Antineoplastic effects of 1,25(OH)₂D₃ and its analogs in breast, prostate and colorectal cancer

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Abstract

The active form of vitamin D₃, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), 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)₂D₃ also exerts antineoplastic effects on various types of cancer. The use of 1,25(OH)₂D₃ itself as treatment against neoplasia is hampered by its calcemic side effects. Therefore, 1,25(OH)₂D₃-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)₂D₃ in breast, prostate and colorectal cancer (CRC) and the underlying signaling pathways. 1,25(OH)₂D₃ 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)₂D₃ and its analogs. Human studies indicate an inverse association between serum 25(OH)D₃ values and the incidence of certain cancer types. Given the literature, it appears that the epidemiological link between vitamin D₃ 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)₂D₃ and its analogs to induce antineoplastic effects.

Key Words

- ▶ vitamin D
- ▶ analog
- ▶ breast cancer
- ▶ prostate cancer
- ▶ colorectal cancer

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Introduction

Vitamin D₃ is mostly known for its important functions to maintain calcium and phosphate homeostasis. Vitamin D₃ can be obtained from dietary sources, but most vitamin D₃ is generated in the human skin under the influence of sunlight (u.v.-B radiation). During this process 7-dehydrocholesterol is converted to previtamin D₃, an unstable molecule that is rapidly converted to vitamin D₃. However, vitamin D₃ must undergo two subsequent hydroxylations in the liver and kidneys respectively before becoming the active hormone 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). The 25-hydroxylation is executed by

different cytochrome P450 enzymes, including CYP2R1 and CYP27A1, forming the main circulating form 25-hydroxyvitamin D₃ (25(OH)D₃), which in turn undergoes a 1 α -hydroxylation by CYP27B1 in the kidneys to produce 1,25(OH)₂D₃ (Fig. 1). Only one major enzyme degrades 1,25(OH)₂D₃, namely CYP24, which expression is upregulated by 1,25(OH)₂D₃ itself.

CYP27B1 and CYP24A1 expressions in the kidneys are tightly regulated in order to maintain optimal 1,25(OH)₂D₃ levels. However, these metabolizing enzymes are also expressed in almost all nucleated cell types leading

Figure 1

Synthesis of $1,25(\text{OH})_2\text{D}_3$. Vitamin D₃ 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 $1,25(\text{OH})_2\text{D}_3$.

to local $1,25(\text{OH})_2\text{D}_3$ 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).

$1,25(\text{OH})_2\text{D}_3$ 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 D₃ responsive elements (VDREs) in the promoter regions of target genes in order to positively or negatively regulate their transcription. In the absence of $1,25(\text{OH})_2\text{D}_3$, 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 $1,25(\text{OH})_2\text{D}_3$ to its receptor a conformational change in the $1,25(\text{OH})_2\text{D}_3$ /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 $1,25(\text{OH})_2\text{D}_3$ treatment and escape the antiproliferative effects of $1,25(\text{OH})_2\text{D}_3$ (Khanim *et al.* 2004, Ting *et al.* 2007).

Next to the classical effects of $1,25(\text{OH})_2\text{D}_3$ on bone, kidney and intestine, more research has focused on the nonclassical effects of $1,25(\text{OH})_2\text{D}_3$, like cardiovascular, immunomodulatory and antineoplastic effects. However,

using $1,25(\text{OH})_2\text{D}_3$ itself as treatment against neoplasia is hampered due to its calcemic side effects. In order to induce antineoplastic effects, $1,25(\text{OH})_2\text{D}_3$ doses of the nanomolar range are required while normal serum $1,25(\text{OH})_2\text{D}_3$ levels are of the picomolar range. This led to the development of $1,25(\text{OH})_2\text{D}_3$ -derived analogs that are characterized by lower calcemic side effects and stronger antineoplastic effects.

