood pressure and cholesterol serum levels. We analyzed serum 25-hydroxyvitamin D levels in patients under photo-protection, including patients with xeroderma pigmentosum (XP), basal cell nevus syndrome (BCNS), and transplant recipients under immunosuppressive therapy. The serum 25-hydroxyvitamin D levels were decreased in these patients. We conclude that vitamin D serum levels should be monitored carefully in patients under photoprotection, and that vitamin D deficiency should be treated, *e.g. via* oral substitution. We discuss our present knowledge about the relevance of vitamin D deficiency for the increased occurrence of certain malignancies and the possible consequences for sun protection recommendations, that represent a fundamental part of skin cancer prevention programs.

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VITAMIN D SENSITIZES COLON AND PROSTATE CANCER CELLS TO PROGRAMMED CELL DEATH INDUCED BY REACTIVE OXYGEN SPECIES

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Colon and prostate cancer, in addition to breast cancer, are well documented targets for the anticancer activities of vitamin D. Reactive oxygen species (ROS) are major cytotoxic mediators of various anticancer modalities such as radiotherapy, photodynamic therapy and some anticancer drugs. ROS are also important in the anticancer activities of immune cells and cytotoxic cytokines. Superoxide anions are formed in the course of action of many cytotoxic agents. Exposure to H_2O_2 may bring about both programmed cell death (PCD) and accidental cell death. PCD may take the form of apoptosis or necrotic-like cell death. Activation of caspases will lead to apoptotic cell death, while necrotic-like PCD will ensue in the absence of caspase activation. We have previously shown that calcitriol sensitizes breast cancer cells to the action of various ROS- generating anticancer agents and to the action of the pre-formed ROS, H_2O_2. This work assessed if the same holds true for colon and prostate cancer cells, using the HT-29 and LNCaP cell lines as experimental models. The cells were exposed to H_2O_2 using two protocols: a short (1-hour) exposure to high concentrations of H_2O_2 or continuous exposure to the glucose oxidase H_2O_2-generating system. Cell death was observed 24 hours after exposure, this long lag being typical of PCD rather than accidental cell death. The HT-29 cells were much less sensitive to ROS than the LNCaP cells, but pre-exposure to calcitriol significantly increased the cytotoxic effect in both cell lines in both exposure protocols. Cytotoxic concentrations of H_2O_2 induced caspase 3-like activity in LNCaP cells and calcitriol potentiated this hallmark of apoptosis. However, in HT-29 cells, H_2O_2 did not induce caspase activation and, as expected, H_2O_2-induced HT-29 PCD was not affected by a pan-caspase inhibitor. We conclude that vitamin D sensitizes breast, prostate and colon cancer cells to the cytotoxic action of ROS and that this effect may contribute to the *in vivo* anticancer activity of the hormone, both under physiological conditions and in the course of chemo- and radiotherapy.

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SELECTIVE INHIBITORS OF VITAMIN D METABOLISM - CHARACTERISTICS AND PERSPECTIVES

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Synthetic analogs of vitamin D (VD) have a great therapeutic potential in indications ranging from defects in bone metabolism to modulation of the immune and nervous system and to prevention and treatment of proliferative diseases. However, at therapeutic doses, the severe side-effects of analogs still limit their widespread use. At Novartis, we started a promising alternative/supplementary strategy that focuses on CYP24A1 as a key target. In general, the levels of hormonally-active VD are tuned by synthesis *via* CYP27B1 and multi-step oxidation *via* the closely related hydroxylase CYP24A1, which terminates hormone function. Usually, CYP24A1 expression is transient: rapidly induced from basic (constitutive) levels in almost all cells by active VD (analogs), it declines again in parallel with hormone inactivation. However, unbalanced high CYP24A1 expression can occur locally, which reduces active VD to levels incompatible with hormone function, thereby causing/reinforcing disorders: i) Recent reports demonstrate amplification of the CYP24A1 gene in various tumors (*e.g.* breast, prostate, esophagus, colorectal and gastric cancers) pointing to CYP24A1 as an oncogene. ii) Upcoming evidence shows that the capacity to induce CYP24A1 is not restricted to active VD, but may be caused by structurally different endogenous molecules (*e.g.* lithocholic acid, calcitonin; our laboratory found strong induction by retinoic acids). Aiming at increased and stabilized levels of active VD, potent and selective inhibitors of CYP24A1 ("azole-type" compounds) were designed with IC_{50}-values in the low nM range and up to 50-fold selectivity

