Antineoplastic effects of 1,25(OH)$_2$D$_3$ and its analogs in breast, prostate and colorectal cancer

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Abstract

The active form of vitamin D$_3$, 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), is mostly known for its importance in the maintenance of calcium and phosphate homeostasis. However, next to its classical effects on bone, kidney and intestine, 1,25(OH)$_2$D$_3$ also exerts antineoplastic effects on various types of cancer. The use of 1,25(OH)$_2$D$_3$ itself as treatment against neoplasia is hampered by its calcemic side effects. Therefore, 1,25(OH)$_2$D$_3$-derived analogs were developed that are characterized by lower calcemic side effects and stronger antineoplastic effects. This review mainly focuses on the role of 1,25(OH)$_2$D$_3$ in breast, prostate and colorectal cancer (CRC) and the underlying signaling pathways. 1,25(OH)$_2$D$_3$ and its analogs inhibit proliferation, angiogenesis, migration/invasion and induce differentiation and apoptosis in malignant cell lines. Moreover, prostaglandin synthesis and Wnt/b-catenin signaling are also influenced by 1,25(OH)$_2$D$_3$ and its analogs. Human studies indicate an inverse association between serum 25(OH)D$_3$ values and the incidence of certain cancer types. Given the literature, it appears that the epidemiological link between vitamin D$_3$ and cancer is the strongest for CRC, however more intervention studies and randomized placebo-controlled trials are needed to unravel the beneficial dose of 1,25(OH)$_2$D$_3$ and its analogs to induce antineoplastic effects.

Key Words
- vitamin D
- analog
- breast cancer
- prostate cancer
- colorectal cancer

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Introduction

Vitamin D$_3$ is mostly known for its important functions to maintain calcium and phosphate homeostasis. Vitamin D$_3$ can be obtained from dietary sources, but most vitamin D$_3$ is generated in the human skin under the influence of sunlight (u.v.-B radiation). During this process 7-dehydrocholesterol is converted to previtamin D$_3$, an unstable molecule that is rapidly converted to vitamin D$_3$. However, vitamin D$_3$ must undergo two subsequent hydroxylations in the liver and kidneys respectively before becoming the active hormone 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$). The 25-hydroxylation is executed by different cytochrome P450 enzymes, including CYP2R1 and CYP27A1, forming the main circulating form 25-hydroxyvitamin D$_3$ (25(OH)D$_3$), which in turn undergoes a 1α-hydroxylation by CYP27B1 in the kidneys to produce 1,25(OH)$_2$D$_3$ (Fig. 1). Only one major enzyme degrades 1,25(OH)$_2$D$_3$, namely CYP24, which expression is upregulated by 1,25(OH)$_2$D$_3$ itself.

CYP27B1 and *CYP24A1* expressions in the kidneys are tightly regulated in order to maintain optimal 1,25(OH)$_2$D$_3$ levels. However, these metabolizing enzymes are also expressed in almost all nucleated cell types leading

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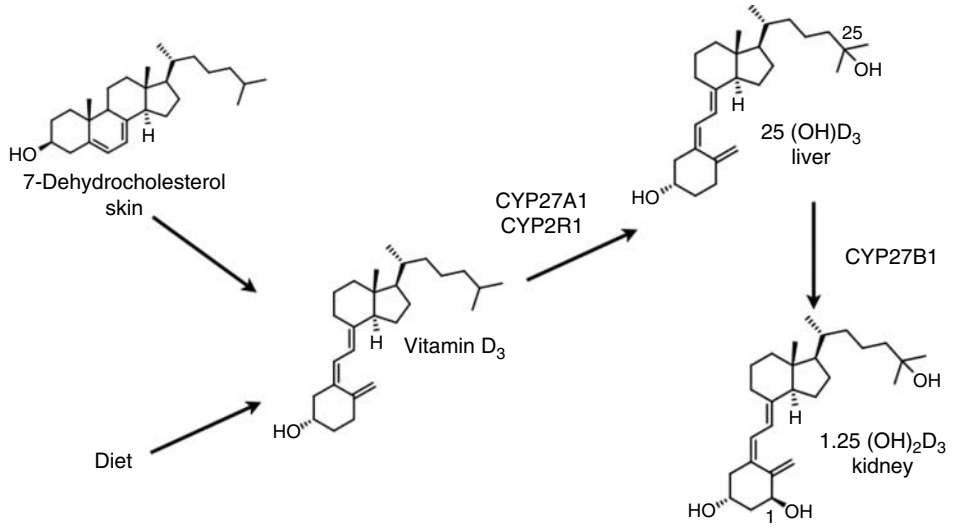

Figure 1
Synthesis of 1,25(OH)$_2$D$_3$. Vitamin D$_3$ is obtained from the diet or generated in the skin from 7-dehydrocholesterol. Two hydroxylation steps are required in the liver and kidneys respectively in order to obtain the hormonally active 1,25(OH)$_2$D$_3$.

to local 1,25(OH)$_2$D$_3$ synthesis (Flanagan *et al.* 2006, Kemmis *et al.* 2006). Locally expressed *CYP27B1* and *CYP24A1* are not regulated by calcium or the parathyroid hormone but are regulated by tissue-specific signals (Young *et al.* 2004, Kallay *et al.* 2005, van Etten *et al.* 2008).

1,25(OH)$_2$D$_3$ binds to the vitamin D receptor (VDR) which is expressed in almost all cell types. After binding the ligand, VDR will heterodimerize with retinoid X receptor and translocate to the nucleus to bind vitamin D$_3$ responsive elements (VDREs) in the promoter regions of target genes in order to positively or negatively regulate their transcription. In the absence of 1,25(OH)$_2$D$_3$, several corepressors block the VDRE of target genes and deacetylate histones in order to keep the chromatin in a dense configuration (Tagami *et al.* 1998). Upon binding of 1,25(OH)$_2$D$_3$ to its receptor a conformational change in the 1,25(OH)$_2$D$_3$/VDR complex occurs, leading to loss of corepressors and attraction of coactivators which will open the chromatin structure, resulting in transcription of target genes. Increased expression of corepressors could be one of the mechanisms by which aggressive cancer cells lose responsiveness to 1,25(OH)$_2$D$_3$ treatment and escape the antiproliferative effects of 1,25(OH)$_2$D$_3$ (Khanim *et al.* 2004, Ting *et al.* 2007).

