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Kolorektal Kanser Tedavisinde Kalsitriol Kullanımı

Sinem TUNÇER ÇAĞLAYAN[1*]

Öne Çıkanlar:
- Vitamin D metabolizması
- Kanser ve kalsitriol
- Kolorektal kanser tedavisinde kalsitriol kullanımı
- Kalsitriolün fiziko-kimyasal dezavantajları

Anahtar Kelimeler:
- Vitamin D
- Kalsitriol
- Kanser

ÖZET:

Kanda daha yüksek konsantrasyonda bulunması ve daha uzun yarılanma ömrü nedeniyle vücut vitamin D düzeyini belirlemede vitamin D'nin ara metaboliti olan kalsidiol değerleri temel alınır. Serum kalsidiol seviyesi ile kanser ilişkisi ilk kez kolorektal kanserde (CRC) ortaya konmuş olup, devam eden çalışmalarda düşük kalsidiol düzeyleri ile CRC'nin yanı sıra, meme, prostat, akciğer, mesane, gastrik ve hemotolojik kanserler için yüksek risk ve kötü prognoz ilişkisi gösterilmiştir. Ancak, CRC ile ilgili olmak üzere yürütülen klinik çalışmalarda, vitamin D takviyesinin kanser oluşum riskini düşürdüğü ya da hastalığın ilerlemesi/prognozu süreçlerinde olumlu katkısını gösterir kuvvetli bulgular elde edilememiştir. Bu çalışmada CRC'de vitamin D yerine, aktif formu olan kalsitriol kullanımının avantajlı olabileceğini öne süren veriler ortaya konmakta ayrıca kalsitriolün farmasötik kullanımındaki kısıtlara işaret edilmektedir. Dolayısıyla, amaca uygun ve yenilikçi olarak tasarlanan taşıyıcı platformlar, gerek vitamin D eksikliği/yetersizliği gerekse anti-kanser etki için kalsitriol faydalanımını arttırabilir.

Use of Calcitriol in Colorectal Cancer Treatment

Highlights:
- Vitamin D metabolism
- Cancer and calcitriol
- Use of calcitriol in colorectal cancer treatment
- Physicochemical disadvantages of calcitriol

Keywords:
- Vitamin D
- Calcitriol
- Cancer

ABSTRACT:

Due to its higher concentration in the blood and longer half-life calcidiol, the intermediate metabolite of vitamin D, is used as the main marker for determining vitamin D levels in the body. The relationship between serum calcidiol levels and cancer was first demonstrated in colorectal cancer (CRC), and subsequent studies have shown a link between low calcidiol levels and a higher risk and poor prognosis not only for CRC but also for breast, prostate, lung, bladder, gastric, and hematological cancers. However, clinical studies related to CRC have not provided strong evidence that vitamin D supplementation reduces cancer risk or positively impacts disease progression or prognosis. This study suggests that administration of calcitriol, the active form of vitamin D, may be more advantageous than vitamin D supplementation in CRC. It also points to the limitations of calcitriol in pharmaceutical applications. Therefore, appropriately designed and innovative carrier platforms could enhance the utilization of calcitriol for both vitamin D deficiency/insufficiency and its anti-cancer effects.

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INTRODUCTION

Colorectal cancer (CRC) is the third most frequently diagnosed malignancy worldwide. By 2035, the number of CRC patients is expected to reach 2.4 million, with an estimated 1.3 million deaths globally. Unfortunately, traditional cancer treatments are associated with significant side effects. In chemotherapy, the drug's poor physicochemical properties, low bioavailability, and limited (or absent) tissue selectivity contribute to these adverse effects. Radiation therapy, in addition to its side effects, can lead to more aggressive tumor emergence in some patients. Inhibitors of growth factors and receptors used in CRC treatment (such as bevacizumab, cetuximab, and panitumumab) can cause serious side effects, including skin irritation (painful fissures), hypertension, embolism risk, and increased susceptibility to infections (Wang et al. 2022). Immunotherapy represents a major advance in cancer treatment; however, it is only effective in a limited number of CRC patients with high microsatellite instability, and some patients may develop acquired resistance (Weng et al. 2022). Furthermore, immunotherapy is not yet economical for routine use and requires time and research and development to create personalized, autologous treatment strategies. Therefore, improving the efficacy and reducing the toxicity of existing drugs by enhancing their cancer-targeting capabilities can be considered a rational strategy (Feldmann 2018).