Several microarray studies on cancer cells treated with $1,25(\text{OH})_2\text{D}_3$ or one of its analogs show that $1,25(\text{OH})_2\text{D}_3$ influences the transcription of a wide variety of genes suggesting a pleiotropic regulatory role for $1,25(\text{OH})_2\text{D}_3$ (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 $1,25(\text{OH})_2\text{D}_3$ causes in different cell types, so therefore $1,25(\text{OH})_2\text{D}_3$ is thought to induce cell-specific gene regulations (Krishnan *et al.* 2004). Clearly, early-stage cancer cells respond better to $1,25(\text{OH})_2\text{D}_3$ or an analog and gene regulation in these cells differs from more malignant cancer cells (Lee *et al.* 2006). The antineoplastic effects of $1,25(\text{OH})_2\text{D}_3$ 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

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)₂D₃**

Mechanisms involved in antineoplastic effects

Effects on proliferation and differentiation The best and earliest described antineoplastic effects of 1,25(OH)₂D₃ 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 G₀/G₁ phase of the cell cycle after 1,25(OH)₂D₃ stimulation (Jensen *et al.* 2001). This antiproliferative effect of 1,25(OH)₂D₃ 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)₂D₃-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)₂D₃ inhibits different cyclins and CDKs resulting in an intact Rb–E2F complex and inhibition of cell proliferation (Wang *et al.* 1997, Park *et al.* 2000b). However, when Rb is knocked out in 1,25(OH)₂D₃-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)₂D₃ since cells losing these pocket proteins will continue cell cycle progression after 1,25(OH)₂D₃ stimulation (Verlinden *et al.* 2007). 1,25(OH)₂D₃ 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)₂D₃ 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)₂D₃ 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)₂D₃ and contributes to the antiproliferative effects of 1,25(OH)₂D₃ (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)₂D₃ 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)₂D₃ (Nazarova *et al.* 2005). A study with CRC cells suggests that 1,25(OH)₂D₃-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 (Ordóñez-Moran *et al.* 2008).

Effects on apoptosis 1,25(OH)₂D₃ 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.* 2000a). Changes in the expression or cellular distribution of B-cell lymphoma 2 antiapoptotic proteins are a possible mechanism of 1,25(OH)₂D₃-mediated apoptosis (James *et al.* 1996, Zhang & Yao 2000, Wagner *et al.* 2003). Apoptosis after 1,25(OH)₂D₃ 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)₂D₃ 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)₂D₃ activates the intrinsic apoptotic pathway, since 1,25(OH)₂D₃ 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)₂D₃ 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)₂D₃ 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)₂D₃ (Zinser *et al.* 2003). Indeed, another study on BC cells shows an increase in intracellular calcium concentrations after 1,25(OH)₂D₃ 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).

Effects on angiogenesis The formation of new blood vessels is necessary for malignant tumor growth. 1,25(OH)₂D₃ inhibits angiogenesis, since treatment of several human cancer cell lines with 1,25(OH)₂D₃ 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)₂D₃ and this inhibition is mediated by an HIF1A-dependent pathway since 1,25(OH)₂D₃ is not able to inhibit VEGF expression in HIF1A knockout (KO) cells (Ben-Shoshan *et al.* 2007). In PC cells 1,25(OH)₂D₃ is able to repress interleukin 8 (IL8), one of the most important angiogenic factors secreted by PC cells (Bao *et al.* 2006a). Moreover, 1,25(OH)₂D₃ also inhibits an upstream regulator of IL8, namely nuclear factor kappa B (NF- κ B), which is thought to be partly responsible for the 1,25(OH)₂D₃-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 D₃ 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)₂D₃, angiogenesis is not influenced (Oades *et al.* 2002) and adding 1,25(OH)₂D₃ 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)₂D₃ 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)₂D₃ 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)₂D₃ 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.* 2006b, Iglesias-Gato *et al.* 2011); and a decreased expression of α 6-integrins,