Next to the classical effects of 1,25(OH)$_2$D$_3$ on bone, kidney and intestine, more research has focused on the nonclassical effects of 1,25(OH)$_2$D$_3$, like cardiovascular, immunomodulatory and antineoplastic effects. However, using 1,25(OH)$_2$D$_3$ itself as treatment against neoplasia is hampered due to its calcemic side effects. In order to induce antineoplastic effects, 1,25(OH)$_2$D$_3$ doses of the nanomolar range are required while normal serum 1,25(OH)$_2$D$_3$ levels are of the picomolar range. This led to the development of 1,25(OH)$_2$D$_3$-derived analogs that are characterized by lower calcemic side effects and stronger antineoplastic effects.

Several microarray studies on cancer cells treated with 1,25(OH)$_2$D$_3$ or one of its analogs show that 1,25(OH)$_2$D$_3$ influences the transcription of a wide variety of genes suggesting a pleiotropic regulatory role for 1,25(OH)$_2$D$_3$ (Swami *et al.* 2003, Pike 2011). The majority of these genes are involved in cell growth, apoptosis, cell signaling, cell adhesion, cell metabolism, immune regulation, redox status, angiogenesis and metastasis. However, significant discrepancies in these microarrays are found when different types of cancer cells are used. This is explained by different molecular mechanisms that 1,25(OH)$_2$D$_3$ causes in different cell types, so therefore 1,25(OH)$_2$D$_3$ is thought to induce cell-specific gene regulations (Krishnan *et al.* 2004). Clearly, early-stage cancer cells respond better to 1,25(OH)$_2$D$_3$ or an analog and gene regulation in these cells differs from more malignant cancer cells (Lee *et al.* 2006). The antineoplastic effects of 1,25(OH)$_2$D$_3$ and its analogs will be reviewed in this paper focusing on breast cancer (BC), prostate cancer (PC) and colorectal cancer (CRC), since most research has been carried out in these

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cancer types. The Pubmed database (2000–2012) was searched with the following keywords: vitamin D or ergocalciferol and BC, PC, CRC or colon cancer.

In vitro antineoplastic effects of 1,25(OH)$_2$D$_3$

Mechanisms involved in antineoplastic effects

Effects on proliferation and differentiation The best and earliest described antineoplastic effects of 1,25(OH)$_2$D$_3$ include the antiproliferative and pro-differentiating effects on cancer cells *in vitro* and *in vivo*. Cell lines expressing the VDR demonstrate higher cell numbers in the G_0/G_1 phase of the cell cycle after 1,25(OH)$_2$D$_3$ stimulation (Jensen *et al*. 2001). This antiproliferative effect of 1,25(OH)$_2$D$_3$ was first described in malignant melanoma cells (Colston *et al*. 1981), and is now widely demonstrated in many other cell types. The exact mechanism of action behind the 1,25(OH)$_2$D$_3$-mediated growth inhibition can differ depending on cell type. The most suggested mechanism influences the complex formation of pocket proteins of the retinoblastoma (Rb) family with E2F transcription factors. This complex dissociates after phosphorylation of Rb proteins by cyclin-dependent kinases (CDK). E2F transcription factors are then able to activate target genes, essential for cell cycle progression (Jensen *et al*. 2001, Verlinden *et al*. 2005). 1,25(OH)$_2$D$_3$ inhibits different cyclins and CDKs resulting in an intact Rb–E2F complex and inhibition of cell proliferation (Wang *et al*. 1997, Park *et al*. 2000*b*). However, when Rb is knocked out in 1,25(OH)$_2$D$_3$-stimulated PC cells other growth inhibitory pathways compensate the loss of Rb (Washington *et al*. 2010). Pocket proteins P107 and P130 are also essential for the growth inhibitory effects of 1,25(OH)$_2$D$_3$ since cells losing these pocket proteins will continue cell cycle progression after 1,25(OH)$_2$D$_3$ stimulation (Verlinden *et al*. 2007). 1,25(OH)$_2$D$_3$ also upregulates CDK inhibitors such as P21 and P27 (Wade *et al*. 2002, Tavera-Mendoza *et al*. 2006). The upregulation of *P27* (*CDKN1B*) by 1,25(OH)$_2$D$_3$ is due to an enhanced *P27* gene transcription and the transcriptional repression of *P45* (*SKP2*), which is implicated in *P27* degradation (Huang & Hung 2006).

1,25(OH)$_2$D$_3$ is also able to modulate cellular growth by influencing other important signaling pathways. The transforming growth factor-β (TGF-β) signalization pathway is activated by 1,25(OH)$_2$D$_3$ and contributes to the antiproliferative effects of 1,25(OH)$_2$D$_3$ (Chen *et al*. 2002) possibly by mediating coassociations between CDK2, P27 and cyclin E (Scaglione-Sewell *et al*. 2000). Inhibition of epidermal growth factor receptor (EGFR) expression by 1,25(OH)$_2$D$_3$ is also thought to aid cell growth inhibition (McGaffin & Chrysogelos 2005, Belochitski *et al*. 2007) as well as the downregulation of survivin, an inhibitor of apoptosis (Li *et al*. 2005, Koike *et al*. 2011) and platelet-derived growth factor down-regulation by 1,25(OH)$_2$D$_3$ (Nazarova *et al*. 2005). A study with CRC cells suggests that 1,25(OH)$_2$D$_3$-mediated antiproliferative effects are dependent on the dual role of the VDR: first, as a transcriptional factor and secondly, as a nongenomic activator of the Rho-ROCK-p38MAPK-MSK signaling pathway (Ordonez-Moran *et al*. 2008).

Effects on apoptosis 1,25(OH)$_2$D$_3$ is able to induce apoptosis in different tumor models, but the exact mechanism behind this effect is not clear (Simboli-Campbell *et al*. 1996, Park *et al*. 2000*a*). Changes in the expression or cellular distribution of B-cell lymphoma 2 antiapoptotic proteins are a possible mechanism of 1,25(OH)$_2$D$_3$-mediated apoptosis (James *et al*. 1996, Zhang & Yao 2000, Wagner *et al*. 2003). Apoptosis after 1,25(OH)$_2$D$_3$ stimulation is also associated with the upregulation of the proapoptotic protein Bcl-2 homologous antagonist/killer (Diaz *et al*. 2000) or could be a result of the interaction between 1,25(OH)$_2$D$_3$ and other signaling pathways such as tumor necrosis factor-α (McGuire *et al*. 2001, Weitsman *et al*. 2004, Golovko *et al*. 2005). A study on PC cells suggests that 1,25(OH)$_2$D$_3$ activates the intrinsic apoptotic pathway, since 1,25(OH)$_2$D$_3$ activates caspase-3 and -9 and stimulates cytochrome *c* release from mitochondria (Guzey *et al*. 2002). Caspase-3 is even thought to cleave and inactivate the VDR during apoptotic induction, however it is not known if this occurs under nonapoptotic circumstances (Malloy & Feldman 2009). Pretreating CRC cells with 1,25(OH)$_2$D$_3$ sensitizes these cells to acute and chronic reactive oxidation species-induced cell death, which may be one of the ways in which 1,25(OH)$_2$D$_3$ exerts its chemopreventive/therapeutic effects (Koren *et al*. 2006). On the other hand, VDR ablation in BC cells abolishes the inhibitory effect on cell growth, while the effects on apoptosis remain the same, suggesting that the VDR does not play a major role in the apoptotic effects of 1,25(OH)$_2$D$_3$ (Zinser *et al*. 2003). Indeed, another study on BC cells shows an increase in intracellular calcium concentrations after 1,25(OH)$_2$D$_3$ stimulation, being a rapid, nongenomic effect that does not involve the VDR. In cancer cells, in contrast to normal mammary cells, this calcium increase induces calpain-mediated apoptosis (Sergeev 2004).