Calcitriol is the active form of vitamin D responsible for both skeletal and extra-skeletal functions. Vitamin D is a prohormone and is inactive. The formation of vitamin D_3 (vitD_3; cholecalciferol) occurs in the epidermis through a non-enzymatic process involving UVB-mediated photolysis. The resulting vitD_3 (referred to hereafter as vitD) binds to vitD binding protein (DBP) in the systemic circulation and is transported to the liver, where it is converted to calcidiol [25(OH)D_3] by the cytochrome P450 enzyme 25-hydroxylase. The final step of active vitD synthesis takes place predominantly in the proximal tubules of the kidney, where calcidiol bound to DBP is taken up by tubular cells via transmembrane proteins that act as surface receptors for DBP in the tubules. It undergoes 1-α hydroxylation by the enzyme 1-α hydroxylase (CYP27B1) to form calcitriol [1,25$(OH)_2D_3$] (Christakos et al. 2019). 1-α hydroxylation also occurs in non-renal sites such as alveolar macrophages, osteoblasts, lymph nodes, colon, breast, keratinocytes, and placenta (Chang and Lee 2019). The genomic effects of vitamin D depend on the activation of the vitamin D receptor (VDR) by calcitriol, leading to changes in the transcriptome and proteome (Carlberg 2022). Following this ligand-receptor interaction, gene transcription (genomic effect) is directly influenced, similar to other steroid hormones. Research indicates that calcitriol regulates 0.8-5% of the genome, affecting genes associated with differentiation, cell growth, apoptosis, DNA repair, metabolism, membrane transport, adhesion, oxidative stress, bone remodeling, xenobiotic detoxification, drug resistance, angiogenesis, lipid synthesis, and immune functions (Carlberg 2022; Díaz et al. 2015; Haussler et al. 2016; Koivisto, Hanel, and Carlberg 2020). Calcitriol also influences intracellular signaling (PKA, cAMP, PLC, PI-3 kinase, and MAP kinase) and directly alters the membrane transport of certain ions (Ca^{+2}, Cl^-). These effects are referred to as the “non-genomic” effects of calcitriol (Díaz et al. 2015; Ferrer-Mayorga et al. 2019; Hii and Ferrante 2016).

Compared to calcitriol, calcidiol levels are used to assess vitD status in the body due to its higher concentration in the blood and longer half-life (Wootton 2005). Serum calcidiol levels are classified as follows: values above 30 ng/mL (75 nmol/L) are defined as “sufficient”, those between 20-30 ng/mL (50-75 nmol/L) are considered “insufficient”, and values below 20 ng/mL (50 nmol/L) are categorized as “deficient” (Öncül Börekçi 2019). Serum calcidiol levels are important because, as previously noted, calcitriol is synthesized from calcidiol by the enzyme 1-α hydroxylase (CYP27B1), which is widely expressed in the epithelial cells of many organ systems (Garland et al. 2009). The association between

low serum calcidiol levels and cancer was first demonstrated in CRC in 1980, and subsequent clinical studies have shown a link between low serum calcidiol levels and a higher risk and poor prognosis for CRC (including metastatic CRC), as well as breast, prostate, lung, bladder, gastric, and hematologic cancers (Carlberg and Muñoz 2022; Jeon and Shin 2018). Based on these epidemiological data, *in vivo* and *in vitro* research into the anti-cancer effects of calcitriol, the active metabolite of vitD, has increased. In summary, calcitriol has anti-cancer effects related to the initiation, development, and progression of cancer, including anti-inflammatory, antioxidant, DNA damage repair, apoptosis, autophagy-induced cell death, inhibition of EMT (Epithelial-Mesenchymal Transition), anti-proliferative, and differentiation-inducing actions (El-Sharkawy and Malki 2020; Jeon and Shin 2018). Additionally, calcitriol has been shown to exert anti-inflammatory and immunomodulatory effects via various inflammatory cytokines involved in CRC progression (Javed et al. 2020), regulate cancer cell metabolism through c-myc, AMPK, mTOR, and TXNIP (Abu el Maaty and Wölfl 2017; Schroll et al. 2018), and suppress the Warburg effect (Huang et al. 2021). Calcitriol has also been reported to enhance the efficacy of many anti-cancer agents in both *in vitro* and *in vivo* settings (Trump 2018).