(CYP24A1/CYP27B1). When tested in human keratinocytes as a model system, these compounds potently stabilized active VD levels generated from $25(OH)D_3$, thereby strongly increasing and extending the hormonal function. In particular, expression of the surrogate marker CYP24 mRNA was amplified and extended and the anti-proliferative activity increased by >2 orders of magnitude. As the first representative out of some 50 selective CYP24 inhibitors, VID400 was preclinically profiled. Selective CYP24A1 inhibitors may become valuable drugs, applied either as single entities (targeted to distinct organs) to increase/extend the local function of endogenous active VD or in combination with low doses of potent, however metabolically labile or toxic, analogs. In all cases, calcemic side-effects will be minimized.

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THE VITAMIN D SYSTEM REPRESENTS A KEY REGULATOR OF THE GROWTH OF CUTANEOUS SQUAMOUS CELL CARCINOMAS (SCC) AND MALIGNANT MELANOMA (MM)

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Increasing evidence points to an important function of vitamin D metabolites in growth regulation in various tissues, and new vitamin D analogs are interesting candidates for the treatment of malignancies, including squamous cell carcinomas (SCC) and malignant melanoma (MM). The expressions of vitamin D receptor (VDR), vitamin D-25-hydroxylase (25-OHase), 25-hydroxyvitamin D-1α-hydroxylase (1α-OHase) and 1,25-dihydroxyvitamin D-24-hydroxylase (24-OHase) were analysed in cutaneous SCC, SCC cell lines and melanoma cell lines. The intensity of VDR immunoreactivity was increased in SCCs as compared to normal human skin (HS). VDR staining did not correlate with histological type or grading, nor with markers for proliferation, differentiation or apoptotic cells. The SCC cell lines (SCL-1, SCL-2) revealed VDR immunoreactivity *in vitro* and incubation of these cells with calcitriol resulted in a dose-dependent suppression of cell proliferation (up to ~ 30%), as measured by a WST-1-based colorimetric assay. For unknown reasons, however, some melanoma tumor cell lines fail to respond to the antiproliferative effects of these compounds. The effects of $25(OH)D_3$, $1,25(OH)_2D_3$, and the vitamin D analog seocalcitol (EB 1089) were studied on the growth of seven melanoma cell lines (IGR, MelJuso, MeWo, SK-Mel-5, SK-Mel-25, SK-Mel-28, SM). As measured by a WST-1-based colorimetric assay, three melanoma cell lines (MeWo, SK-Mel-28, SM) responded to the antiproliferative effects of the active vitamin D analogs, while the others were resistant. A strong induction (up to 7000-fold) of 1,25-dihydroxyvitamin D-24-hydroxylase (24-OHase) mRNA was detected in responsive cell lines along with $1,25(OH)_2D_3$-treatment, indicating the functional integrity of vitamin D receptor (VDR)-mediated transcription. In contrast, induction of 24-OHase was much lower in resistant melanoma cells (up to 70-fold). VDR mRNA was induced up to 3-fold, both in responsive and resistant cell lines, with $1,25(OH)_2D_3$ treatment. RNA levels for VDR, 25-OHase, 1α-OHase and 24-OHase were significantly elevated in SCCs as compared to HS, as measured by real-time PCR. In conclusion, our findings demonstrate that alterations in VDR expression, as well as in local synthesis or metabolism of vitamin D metabolites, may be of importance for growth regulation of SCCs and MMs. Additionally, SCCs represent potential targets for therapy with new vitamin D analogs that exert few calcemic side-effects, or for the pharmacological modulation of calcitriol synthesis/metabolism in these tumors.