β 4-integrins (Sung & Feldman 2000) and intracellular adhesion molecule 1 (Stio *et al.* 2011) after treating cancer cells with 1,25(OH)₂D₃/analog. 1,25(OH)₂D₃ 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)₂D₃ 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)₂D₃ through blockage of NF- κ B (Chen *et al.* 2010). 1,25(OH)₂D₃ 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)₂D₃ 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)₂D₃-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 D₃ deficiency promotes the growth of BC cells in an *in vivo* model for bone metastasis (Ooi *et al.* 2010). A high vitamin D₃ 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)₂D₃ is able to reduce metastasis formation with 50% (Spina *et al.* 2007). All these results suggest that 1,25(OH)₂D₃ 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

intraepithelial neoplasia (PIN) and PC (De Marzo *et al.* 2007). It is already well known that $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$, transcript levels of *IL1*, *IL6* and *IL17* pathway members are suppressed (Kovalenko *et al.* 2010). $1,25(\text{OH})_2\text{D}_3$ 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, $1,25(\text{OH})_2\text{D}_3$ inhibits NF- κB signaling by acting on different members of this pathway (Bao *et al.* 2006a). $1,25(\text{OH})_2\text{D}_3$ 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, *I κ Ba* (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, $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ is added to cancer cell lines, most studies agree that lower concentrations of prostaglandin are found compared with vehicle-stimulated cells. Indeed, $1,25(\text{OH})_2\text{D}_3$ decreases the levels of COX2 and induces 15-prostaglandin dehydrogenase, which results in a reduction of local prostaglandin concentrations. Moreover, $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$. $1,25(\text{OH})_2\text{D}_3$ is able to modulate several genes and pathways involved in cell proliferation, apoptosis, angiogenesis, invasion and inflammation. Moreover, $1,25(\text{OH})_2\text{D}_3$ influences the production of prostaglandin and interferes with Wnt/b-catenin signaling.

Wnt/b-catenin signaling The molecular mechanisms behind the antineoplastic effects of 1,25(OH)₂D₃ have been extensively studied in CRC. 1,25(OH)₂D₃ 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 TGFβ pathway eventually result in the progression of early aberrant crypt foci to colon adenocarcinoma. 1,25(OH)₂D₃ suppresses b-catenin/TCF transcriptional activity and their target genes via several mechanisms. 1,25(OH)₂D₃ 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)₂D₃/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)₂D₃ (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)₂D₃ (Barbachano et al. 2010). 1,25(OH)₂D₃ 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)₂D₃/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 D₃ deficient or VDR KO mice for a better understanding of the link between vitamin D₃ and the development and progression of cancer. A vitamin D₃-deficient diet leading to 25(OH)D₃ 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 D₃-deficient diet and afterward injected with cancer cells (Tangpricha et al. 2005). Also, a vitamin D₃-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 D₃ 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 D₃ reverses these effects (Yang et al. 2008a,b, Newmark et al. 2009). Moreover, the Western diet supplemented with calcium and vitamin D₃ 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)₂D₃ and its analogs on tumor development in rodents with BC, PC or CRC. Most studies agree that 1,25(OH)₂D₃ 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)₂D₃ 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)₂D₃ and its analogs clearly affect proliferation, differentiation, apoptosis, angiogenesis, invasion and inflammation of malignant cells. *In vivo* data mostly indicate that 1,25(OH)₂D₃ 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