Numerous studies have been conducted that demonstrate the negative correlation between CRC and serum calcidiol concentration, the relationship between high calcidiol levels (≥30 ng/mL) and good prognosis among CRC patients, and the higher prevalence of vitD deficiency/insufficiency in CRC patients compared to healthy individuals, as summarized by Peixoto et al. (2022) and Na *et al*. (2022) (Na et al. 2022; Peixoto et al. 2022). Meta-analyses across different countries, regions, ethnicities, and age and gender groups consistently reveal an inverse association between calcidiol levels and the risk of colorectal cancer (CRC) as well as CRC-related mortality. In this context, one might consider that "vitD_3 supplementation could reduce CRC incidence and serve as a potential treatment approach". However, clinical studies regarding vitD_3 supplementation's impact on CRC incidence, disease-free survival, polyp formation risk, and cancer recurrence risk (Table 1) (Peixoto et al. 2022) have not established a strong relationship in favor of the supplementation's benefits (Na et al. 2022; Peixoto et al. 2022).

Table 1. Clinical studies on the effect of vitD supplementation on CRC (n: number of participants, HR: Hazard Ratio and CI: Confidence Interval)

Reference	Population	Intervention	Control	Result-HR (95% CI)
(Song et al. 2020)	Men and women aged 50 and older without a history of cardiovascular disease or cancer, USA n= 12,786 men n= 13,085 women	2000 IU (50 µg) vitD_3 + 1 g ω−3 fatty acids daily	Placebo	Colon polyp risk: 1.08 (0.92-1.27) Serrated polyp risk: 1.02 (0.82-1.26) CRC risk: 1.09 (0.73-1.62)
(Wactawski-Wende et al. 2006)	Postmenopausal women, USA n=18176 vitD_3 and calcium n=18106 plasebo	Daily 1000 mg elemental calcium and 400 IU (10 µg) vitD_3	Placebo	Invasive CRC incidence: 1.08 (0.86-1.34)
(Urashima et al. 2019)	Patients aged 30-90 with gastrointestinal cancers (stage I-III), Japan n=251 vitD_3 n=166 placebo	2000 IU (50 µg) vitD_3 daily	Placebo	Recurrence or death risk: 0.76 (0.50-1.14)
(Ng et al. 2019)	Patients with advanced or metastatic CRC, USA n=69 high-dose vitD_3 n=70 standard-dose vitD_3	Chemotherapy drug combination and bevacizumab with an initial dose of 8000 IU (200 µg)	Daily for the first cycle, then 4000 IU (100 µg) daily for subsequent cycles	Progression-free survival (PFS) or death: 13 vs 11 (control) months 0.64 (0-0.90; p =0.02)

Table 1 (Continued). Clinical studies on the effect of vitD supplementation on CRC (n: number of participants, HR: Hazard Ratio and CI: Confidence Interval)

(Baron et al. 2015)	Recently diagnosed adenomas and patients without known colorectal polyps after full colonoscopy, USA n=2259	All-factor randomization and two-group randomization. 1000 IU (25 µg) $vitD_3$, 1200 mg calcium carbonate, both agents, or placebo; daily	Placebo	Recurrence of adenomas: $vitD_3$ vs non-$vitD_3$ 0.99 (0.89-1.09), calcium vs non-calcium 0.95 (0.85-1.06), both agents vs neither 0.93 (0.80-1.08)
(Ng K et al. 2024)	A multicenter, double-blind, phase III randomized clinical trial evaluating vitD combined with standard chemotherapy and bevacizumab in patients with previously untreated metastatic colorectal cancer n=455	A phase 3 trial comparing high (8000 IU/day x 14 days then 4000 IU/day) and standard doses of $vitD_3$ (400 IU/day) in patients receiving chemotherapy drug combinations and bevacizumab for advanced and metastatic CRC (SOLARIS; Alliance A021703)	Metastatic CRC patients received standard doses of $vitD_3$ (400 IU/day)	Improved progression-free survival in patients with left-sided metastatic CRC receiving high-dose versus standard-dose $VitD_3$ (HR 0.74, 95% CI 0.55-1.00; p-interaction=0.02)