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Effects on angiogenesis The formation of new blood vessels is necessary for malignant tumor growth. $1,25(OH)_2D_3$ inhibits angiogenesis, since treatment of several human cancer cell lines with $1,25(OH)_2D_3$ results in a decrease in hypoxia-inducible factor-1 α (HIF1A) expression, which is the most important transcription factor in angiogenesis. Also its target genes, such as vascular endothelial growth factor (VEGF), are inhibited by $1,25(OH)_2D_3$ and this inhibition is mediated by an HIF1A-dependent pathway since $1,25(OH)_2D_3$ is not able to inhibit VEGF expression in HIF1A knockout (KO) cells (Ben-Shoshan *et al.* 2007). In PC cells $1,25(OH)_2D_3$ is able to repress interleukin 8 (IL8), one of the most important angiogenic factors secreted by PC cells (Bao *et al.* 2006*a*). Moreover, $1,25(OH)_2D_3$ also inhibits an upstream regulator of IL8, namely nuclear factor kappa B (NF-κB), which is thought to be partly responsible for the $1,25(OH)_2D_3$-mediated IL8 inhibition. The parathyroid hormone-related protein augments intratumoral vessel density and VEGF expression in PC cells, but these effects are reversed when cells are treated with the EB1089 vitamin D_3 analog (Bhatia *et al.* 2009). Moreover, when tumor-derived endothelial cells are injected into VDR KO mice, the resulting tumors are characterized by larger blood vessels, more vascular leaking and a higher expression of HIF1A and VEGF (Chung *et al.* 2009). The loss of VDR eventually leads to abnormal tumor angiogenesis and aberrant angiogenic signaling. However, when different rodent strains with PC are treated with $1,25(OH)_2D_3$, angiogenesis is not influenced (Oades *et al.* 2002) and adding $1,25(OH)_2D_3$ to the SW480-ADH CRC cell line increases VEGF levels, in contrast to the earlier mentioned studies. These data suggest the possibility that the effects of $1,25(OH)_2D_3$ on angiogenesis of tumor cells may be tumor and cell type dependent (Fernandez-Garcia *et al.* 2005).

Effects on invasion and migration Invasion of a tumor in the surrounding tissues is an important hallmark of cancer and research on different cell types shows that $1,25(OH)_2D_3$ and its analogs inhibit the invasiveness of human cancer cells (Chen *et al.* 2007). In LNCaP cells, the activation of the c-Jun N-terminal kinases/stress-activated protein kinases, mitogen-activated protein kinase (JNK/SAPK MAPK) signaling pathway by $1,25(OH)_2D_3$ is essential for its antiinvasive effects (Larsson *et al.* 2008). Other studies find decreased matrix metalloproteinase-2 and -9 (enzymes involved in the breakdown of the extracellular matrix) and cathepsin (a proteinase) activity (Tokar & Webber 2005, Bao *et al.* 2006*b*, Iglesias-Gato *et al.* 2011); and a decreased expression of α6-integrins, β4-integrins (Sung & Feldman 2000) and intracellular adhesion molecule 1 (Stio *et al.* 2011) after treating cancer cells with $1,25(OH)_2D_3$/analog. $1,25(OH)_2D_3$ regulates different components of the plasminogen activator system, which controls fibrin degradation in malignant cells (Koli & Keski-Oja 2000). Tissue-type plasminogen activator is stimulated by $1,25(OH)_2D_3$ in osteosarcoma cells via VDREs in the human tissue-type plasminogen activator enhancer (Merchiers *et al.* 1999). Plasminogen activator inhibitor-1 on the other hand is downregulated by $1,25(OH)_2D_3$ through blockage of NF-κB (Chen *et al.* 2010). $1,25(OH)_2D_3$ also mediates the inhibition of vimentin, an intermediate filament protein that is associated with loss of differentiation and acquisition of motility (Tokar & Webber 2005). E-cadherin, on the other hand, is upregulated by $1,25(OH)_2D_3$ in SW480-ADH cells. Phosphatidylinositol 5-phosphate 4-kinase type IIB is required for this induction and this kinase is known to play a role in $1,25(OH)_2D_3$-mediated inhibition of cellular motility (Kouchi *et al.* 2011). Loss of E-cadherin induces epithelial–mesenchymal cell transition via disruption of cell adhesion. Similar findings are reported in a study where increased levels of E-cadherin expression are accompanied with repressed cell rolling and reduced adhesion of the cancer cells to the endothelium (Hsu *et al.* 2011).

Moreover, vitamin D_3 deficiency promotes the growth of BC cells in an *in vivo* model for bone metastasis (Ooi *et al.* 2010). A high vitamin D_3 diet does not change the incidence of metastasis in a CRC rat model, however supplementing the diet with an analog (Ro 25-9022 or Ro 25-5317) significantly decreases metastasis (Evans *et al.* 2000). When immune compromised mice are transplanted with human BC cells, the formation of metastasis is completely inhibited when mice are treated i.p. with the 'Deuterated Gemini' analog, while $1,25(OH)_2D_3$ is able to reduce metastasis formation with 50% (Spina *et al.* 2007). All these results suggest that $1,25(OH)_2D_3$ and its analogs reduce the invasive and migration capacities of cancer cells by mediating changes in the tumor cell–extracellular matrix interaction as well as by promoting cell–cell contact.