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1,25-DIHYDROXYVITAMIN D_3 REGULATES CLUSTERIN EXPRESSION IN MELANOMA CELLS

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Clusterin is a glycoprotein that is implicated in various cell functions including cell growth, cell adhesion and apoptosis. There are two known clusterin isoforms that are obtained by alternate splicing, the nuclear (nCLU) and the secretory (sCLU). The pro-apoptotic nCLU has been shown to be involved in the regulation of cell cycle progression and apoptosis. We analyzed immunohistochemically paraffin sections of primary cutaneous malignant melanomas (n=25), metastases of malignant melanomas (n=18) and acquired melanocytic nevi (n=30) using clusterin-specific antibodies that detect proapoptotic nCLU and secretory sCLU and a streptavidin-peroxidase technique. Both the proapoptotic nCLU and the antiapoptotic sCLU were detected in a proportion of malignant melanomas and metastases of malignant melanoma, but not in acquired melanocytic nevi. Additionally, we analysed the expression of clusterin in various melanoma cell lines (MeWo, SkMel28). All melanoma cell lines analysed revealed strong expression of

clusterin mRNA and protein. Interestingly, the clusterin mRNA and protein levels were regulated time-dependently by treatment of melanoma cells with 1,25-dihydroxyvitamin D_3. Regulation of clusterin mRNA expression by 1,25-dihydroxyvitamin D_3 was confirmed by measuring clusterin promoter activity (luciferase assay). Our findings indicate that: (i) in contrast to benign acquired melanocytic nevi, clusterin is expressed in primary cutaneous malignant melanomas, metastases of malignant melanoma and melanoma cell lines; (ii) clusterin expression is regulated time-dependently by 1,25-dihydroxyvitamin D_3, indicating that antiproliferative effects of 1,25-dihydroxyvitamin D_3 on melanoma cell lines may be at least in part mediated *via* regulation of clusterin expression; (iii) clusterin may be of importance for the growth characteristics of melanoma cells.

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25-HYDROXYVITAMIN D IN PATIENTS WITH COLORECTAL CARCINOMA AND ADENOMA

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Vitamin D inhibits the proliferation of human colon cancer cell lines. Premature cancer mortality in areas with little sunlight may be due to inadequate exposure to ultraviolet-B radiation. Serum 25-hydroxyvitamin D [25(OH)D] is an indicator of vitamin D sufficiency. Therefore, the correlation between serum 25(OH)D and the risk of colorectal neoplasia was determined in this study. *Materials and Methods*: In a prospective open study, serum 25(OH)D was determined in patients who had undergone total colonoscopy in ten practices of gastroenterology in southwest Germany. Only patients with normal colon mucosa (controls), large adenomas (> 1cm) and colorectal carcinoma (CRC) were included into this study. Serum 25(OH)D was determined on the automated instrument Nichols Advantage (Nichols Institute Diagnostics, San Juan Capistrano, CA 92675, USA). All groups of patients were examined separately in summer (May to October) and winter (November to April). The patients answered a questionnaire about sunlight exposure, nutrition and intake of vitamins. *Results*: 25(OH)D was determined in 264 patients in the summer (155 males, 109 females) and in 275 patients in the winter (171 males and 104 females). The results in controls (n=112 in summer and 127 in winter) and patients with adenomas (n=107 in summer and 95 in winter) and CRC (n=45 in summer and 53 in winter) are shown in the table. Patients with CRC showed significantly lower serum 25(OH)D compared to controls and adenomas. The median 25(OH)D levels in patients with CRC were 19, 18, 16 and 14 μg/l (stage I, II, III and IV, resp.). Approximately half of the controls in the summer and winter had serum 25(OH)D levels below 25 μg/l.

Table. *Mean serum 25(OH)D levels (μg/l) in summer and winter in controls and patients with large adenomas and CRC.*

	Summer		Winter	
	Mean±S.D.	*t*-test	Mean±S.D.	*t*-test
Controls	25±15		26±14	
Adenomas	23±10	ns	24±11	n.s.
CRC	16±7	$p=0.0001$	21±11	

t-tests: Controls *versus* adenomas and CRC

Conclusion: Patients with CRC had significantly lower serum 25(OH)D levels compared to controls. Patients with large adenomas also had lower levels compared to controls, though the difference, was not statistically significant. Vitamin D may play a role in colorectal cancer prevention. Since nearly half of the controls had 25(OH)D levels below 25 μg/l, an effort should be taken to detect and treat vitamin D deficiency in Germany.