When reviewing these clinical studies, two main factors appear likely to influence study outcomes and could lead to inconsistencies in the data. First, clinical trials have used vitD ($vitD_3$) rather than calcitriol, and second, supplementation strategies in these trials vary. Regarding the first point, $vitD_3$ (cholecalciferol) requires sequential hydroxylation in the liver and kidneys to become the biologically active form, calcitriol. Differences in individual metabolism, liver and kidney function, and other physiological factors may affect this conversion, potentially influencing study outcomes. To date, there are no clinical studies on the effects of calcitriol supplementation in CRC. It should be emphasized that serum calcidiol level is a clinical parameter used to measure endogenous or dietary vitD status. The active metabolite of vitD, however, is calcitriol, which is the bioactive form responsible for vitD's wide range of biological effects, including anti-neoplastic actions (Díaz et al. 2015). When vitD is supplemented, various factors -such as age (e.g., reduced vitD activation in the kidneys in geriatric populations), mutations/SNPs in genes involved in vitD metabolism and transport, medical history (e.g., metabolic issues, liver failure, nephrotic syndrome, chronic kidney disease), certain medications (e.g., glucocorticoids), chemotherapy, and calcium intake- determine how effectively an individual benefits from supplementation. Thus, demographic, biological, and genetic factors contribute to individual variation in vitD supplementation efficacy. Baseline calcidiol levels are also affected by factors like sex, body mass index/body fat percentage (calcidiol and vitD are stored in adipose tissue), ethnicity, estrogen use, dietary fat content, and composition (Ammar et al. 2022; Jiang, Kiel, and Kraft 2019; Mazahery and von Hurst 2015; Waterhouse et al. 2014; Wei, Zhu, and Ji 2019). Additionally, calcitriol regulates vitD metabolism through negative feedback involving PTH (parathyroid hormone) and FGF-23 (fibroblast growth factor-23) (Jeon and Shin 2018). Also, vitD metabolism can change in cancer (Jeon and Shin 2018). Therefore, using the vitD form in anti-cancer studies may limit effectiveness and contribute to vitD resistance (Jeon and Shin 2018). As for the second point -variability in supplementation strategies- differences in dosage, frequency, and duration of $vitD_3$ administration across studies could contribute to inconsistencies in the clinical data. While calcitriol's anti-cancer effects are

dose-dependent (Trump 2018), most clinical trials have not used high doses. Although a daily dose of 2000 IU has been shown effective in healthy individuals (Dědečková et al. 2023), the highest dose in many studies was often 2000 IU or less. Notably, these clinical studies did not group patients according to baseline calcidiol levels (Brown 2019). Additionally, in some studies, $vitD_3$ was supplemented with ω−3 fatty acids or calcium, and the anti-cancer drugs and administration models for patients differed. A recent phase 3 study, funded by the "Alliance for Clinical Trials in Oncology" and backed by the National Cancer Institute (NCI), is evaluating the efficacy of high-dose $vitD_3$ (8000 IU) in combination with standard chemotherapy and bevacizumab in patients with previously untreated metastatic colorectal cancer (mCRC). The results of this study are discussed in detail in the "Results and Discussion" section.

Although supplementation with high doses of vitD may aim for an anti-cancer effect in CRC, changes in vitD metabolism due to cancer may restrict calcitriol production and thus limit the utilization of calcitriol. This study presents data suggesting that using calcitriol, instead of vitD, can be advantageous for achieving anti-cancer effects in CRC.

MATERIALS AND METHODS

In silico analyses

Publicly available CRC transcriptomic datasets were retrieved from the NCBI Gene Expression Omnibus (GEO) database for *in silico* analysis of genes involved in vitD metabolism. Two independent cohorts were analyzed. The GSE44076 dataset (98 tumors, 98 paired adjacent noncancerous mucosa, and 50 healthy controls) was generated on the Affymetrix Human Genome U219 Array. This dataset includes newly diagnosed, treatment-naïve colorectal cancer patients, the majority of whom were stage II, and contains tumor tissue, patient-matched adjacent normal mucosa, and healthy mucosa from non-cancer individuals. The GSE37364 dataset (70 colorectal carcinomas, 29 dysplastic adenomas, and 31 healthy mucosa) was generated on the Affymetrix Human Genome U133 Plus 2.0 Array. This dataset includes healthy controls, adenomatous polyps with dysplasia, and carcinomas stratified by Dukes stage (A/B and C/D).