actual tumor formation seems less influenced. Also, a locally low vitamin D₃ status may influence tissues in a way that these tissues are more sensitive to early procarcinogenic events. Using 1,25(OH)₂D₃ or its analogs alone as cancer treatment on the other hand is not sufficient, since 1,25(OH)₂D₃ is not able to eradicate tumor cells. Therefore, 1,25(OH)₂D₃ 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)₂D₃ 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)₂D₃ 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)₂D₃ concentrations decrease since less 1,25(OH)₂D₃ 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)₂D₃-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)₂D₃ production. Recent studies in the United States have shown that 25(OH)D₃ 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 D₃ intake and the risk of developing cancer (John *et al.* 1999, Lin *et al.* 2007, Oh *et al.* 2007). However, measuring 25(OH)D₃ serum levels is currently the golden standard for evaluation of the vitamin D status since concentrations of 1,25(OH)₂D₃ are tightly regulated by the renal metabolizing enzymes in order to maintain calcium homeostasis (Millen *et al.* 2010). Synthesis of 25(OH)D₃ on the other hand is not strictly regulated and combines the exposure to sunlight as well as the dietary/supplemental intake of vitamin D₃. Moreover, while the half-life of 1,25(OH)₂D₃ is only 4–6 h, 25(OH)D₃ has a half-life of 3 weeks. Several studies investigated the relationship between serum 25(OH)D₃ levels and the risk of developing cancer. For most cancer types, the results are conflicting. However, the majority of

observational, postdiagnostic studies on CRC report a significant inverse association between 25(OH)D₃ serum levels and the risk for CRC or colorectal adenoma (Fedirko *et al.* 2010a, 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)D₃ values during cancer initiation and early progression. This can be overcome by measuring 25(OH)D₃ concentrations before cancer diagnosis. A prediagnostic study reports that CRC patients with higher 25(OH)D₃ values tend to have a better outcome prognosis than CRC patients with lower 25(OH)D₃ levels (Ng *et al.* 2008). Most prediagnostic studies in the United States and Europe find an inverse association between 25(OH)D₃ 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)D₃ 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)D₃ levels are elevated (>30 ng/ml), however this study only included male smokers and mean 25(OH)D₃ 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)D₃ values. For BC and PC the association with lower 25(OH)D₃ 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)D₃ 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)D₃ levels and increased risk of recurrence in BC survivors (Jacobs *et al.* 2010). A limited number of studies compared prediagnostic 25(OH)D₃ serum levels with BC risk but results remain conflicting. The Nurses Health Study finds an inverse association between 25(OH)D₃ 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)D₃ 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)D₃ values above 23.5 ng/ml compared with 25(OH)D₃ levels lower

than 18.3 ng/ml. A Danish study showed that women with 25(OH)D₃ 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)D₃ 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)D₃ values in this study were very high (35.5 ng/ml) and the cutoff between low and high 25(OH)D₃ serum levels was relatively high (30 ng/ml). For PC, the link between low 25(OH)D₃ levels and augmented cancer risk is also not clear. In most prediagnostic Nordic studies, an inverse association is found between 25(OH)D₃ 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 D₃ deficient compared with 20% in the US studies (Ahn *et al.* 2008). It appears that only subjects with very low 25(OH)D₃ serum levels are at higher risk for PC. In contrast, some studies suggest that also higher 25(OH)D₃ levels increase the risk of developing PC (Tuohimaa *et al.* 2004, Shui *et al.* 2012). Other prediagnostic studies find that lower 25(OH)D₃ 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)D₃ serum values since diverse assays to measure 25(OH)D₃ 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)D₃ measurement, while this may not be reflective for long-term levels of circulating 25(OH)D₃. The exact time frame in which 25(OH)D₃ 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)D₃ 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,

nausea, etc.) can result in lower 25(OH)D₃ values. It is also not clear to what extent 25(OH)D₃ serum values are representative for the local tissue vitamin D status. Taken together, these studies indicate that the inverse association between serum 25(OH)D₃ 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 D₃ levels and the incidence of cancer. Future prediagnostic observational studies should include several 25(OH)D₃ serum measurements and longer follow-up periods in order to determine the exact time frame in which vitamin D₃ levels are crucial for cancer initiation or progression. Furthermore, it is of interest to establish the local tissue 25(OH)D₃/1,25(OH)₂D₃ levels to investigate if 25(OH)D₃ serum measurements are representative for the local vitamin D status in tissues.