Effects on inflammation and inflammatory pathways Patients suffering from chronic inflammatory conditions are at higher risk of developing cancer, such as inflammatory bowel disease patients who have an increased risk of developing CRC (Dyson & Rutter 2012) or lesions in the prostate called proliferative inflammatory atrophy, which are associated with acute or chronic inflammation and are thought to precede prostate

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intraepithelial neoplasia (PIN) and PC (De Marzo *et al.* 2007). It is already well known that 1,25(OH)$_2$D$_3$ exerts immunomodulatory effects, such as stimulation of the native immune system and inhibition of the adaptive immune system. When immortalized PC cells are treated with 1,25(OH)$_2$D$_3$, transcript levels of *IL1*, *IL6* and *IL17* pathway members are suppressed (Kovalenko *et al.* 2010). 1,25(OH)$_2$D$_3$ also inhibits the expression of IL6 in adenocarcinoma PC cells (Nonn *et al.* 2006) and the vitamin D analog BXL-628 inhibits the production of proinflammatory cytokines and chemokines in human benign prostatic hyperplasia cells (Adorini *et al.* 2007). Moreover, when mice are given a modified diet with more fat and less vitamin D, calcium and fibers, augmented serum levels of IL1B and its targets are measured. Supplementing these mice with vitamin D and calcium prevents or mitigates this effect (Bastie *et al.* 2012). As mentioned before, 1,25(OH)$_2$D$_3$ inhibits NF-κB signalization by acting on different members of this pathway (Bao *et al.* 2006*a*). 1,25(OH)$_2$D$_3$ strongly represses the *P65* (*RELA*) subunit transactivation in BC, PC and CRC cells while it also induces the expression of the NF-κB pathway inhibitor, IκBa (Sun *et al.* 2008, Tse *et al.* 2010).

Interference with other signaling pathways

Effects on prostaglandin synthesis Next to the effects on proliferation, apoptosis, angiogenesis, cell invasion and inflammation, 1,25(OH)$_2$D$_3$ can also influence prostaglandin synthesis (Fig. 2). Prostaglandin promotes carcinogenesis and facilitates cancer progression. In BC cells higher levels of cyclooxygenase 2 (COX2), the enzyme responsible for the synthesis of prostaglandins, and lower expression of 15-prostaglandin dehydrogenase, the enzyme responsible for degrading prostaglandin, are found (Thill *et al.* 2009). Moreover, in these cells lower VDR expression seems to be associated with higher COX2 expression. In human BC samples higher levels of COX2 and lower levels of VDR are found in malignant tumors (Thill *et al.* 2010). When 1,25(OH)$_2$D$_3$ is added to cancer cell lines, most studies agree that lower concentrations of prostaglandin are found compared with vehicle-stimulated cells. Indeed, 1,25(OH)$_2$D$_3$ decreases the levels of COX2 and induces 15-prostaglandin dehydrogenase, which results in a reduction of local prostaglandin concentrations. Moreover, 1,25(OH)$_2$D$_3$ treatment leads to a reduced expression of prostaglandin receptors (Moreno *et al.* 2005, Krishnan *et al.* 2007).

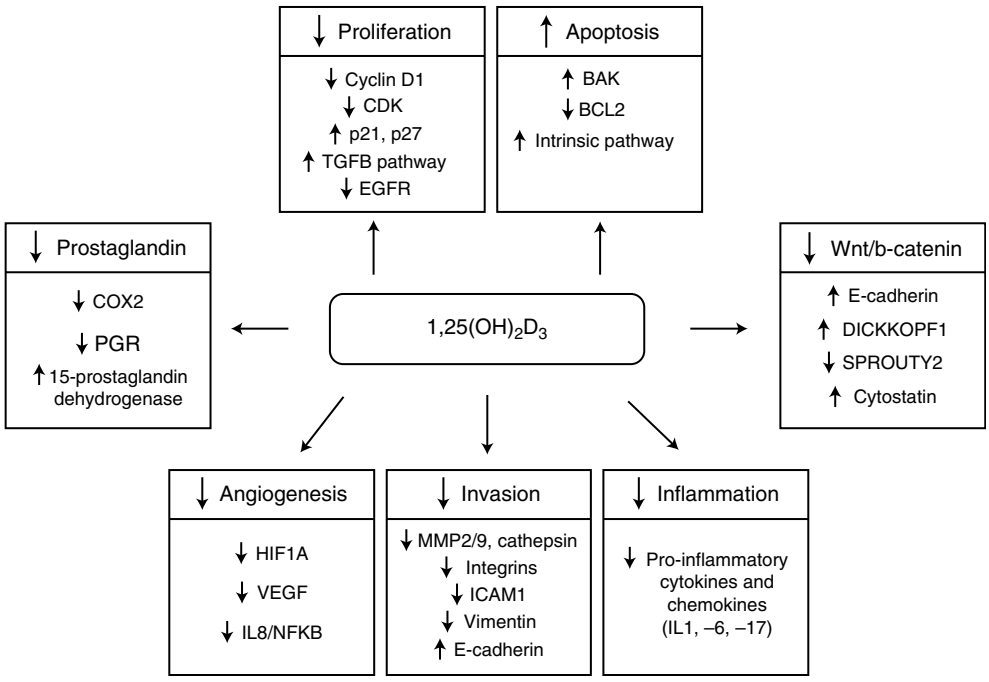

Figure 2
Schematic overview of several antineoplastic effects of 1,25(OH)$_2$D$_3$. 1,25(OH)$_2$D$_3$ is able to modulate several genes and pathways involved in cell proliferation, apoptosis, angiogenesis, invasion and inflammation. Moreover, 1,25(OH)$_2$D$_3$ influences the production of prostaglandin and interferes with Wnt/b-catenin signaling.

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Wnt/b-catenin signaling The molecular mechanisms behind the antineoplastic effects of 1,25(OH)$_2$D$_3$ have been extensively studied in CRC. 1,25(OH)$_2$D$_3$ blocks the main deregulated pathway in CRC, namely the Wnt/b-catenin pathway. The tumor suppressor gene adenomatous polyposis coli (*APC*), which is considered as the gatekeeper gene during CRC development (Wasan *et al*. 1998), is bound to a b-catenin complex in the absence of a Wnt ligand and is degraded by the proteasome. After Wnt binds to its receptor or in case of an activating mutation of *APC*, β-catenin accumulates in the cell cytoplasm and translocates to the nucleus where it binds T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors and influences the expression of genes such as *c-MYC* (*MYC*). Additional mutations in the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*), *P53* gene and TGFB pathway eventually result in the progression of early aberrant crypt foci to colon adenocarcinoma. 1,25(OH)$_2$D$_3$ suppresses b-catenin/TCF transcriptional activity and their target genes via several mechanisms. 1,25(OH)$_2$D$_3$ induces E-cadherin expression which can bind b-catenin and thus suppresses the translocation of b-catenin to the nucleus. Secondly, the 1,25(OH)$_2$D$_3$/VDR complex also competes with TCF4 transcription factors to bind b-catenin (Palmer *et al*. 2001), resulting in lower expression of *c-MYC*. DICKKOPF 1, an extracellular Wnt antagonist, is stimulated by 1,25(OH)$_2$D$_3$ (Aguilera *et al*. 2007), while SPROUTY 2, a protein that is upregulated in high-grade tumors and inhibits E-cadherin expression, is inhibited by 1,25(OH)$_2$D$_3$ (Barbachano *et al*. 2010). 1,25(OH)$_2$D$_3$ also induces cytostatin D expression which inhibits cell proliferation, migration, Wnt/b-catenin signaling and induces E-cadherin and other adhesion molecules (Alvarez-Diaz *et al*. 2009). *Apc*$^{min/+}$ mice spontaneously develop tumors in the small and large intestine and are a commonly used model for intestinal cancer. Treating these mice with 1,25(OH)$_2$D$_3$/analogs decreases the nuclear translocation of b-catenin and the expression of TCF1 transcription factors, while the tumor suppressor activity of E-cadherin is enhanced (Xu *et al*. 2010).