Expression values were obtained from GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/), which uses the authors' processed series matrix files, and analyses were performed within GEO2R. Probe sets corresponding to vitamin D metabolic genes were identified from the platform annotation files and used for expression extraction. Since the absolute values are not directly comparable across datasets, the interpretations are performed based on relative differences within each cohort in this study.

Data are presented as violin plots and median expressions are indicated (red dashed line). For statistical testing, ordinary one-way ANOVA (with multiple comparisons) was used.

Cell culture, calcitriol treatment, and cell proliferation experiments

HCT-116 human CRC cells (German Cancer Research Center-DKFZ, Germany) were cultured in T25 culture flasks in high-glucose DMEM medium (Capricorn Scientific, Germany) without phenol red, supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS), and 1% penicillin/streptomycin, in a 5% CO_2 incubator, at 37°C (Tunçer et al. 2016). Cells were passaged using 0.25% Trypsin-EDTA when flask confluency reached a maximum of 70%.

To investigate the effect of calcitriol on cell proliferation, cells were seeded in 96-well plates as $5x10^3$ cells per well one day prior to the treatments. Calcitriol (MedChemExpress, USA) was prepared in ethanol (EtOH) as a 2.5 mM stock solution, then aliquoted and stored at -80°C. The day after cell seeding, the medium was removed, and cells were treated with either 100 nM calcitriol, the solvent (EtOH) as a vehicle (0.004%, v/v), or medium alone (untreated control). After 48 hours, cell proliferation, viability, and cytotoxicity were determined using the MTT assay, a method that monitors

metabolic activity, as previously described (Karaçam and Tunçer 2022). Briefly, at the end of the incubation period, the medium was removed, and 100 µL of MTT (BioVision, USA) at a final concentration of 0.5 mg/mL in medium was added to each well. Plates were incubated for 4 hours at 37°C, after which 100 µL of SDS-HCl solution (10 mL of 0.01 M HCl containing 1 g SDS) was added to each well. Following overnight incubation at 37°C, absorbance was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA). Relative cell viability (%) was calculated as a percentage relative to the untreated control group.

Statistical analyses

GraphPad Prism 8.01 (GraphPad Software, USA) was used for graph preparation and statistical analyses. Statistical significance was assessed using t-tests or one-way ANOVA. Differences were considered significant at $p \leq 0.05$ (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$). Non-significant comparisons were indicated as "ns".

RESULTS AND DISCUSSION

Expression of genes related to calcitriol synthesis is altered in colorectal cancer

Analysis of two independent colorectal cancer transcriptomic cohorts (GSE44076 and GSE37364) revealed consistent disruptions in vitD metabolism across the adenoma-carcinoma sequence. The genes interrogated in these datasets represent key components of the vitD pathway: *CYP2R1* and *CYP27A1* encode 25-hydroxylases that catalyze the conversion of cholecalciferol (vitamin D_3) to 25-hydroxyvitamin D_3 (calcidiol); *CYP27B1* encodes the 1α-hydroxylase that converts calcidiol to the active hormone 1,25-dihydroxyvitamin D_3 (calcitriol); *CYP24A1* encodes the 24-hydroxylase responsible for calcitriol degradation; and VDR encodes the nuclear receptor that mediates calcitriol-dependent transcriptional responses.