Clinical trials

If a low vitamin D₃ status increases the risk of developing cancer, then clinical randomized trials should reveal a decrease in cancer risk when subjects are supplemented with vitamin D₃ (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 D₃ or a placebo. After a mean follow-up of 7 years, the calcium and vitamin D₃ 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 D₃ 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 D₃ 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 D₃ 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 D₃ per day or a placebo. Here, different markers were evaluated in the normal rectal mucosa of these patients. Daily supplementation with vitamin D₃ induces beneficial changes in the normal rectal tissue of these patients indicating that vitamin D₃ 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 <i>et al.</i> (2006)	36 282	Postmenopausal women	400 IU/day and 1000 mg calcium/day	Mean: 7 years	No effect on incidence of CRC
Chlebowski <i>et al.</i> (2008)	36 282	Postmenopausal women	400 IU/day and 1000 mg calcium/day	Mean: 7 years	No effect on incidence of invasive BC
Avenell <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (2009a)	92	Patients with colorectal adenoma	800 IU/day and/or 2.0 g calcium/day	6 Months	Increased apoptosis markers in normal mucosa
Fedirko <i>et al.</i> (2009b)	92	Patients with colorectal adenoma	800 IU/day and/or 2.0 g calcium/day	6 Months	Increased differentiation markers in normal mucosa
Fedirko <i>et al.</i> (2010b)	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 <i>et al.</i> (2012)	48	PC patients with adenocarcinoma	4000 IU/day	12 Months	No change in PSA; decrease in Gleason score; no adverse effects
Morris <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (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.* 2010b) and enhanced colorectal epithelial cell differentiation (Fedirko *et al.* 2009b) and apoptosis (Fedirko *et al.* 2009a).

Vitamin D₃ 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 D₃ 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 D₃ analog paricalcitol (Schwartz *et al.* 2005), a 19-nor analog of 1,25(OH)₂D₂ (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 D₃ is often combined with other standard cancer therapies. Most of these regimens are well tolerated and the use of vitamin D₃ gives no additional toxicity compared with the standard therapies alone. However, most of these studies find no beneficial effect of vitamin D₃ (Morris *et al.* 2004). It is possible that the used concentrations of vitamin D₃ (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)₂D₃ 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 D₃ 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 D₃ intake

A great percentage of the population and especially cancer patients have a low vitamin D₃ status (Napoli *et al.* 2010, Choo *et al.* 2011). The minimum uptake of vitamin D₃ in order to obtain sufficient serum 25(OH)D₃ levels remains a

controversial topic. The US Institute of Medicine considers serum 25(OH)D₃ levels of 20 ng/ml (or 50 nmol/l) as normal, while the US Endocrine Society defines serum 25(OH)D₃ levels under 20 ng/ml as vitamin D₃ deficient, levels between 20 and 30 ng/ml as vitamin D₃ insufficient and levels above 30 ng/ml (or 75 nmol/l) as vitamin D₃ sufficient. Concentrations of 20 ng/ml are believed to be sufficient for normal skeletal health (Bouillon 2011), however for the antineoplastic effects of vitamin D₃ concentrations above 30 ng/ml may be required because many intervention studies could not find beneficial effects of vitamin D₃ supplements on cancer risk when people were supplemented with <1000 IU/day (Rohan et al. 2009). To obtain serum 25(OH)D₃ levels above 30 ng/ml a daily intake of 1000 IU vitamin D₃ is necessary (Pramyothin & Holick 2012). Supplementations of 1000 IU/day or more result in an average serum 25(OH)D₃ 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 D₃ reduces the incidence of CRC with minimal risks (Gorham et al. 2007). Therefore, many scientists argue for serum 25(OH)D₃ levels of 30 ng/ml or more (von Dörmann 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 D₃ 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 D₃ 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)D₃ levels above 20 ng/ml are beneficial for human health.

General conclusions

The active hormone 1,25(OH)₂D₃ exerts next to its classical effects on bone and calcium homeostasis also antineoplastic effects. 1,25(OH)₂D₃ 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 pre-diagnostic 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)D₃ 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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