In vivo studies

Many studies have used vitamin D$_3$ deficient or VDR KO mice for a better understanding of the link between vitamin D$_3$ and the development and progression of cancer. A vitamin D$_3$-deficient diet leading to 25(OH)D$_3$ serum levels <6 ng/ml promotes the growth of human BC cells in the bones of nude mice (Ooi *et al*. 2010). Similar results are obtained in *Balb/C* mice, which were given a vitamin D$_3$-deficient diet and afterward injected with cancer cells (Tangpricha *et al*. 2005). Also, a vitamin D$_3$-deficient diet induces more proliferation and less apoptosis (Kovalenko *et al*. 2011) as well as a higher tumor growth in prostatic tissue (Ray *et al*. 2012). Since the Western diet is believed to play a role in the development of cancer and especially in that of CRC, a rodent diet with high fat and low calcium and vitamin D$_3$ levels was created to mimic human Western dietary habits. Feeding rodents with this Western diet promotes colonic tumor formation, however supplementing these animals with sufficient levels of calcium and vitamin D$_3$ reverses these effects (Yang *et al*. 2008*a*,*b*, Newmark *et al*. 2009). Moreover, the Western diet supplemented with calcium and vitamin D$_3$ leads to less hyperproliferation and hyperplasia in breast glands of mice (Kurihara *et al*. 2008). Also, supplementing the diet with 5000 IU vitamin D/kg diet inhibits tumor growth in xenograft models of PC and BC (Swami *et al*. 2012).

VDR KO mice show higher levels of proliferation and oxidative stress in the distal part of the colon (Kallay *et al*. 2001) and are more sensitive to carcinogenic products (Zinser *et al*. 2003). The progression of long probasin promoter-large T-antigen prostate tumors was compared in VDR KO and WT mice, revealing that VDR KO mice develop PC more quickly than their VDR WT/LPB-Tag littermates and that these VDR KO tumors display more proliferation (Mordan-McCombs *et al*. 2010). Crossing VDR KO mice with *Apc*$^{min/+}$ mice does not lead to the formation of more intestinal malignancies, however the tumor size is bigger compared with VDR WT/*Apc*$^{min/+}$ mice (Larriba *et al*. 2011, Zheng *et al*. 2011). Many studies investigated the effect of 1,25(OH)$_2$D$_3$ and its analogs on tumor development in rodents with BC, PC or CRC. Most studies agree that 1,25(OH)$_2$D$_3$ and its analogs are able to inhibit tumor cell growth (Verlinden *et al*. 2000, Oades *et al*. 2002, Milliken *et al*. 2005, Lee *et al*. 2008, 2010, Okamoto *et al*. 2011) without effects on tumor formation. However, some studies suggest that 1,25(OH)$_2$D$_3$ is also able to inhibit the formation of premalignant lesions *in vivo* like aberrant crypt foci in CRC (Xu *et al*. 2010, Hummel *et al*. 2012) and PIN (Banach-Petrosky *et al*. 2006).

In vitro data demonstrate that 1,25(OH)$_2$D$_3$ and its analogs clearly affect proliferation, differentiation, apoptosis, angiogenesis, invasion and inflammation of malignant cells. *In vivo* data mostly indicate that 1,25(OH)$_2$D$_3$ and its analogs are able to inhibit tumor growth due to its antiproliferative and prodifferentiating effects as well as by influencing other important processes such as angiogenesis, invasion and inflammation, while

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actual tumor formation seems less influenced. Also, a locally low vitamin D_3 status may influence tissues in a way that these tissues are more sensitive to early procarcinogenic events. Using 1,25(OH)$_2$D$_3$ or its analogs alone as cancer treatment on the other hand is not sufficient, since 1,25(OH)$_2$D$_3$ is not able to eradicate tumor cells. Therefore, 1,25(OH)$_2$D$_3$ and its analogs could be combined with cytotoxic products when used for cancer treatment.

Human studies

VDR, CYP27B1 and CYP24A1 expressions in cancer

Locally produced 1,25(OH)$_2$D$_3$ does not contribute to calcium homeostasis, but is believed to exert autocrine/paracrine effects. Elevated as well as decreased CYP24A1 or CYP27B1 expressions are reported in different cancer cell lines (Whitlatch *et al*. 2002, Fischer *et al*. 2009, Matilainen *et al*. 2010). On the contrary, most studies on human cancer biopsies agree with the following hypothesis. The expression of VDR and CYP27B1 increases initially when a tumor develops, but while the tumor becomes more malignant and starts to dedifferentiate, the expression of VDR and CYP27B1 decreases while the expression of CYP24A1 strongly increases in human tissues of BC and CRC (Bareis *et al*. 2001, Bises *et al*. 2004, Matusiak & Benya 2007, Lopes *et al*. 2010). This suggests that during early tumorigenesis the synthesis and signaling of 1,25(OH)$_2$D$_3$ are upregulated as a physiological defense system against epithelial tumor progression. When tumors dedifferentiate, VDR and CYP27B1 levels drop while CYP24A1 expression increases, implicating that local 1,25(OH)$_2$D$_3$ concentrations decrease since less 1,25(OH)$_2$D$_3$ is synthesized while more is metabolized. The sequential acquisition of mutations that occur during tumor progression and metastasis could possibly negatively influence the expression of 1,25(OH)$_2$D$_3$-metabolizing enzymes (Cross *et al*. 2001). Changes have also been reported in the adjacent, normal tissue of cancer patients. Studies using CRC or BC samples report a decrease in *CYP27B1* expression in normal tissue adjacent to the tumor (Ogunkolade *et al*. 2002, McCarthy *et al*. 2009). It is possible that tumors secrete endocrine/paracrine factors, which influence *CYP27B1* expression, but other studies suggest that this downregulation of *CYP27B1* is caused by hypermethylation of its promoter (Shi *et al*. 2002). Decreased VDR expression ratios are found in the nucleus, compared with the cytoplasm of neoplastic lesions, which suggests that less VDR translocates to the nucleus during tumor progression (Matusiak *et al*. 2005). Moreover, when oncogenes are introduced into mammary epithelial cells, CYP27B1 and VDR expressions decrease (Kemmis & Welsh 2008). Most of these studies are based on mRNA, western blot and immunohistochemistry techniques, while not many studies investigate the enzymatic activity of CYP27B1 and CYP24A1. Also, these observational studies cannot distinguish if changes in VDR, CYP27B1 and CYP24A1 are a cause or rather a consequence of carcinogenesis (Whitlatch *et al*. 2002).