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VITAMIN D COMPOUNDS: CLINICAL DEVELOPMENT AS CANCER THERAPY AND PREVENTION AGENTS

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Vitamin D compounds have biologic activities which indicate that they may have a role in cancer treatment and prevention; they induce apoptosis, inhibit progression through cell cycle (G_1) arrest, induce differentiation, inhibit invasion and motility and reduce angiogenesis. It is argued that these effects cannot be achieved *in vivo* or in the clinic because of the toxicity of these agents. There is increasing evidence that this is not the case. Approaches to averting vitamin D toxicity *in vivo* include the use of potentially less toxic analogs, concomitant use of agents such as glucocorticoids or bisphosphonates, inhibiting intra-tumoral catabolism of vitamin D and modified dosing regimens and routes of administration. Each of these approaches has been developed preclinically and is being tested in clinical trials. Calcitriol can be given by mouth at doses as high as 28 μg QDx3 weekly (Trump *et al.*) and 2.6 μg/kg weekly (Beer *et al.*)

without any toxicity. Dosing is limited by apparently saturable absorption, not toxicity. Intravenous administration 96 μg weekly + gefitinib causes no toxicity. Our data indicate that AUC ~ 40-50 ng h/ml is an exposure associated with antitumor effects in preclinical models. This AUC is achieved at ~ 100 μg calcitriol *i.v.* Improved oral formulations, modulation of reduced bioavailablility and exposure through CYP24 inhibition and combinations of calcitriol and analogs with platinum analogs, EFG rTK inhibitors and taxanes antimetabolites are being evaluated in the laboratory and in the clinic. (Supported by NCI grants CA-95045, CA-67267 and CA-85142).

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THE EFFECT OF CALCITRIOL ANALOG ON BETA3 INTEGRIN-POSITIVE TUMORS

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The results of recent studies indicate that the new analogs of calcitriol will find an application in antitumor therapy, especially in combination with other treatment strategies. However, there is a need for further preclinical studies towards a better understanding of the mechanisms of calcitriol's biological activity before these compounds can be introduced into clinical studies. Integrins are cell surface receptors engaged in basic cell processes: adhesion, migration, proliferation and differentiation and, by consequence, in tumor progression. It has been shown that the vitamin D_3 compounds can influence integrin expression. The effects of calcitriol and its analog 1,24-$(OH)_2D_3$ [1,24-D_3], synthesized in order to avoid the hypercalcemic effect of calcitriol, were evaluated on αvβ3 integrin-positive mouse WEHI-3 leukemia, B16 melanoma and LLC lung cancer and human HS294T melanoma and Du-145 prostate cancer invasive properties. All the cells applied expressed a high level of αvβ3 (but not of αIIβ3) integrin. This expression was significantly reduced by the *in vitro* treatment of LLC and WEHI-3 (not B16, HS294T and Du-145) cells with calcitriol or 1,24-D_3. Both compounds inhibited proliferation of LLC and WEHI-3 cells after 72 or 96 hours of *in vitro* treatment. Moreover, the vitamins diminished the adhesion of LLC and WEHI-3 cells to fibrinogen (ligand for β3 integrin). Further, *in vitro* incubation of LLC cells with both agents retarded the growth of subcutaneous tumors in mice. Also, the [1,24-D_3] treatment of mice bearing LLC tumors retarded the growth of subcutaneous tumors and diminished the number of lung metastatic foci. Calcitriol did not affect subcutaneous tumor growth, but also diminished the number of lung metastatic foci. In all experiments, the biological activity of 1,24-D_3 was similar to or higher than that of calcitriol, which is in accordance with our previous experiments, showing higher antiproliferative but lower calcemic activities, and lower lethal toxicity of the new analog as compared to the parental drug.

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