Observational epidemiological studies

Garland & Garland (1980) were the first to report that CRC mortality in the United States is higher in areas where people are less exposed to natural sunlight. Since this observation, several studies in different regions of the world have confirmed that the risk of BC (Mohr *et al*. 2008, Anderson *et al*. 2011), PC (John *et al*. 2007, Gilbert *et al*. 2009) and CRC (Grant 2002, Boscoe & Schymura 2006) augments when people are less exposed to sunlight and u.v.-B radiation or when the area of residence lies at higher latitudes where less solar exposure may lead to vitamin D deficiency (Grant 2011). Besides u.v.-B exposure, also skin pigmentation influences vitamin D status. Higher pigmentation protects against u.v.-B radiation and is correlated with latitude, leading to a decreased 1,25(OH)$_2$D$_3$ production. Recent studies in the United States have shown that 25(OH)D$_3$ serum levels are lower in subjects with African ancestry compared with subjects with a Caucasian ancestry (Murphy *et al*. 2012, Yao *et al*. 2012). African Americans are also at higher risk of developing BC, CRC and PC as well as more aggressive and advanced tumors (Reddy *et al*. 2003, Fiscella *et al*. 2010, Yao & Ambrosone 2012). Other studies also suggest an inverse association between vitamin D_3 intake and the risk of developing cancer (John *et al*. 1999, Lin *et al*. 2007, Oh *et al*. 2007). However, measuring 25(OH)D$_3$ serum levels is currently the golden standard for evaluation of the vitamin D status since concentrations of 1,25(OH)$_2$D$_3$ are tightly regulated by the renal metabolizing enzymes in order to maintain calcium homeostasis (Millen *et al*. 2010). Synthesis of 25(OH)D$_3$ on the other hand is not strictly regulated and combines the exposure to sunlight as well as the dietary/supplemental intake of vitamin D_3. Moreover, while the half-life of 1,25(OH)$_2$D$_3$ is only 4–6 h, 25(OH)D$_3$ has a half-life of 3 weeks. Several studies investigated the relationship between serum 25(OH)D$_3$ levels and the risk of developing cancer. For most cancer types, the results are conflicting. However, the majority of

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observational, postdiagnostic studies on CRC report a significant inverse association between 25(OH)D_3 serum levels and the risk for CRC or colorectal adenoma (Fedirko *et al*. 2010*a*, Jenab *et al*. 2010, Lee *et al*. 2011). Some of these studies find that this association is even stronger for more advanced cancers or for distal and rectal tumors (Wei *et al*. 2008, Lee *et al*. 2011). However, postdiagnostic measurements may not represent the 25(OH)D_3 values during cancer initiation and early progression. This can be overcome by measuring 25(OH)D_3 concentrations before cancer diagnosis. A prediagnostic study reports that CRC patients with higher 25(OH)D_3 values tend to have a better outcome prognosis than CRC patients with lower 25(OH)D_3 levels (Ng *et al*. 2008). Most prediagnostic studies in the United States and Europe find an inverse association between 25(OH)D_3 levels and CRC risk (Wu *et al*. 2007, Freedman *et al*. 2010, Woolcott *et al*. 2010). In a European study a 40% reduced chance of developing CRC is found when 25(OH)D_3 levels are above 33.4 ng/ml compared with levels under 16.1 ng/ml (Jenab *et al*. 2010). In contrast, a Finnish study reports an increased colon cancer risk when serum 25(OH)D_3 levels are elevated (>30 ng/ml), however this study only included male smokers and mean 25(OH)D_3 levels were relatively low compared with the other prediagnostic studies (Weinstein *et al*. 2011). Others only describe an augmented risk for rectal cancer (Otani *et al*. 2007) or cancer in the distal part of the colon (Feskanich *et al*. 2004) for subjects with lower 25(OH)D_3 values. For BC and PC the association with lower 25(OH)D_3 levels is not so clear. One postdiagnostic BC study reports a stronger association in women with estrogen receptor (ER)-negative tumors (Yao *et al*. 2011). Other studies find an inverse association between serum 25(OH)D_3 levels and the recurrence of BC or BC mortality (Goodwin *et al*. 2009, Vrieling *et al*. 2011) or the size of the tumor (Hatse *et al*. 2012). Another study did not find associations between lower serum 25(OH)D_3 levels and increased risk of recurrence in BC survivors (Jacobs *et al*. 2010). A limited number of studies compared prediagnostic 25(OH)D_3 serum levels with BC risk but results remain conflicting. The Nurses Health Study finds an inverse association between 25(OH)D_3 levels and BC risk which is more pronounced in women of 60 years or older (Bertone-Johnson *et al*. 2005). Two other prospective studies with postmenopausal women in the United States did not find evidence that higher 25(OH)D_3 levels lead to a decreased BC risk (Freedman *et al*. 2008, McCullough *et al*. 2009). However, one of these studies found a nonsignificant decreased BC risk for women with 25(OH)D_3 values above 23.5 ng/ml compared with 25(OH)D_3 levels lower than 18.3 ng/ml. A Danish study showed that women with 25(OH)D_3 levels of 33.5 ng/ml or more have a 48% reduced risk of BC compared with women with levels lower than 24 ng/ml (Rejnmark *et al*. 2009). This reduced BC risk was even more pronounced in premenopausal women. Another European study also found an inverse association between BC risk and 25(OH)D_3 serum levels after a follow-up of ~10 years, which was also more pronounced in younger women (Engel *et al*. 2010). On the other hand, a Swedish study found a weak association after a follow-up of 10–15 years (Almquist *et al*. 2010). The mean 25(OH)D_3 values in this study were very high (35.5 ng/ml) and the cutoff between low and high 25(OH)D_3 serum levels was relatively high (30 ng/ml). For PC, the link between low 25(OH)D_3 levels and augmented cancer risk is also not clear. In most prediagnostic Nordic studies, an inverse association is found between 25(OH)D_3 levels and PC (Ahonen *et al*. 2000). In contrast, several prediagnostic studies in the United States do not find this association (Travis *et al*. 2009, Barnett *et al*. 2010). Then again, in the Nordic studies almost half of the subjects were vitamin D_3 deficient compared with 20% in the US studies (Ahn *et al*. 2008). It appears that only subjects with very low 25(OH)D_3 serum levels are at higher risk for PC. In contrast, some studies suggest that also higher 25(OH)D_3 levels increase the risk of developing PC (Tuohimaa *et al*. 2004, Shui *et al*. 2012). Other prediagnostic studies find that lower 25(OH)D_3 values are associated with a higher risk for aggressive PC (Li *et al*. 2007) or with lethal PC (Fang *et al*. 2011). Yet, it is still rather difficult to compare different observational studies due to substantial differences in 25(OH)D_3 serum values since diverse assays to measure 25(OH)D_3 are currently available on the market and because control subjects are selected in different ways. Moreover, disparities between cutoff points exist and could be due to differences in sun exposure and latitude of the study but also to differences in food fortification. In addition, most studies base their results on a single 25(OH)D_3 measurement, while this may not be reflective for long-term levels of circulating 25(OH)D_3. The exact time frame in which 25(OH)D_3 plays an important role for cancer development and progression is not known. Prediagnostic measurements can be taken too early, but on the other hand, postdiagnostic measurements can be taken too late and can be prone to inverse causality since it is not clear if low 25(OH)D_3 levels are a causative effect or a result of cancer. When diagnosed, chemotherapy and behavioral changes of the patients (less sun exposure and physical activity, less food intake,

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nausea, etc.) can result in lower 25(OH)D_3 values. It is also not clear to what extent 25(OH)D_3 serum values are representative for the local tissue vitamin D status. Taken together, these studies indicate that the inverse association between serum 25(OH)D_3 levels and cancer risk is probably the strongest for CRC, while for other cancers results are inconsistent. Moreover, only randomized clinical trials are able to investigate if there is a causal relationship between vitamin D_3 levels and the incidence of cancer. Future prediagnostic observational studies should include several 25(OH)D_3 serum measurements and longer follow-up periods in order to determine the exact time frame in which vitamin D_3 levels are crucial for cancer initiation or progression. Furthermore, it is of interest to establish the local tissue 25(OH)D_3/1,25(OH)$_2$-D_3 levels to investigate if 25(OH)D_3 serum measurements are representative for the local vitamin D status in tissues.

Clinical trials

If a low vitamin D_3 status increases the risk of developing cancer, then clinical randomized trials should reveal a decrease in cancer risk when subjects are supplemented with vitamin D_3 (Tables 1 and 2). The Women's Health Initiative designed a randomized placebo-controlled clinical trial where 36 282 women were either supplemented daily with 1 g calcium and 400 IU (10 μg) vitamin D_3 or a placebo. After a mean follow-up of 7 years, the calcium and vitamin D_3 supplementations have no effect on CRC risk, BC risk or overall mortality (Wactawski-Wende *et al.* 2006, Chlebowski *et al.* 2008, LaCroix *et al.* 2009). However, personal supplementation of calcium and vitamin D_3 was not forbidden during the trial and 57% of the subjects in the placebo arm took personal supplements. When analysis is restricted to the women who did not take any personal supplements, the regimen of 1 g calcium plus 400 IU vitamin D_3 decreases the risk for CRC, BC and total cancer with 14–20% (Bolland *et al.* 2011). A recent trial with a daily supplementation of 800 IU vitamin D_3 alone or in combination with 1 g calcium did not affect cancer mortality or cancer incidence (Avenell *et al.* 2011). In another randomized placebo-controlled clinical trial patients with colorectal adenoma were supplemented during 6 months with 2 g calcium and/or 800 IU vitamin D_3 per day or a placebo. Here, different markers were evaluated in the normal rectal mucosa of these patients. Daily supplementation with vitamin D_3 induces beneficial changes in the normal rectal tissue of these patients indicating that vitamin D_3 could promote antineoplastic pathways such as higher activity of DNA mismatch repair

Table 1 Overview of randomized placebo-controlled clinical trials.

	Sample size	Subjects	Dosage vitamin D	Duration of intervention	Outcome
Wactawski-Wende *et al.* (2006)	36 282	Postmenopausal women	400 IU/day and 1000 mg calcium/day	Mean: 7 years	No effect on incidence of CRC
Chlebowski *et al.* (2008)	36 282	Postmenopausal women	400 IU/day and 1000 mg calcium/day	Mean: 7 years	No effect on incidence of invasive BC
Avenell *et al.* (2011)	5292	85% of subjects is at least 70 years with previous low-trauma fracture	800 IU/day and/or 1000 mg calcium/-day	Mean: 6.2 years	No effect on cancer mortality or cancer incidence
Scher *et al.* (2011)	953	Patients with metastatic, androgen-independent PC	45 μg DN-101 + chemotherapy	Up to 30 weeks	Treatment arm was associated with shorter survival: trial stopped
Sidelnikov *et al.* (2010)	92	Patients with colorectal adenoma	800 IU/day and/or 2.0 g calcium/day	6 Months	Increased DNA mismatch repair markers in normal mucosa
Fedirko *et al.* (2009*a*)	92	Patients with colorectal adenoma	800 IU/day and/or 2.0 g calcium/day	6 Months	Increased apoptosis markers in normal mucosa
Fedirko *et al.* (2009*b*)	92	Patients with colorectal adenoma	800 IU/day and/or 2.0 g calcium/day	6 Months	Increased differentiation markers in normal mucosa
Fedirko *et al.* (2010*b*)	92	Patients with colorectal adenoma	800 IU/day and/or 2.0 g calcium/day	6 Months	Decreased oxidative DNA damage marker in normal mucosa

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Table 2 Overview of clinical trials with vitamin D supplementation.

	Sample size	Subjects	Dosage vitamin D	Duration of intervention	Outcome
Marshall *et al.* (2012)	48	PC patients with adenocarcinoma	4000 IU/day	12 Months	No change in PSA; decrease in Gleason score; no adverse effects
Morris *et al.* (2004)	31	PC patients with increasing PSA levels who completed local treatment and/or patients with metastasis	Calcitriol escalating dose: 4–30 μg 3 times/week	Median: 12 months	Regimen well tolerated; minimal antitumor effects
Beer *et al.* (2003)	22	PC patients with rising serum PSA after prostatectomy and/or radiotherapy	0.5 μg/kg 1 time/week	Median: 10 months	Regimen well tolerated; declines in PSA levels and increased PSA doubling time
Schwartz *et al.* (2005)	18	Patients with androgen-independent PC	Paricalcitol i.v. escalating dose: 5–25 μg 3 times/week	12 Weeks	Declines in PSA levels; regimen well tolerated
Woo *et al.* (2005)	15	PC patients with increasing PSA levels who completed local treatment	2000 IU/day	Mean: 8 months	Decrease in the rate of PSA rise; no toxicities

mechanisms (Sidelnikov *et al.* 2010), a decrease in oxidative DNA damage (Fedirko *et al.* 2010*b*) and enhanced colorectal epithelial cell differentiation (Fedirko *et al.* 2009*b*) and apoptosis (Fedirko *et al.* 2009*a*).

Vitamin D_3 as a single high dose or as a repeated lower dose is often used in combination with standard cancer therapies during clinical trials. Administering 0.5 μg/kg vitamin D_3 once a week to PC patients whose prostate-specific antigen (PSA) increased after surgery and/or irradiation is well tolerated, however none of the patients reach a 50% reduction of the PSA levels, but some patients demonstrate decreased PSA levels and increased PSA doubling times (Beer *et al.* 2003). Similar results were obtained in PC studies where patients were treated with the vitamin D_3 analog paricalcitol (Schwartz *et al.* 2005), a 19-nor analog of 1,25(OH)$_2$D$_2$ (Woo *et al.* 2005) or 4000 IU/day vitamin D (Marshall *et al.* 2012).

Most trials have focused on androgen-independent PC patients where vitamin D_3 is often combined with other standard cancer therapies. Most of these regimens are well tolerated and the use of vitamin D_3 gives no additional toxicity compared with the standard therapies alone. However, most of these studies find no beneficial effect of vitamin D_3 (Morris *et al.* 2004). It is possible that the used concentrations of vitamin D_3 (up to 90 μg/week or a daily dose of 0.5 μg) are still too low to induce antineoplastic effects or that the treatment length in these trials is too short. The ASCENT study combined docetaxel and 45 μg DN-101, a high-dose formulation of 1,25(OH)$_2$D$_3$ that is specifically designed for cancer treatment, or placebo per week in PC patients and results were very promising. Addition of DN-101 to the regimen augments survival of the patients and decreases PSA (Beer *et al.* 2007). These data suggest that DN-101 might enhance the antitumor effects of docetaxel. However, the following phase III study was ceased due to higher mortality in the docetaxel + DN-101 arm compared with the docetaxel + placebo group. On the other hand, most deaths in the DN-101 arm of the study are due to PC progression. Moreover, subjects in the control arm only received docetaxel once in every 3 weeks, while the DN-101 arm subjects received docetaxel once in a week (Scher *et al.* 2011).

Since randomized clinical trials do not confirm the inverse association found in the observational studies, it has already been hypothesized that vitamin D_3 status would reflect the propensity of an individual to develop cancer instead of being one of the causes of cancer (Gandini *et al.* 2010).

Optimal vitamin D_3 intake

A great percentage of the population and especially cancer patients have a low vitamin D_3 status (Napoli *et al.* 2010, Choo *et al.* 2011). The minimum uptake of vitamin D_3 in order to obtain sufficient serum 25(OH)D_3 levels remains a

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controversial topic. The US Institute of Medicine considers serum 25(OH)D_3 levels of 20 ng/ml (or 50 nmol/l) as normal, while the US Endocrine Society defines serum 25(OH)D_3 levels under 20 ng/ml as vitamin D_3 deficient, levels between 20 and 30 ng/ml as vitamin D_3 insufficient and levels above 30 ng/ml (or 75 nmol/l) as vitamin D_3 sufficient. Concentrations of 20 ng/ml are believed to be sufficient for normal skeletal health (Bouillon 2011), however for the antineoplastic effects of vitamin D_3 concentrations above 30 ng/ml may be required because many intervention studies could not find beneficial effects of vitamin D_3 supplements on cancer risk when people were supplemented with $<$1000 IU/day (Rohan *et al.* 2009). To obtain serum 25(OH)D_3 levels above 30 ng/ml a daily intake of 1000 IU vitamin D_3 is necessary (Pramyothin & Holick 2012). Supplementations of 1000 IU/day or more result in an average serum 25(OH)D_3 level of 33 ng/ml and these patients have a 50% lower incidence for developing CRC compared with reference values (Gorham *et al.* 2005). A meta-analysis concluded that a daily intake of 1000–2000 IU of vitamin D_3 reduces the incidence of CRC with minimal risks (Gorham *et al.* 2007). Therefore, many scientists argue for serum 25(OH)D_3 levels of 30 ng/ml or more (von Domarus *et al.* 2011) and daily intakes of 2000 IU or more in order to guarantee at least bone health and possibly protection against cancer (Bischoff-Ferrari 2008, Hollis 2009, Leidig-Bruckner *et al.* 2010). The US Endocrine Society's Clinical Practical Guideline also suggests a daily vitamin D_3 intake between 1500 and 2000 IU for adults (Pramyothin & Holick 2012). However, the long-term safety effect of daily intake of such doses of vitamin D_3 in randomized placebo-controlled clinical trials is not yet proven. The Institute of Medicine recommends daily doses of 600 IU, since there is still no conclusive evidence that serum 25(OH)D_3 levels above 20 ng/ml are beneficial for human health.

General conclusions

The active hormone 1,25(OH)$_2$D$_3$ exerts next to its classical effects on bone and calcium homeostasis also antineoplastic effects. 1,25(OH)$_2$D$_3$ influences the proliferation, apoptosis, angiogenesis, invasion and migration of a tumor, while it also modulates several intracellular signaling pathways. The epidemiological link between vitamin D and cancer is the strongest for CRC, however more prediagnostic studies and randomized placebo-controlled clinical trials are needed. Guidelines on vitamin D supplementation exist to maintain bone homeostasis, however it is unclear if these doses are sufficient to induce antineoplastic effects. Future randomized placebo-controlled clinical trials with vitamin D doses above 800 IU are required in order to investigate antineoplastic effects. Also, the time point at which vitamin D status is important for tumor inhibition should be investigated in more detail. Serum 25(OH)D_3 levels measurements should be taken several times during clinical studies and should be standardized by using liquid chromatography–tandem mass spectrometry. Finally, special attention should be given to the effect of vitamin D supplementation in relation to cancer in severely vitamin D-deficient people.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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