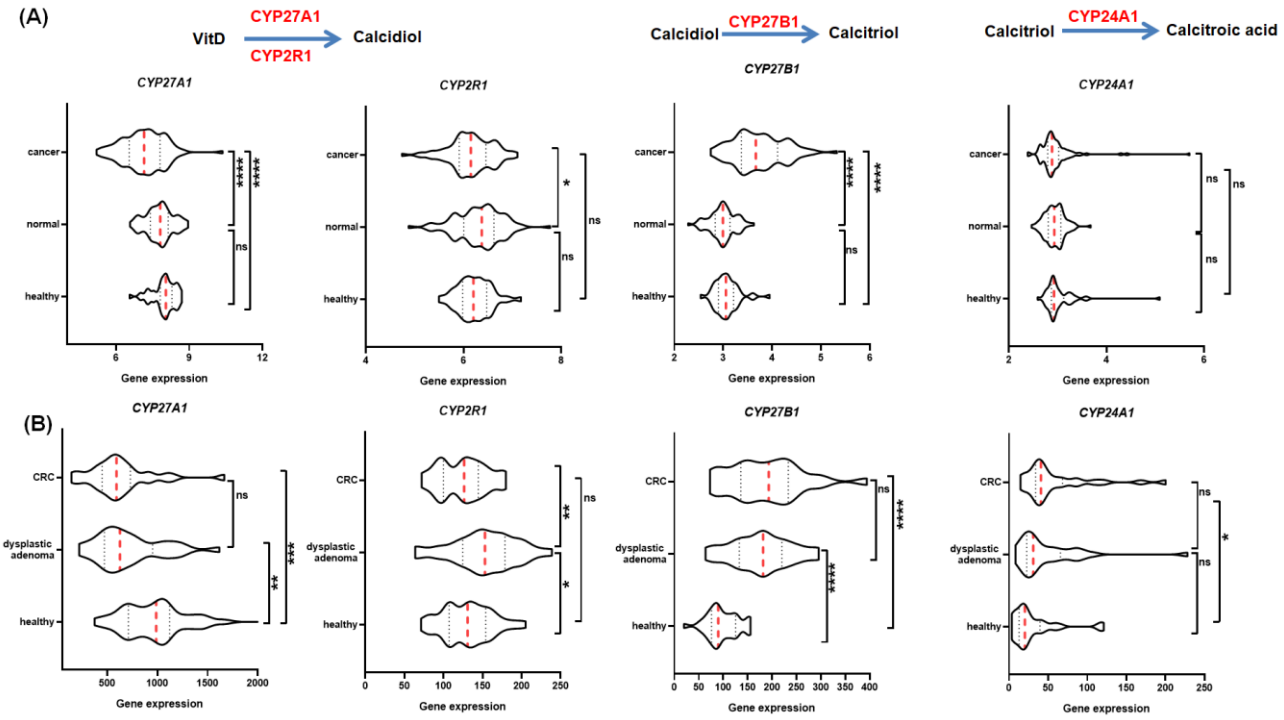

Figure 1. Expression of genes related to vitamin D metabolism in colorectal cancer. (A) Expression analysis from the GSE44076 cohort showing *CYP27A1* (probe ID: 11731430_a_at), *CYP2R1* (probe ID: 11722910_s_at), *CYP27B1* (probe ID: 11722122_at), and *CYP24A1* (probe ID: 11737968_a_at) in healthy mucosa, adjacent noncancerous mucosa, and tumor tissues from newly diagnosed CRC patients (mostly stage II). (B) Expression analysis from the GSE37364 cohort showing *CYP27A1* (probe ID: 203979_at), *CYP2R1* (probe ID: 207786_at), *CYP27B1* (probe ID: 205676_at), and *CYP24A1* (probe ID: 206504_at) in healthy mucosa, dysplastic adenomas, and colorectal carcinomas. Ordinary one-way ANOVA is used for comparisions

In GSE44076, which includes healthy mucosa, paired adjacent noncancerous mucosa, and tumors from newly diagnosed patients (mostly stage II), *CYP27A1* expression was significantly decreased in tumors, while *CYP2R1* tended to be lower. These changes indicate that substrate hydroxylation capacity is already compromised within tumors. In contrast, *CYP27B1* was specifically upregulated in tumors compared with both healthy and adjacent tissues, reflecting a compensatory attempt to sustain calcitriol synthesis. *CYP24A1* expression remained unchanged, suggesting that catabolism was not yet a dominant feature in this early-stage cohort (Figure 1A).

The GSE37364 dataset, which includes healthy controls, dysplastic adenomas, and colorectal carcinomas, provided further insight into vitD metabolism across the adenoma-carcinoma sequence (Figure 1B). *CYP27A1* expression was highest in healthy mucosa and declined in dysplastic adenomas, with tumors showing a tendency toward further reduction. *CYP2R1* showed an initial increase in adenomas but returned to healthy levels in carcinomas, indicating that any early compensatory rise was not sustained during cancer progression. *CYP27B1* was markedly elevated in adenomas compared with healthy mucosa and tended to be higher in carcinomas. Thus, in adenomas, early compensatory changes occurred, including *CYP2R1* and *CYP27B1* upregulation, which may represent an "escape attempt" to sustain calcitriol synthesis despite declining *CYP27A1*. In contrast, *CYP24A1* expression was lowest in healthy mucosa, tended to increase in adenomas, and was significantly elevated in carcinomas.

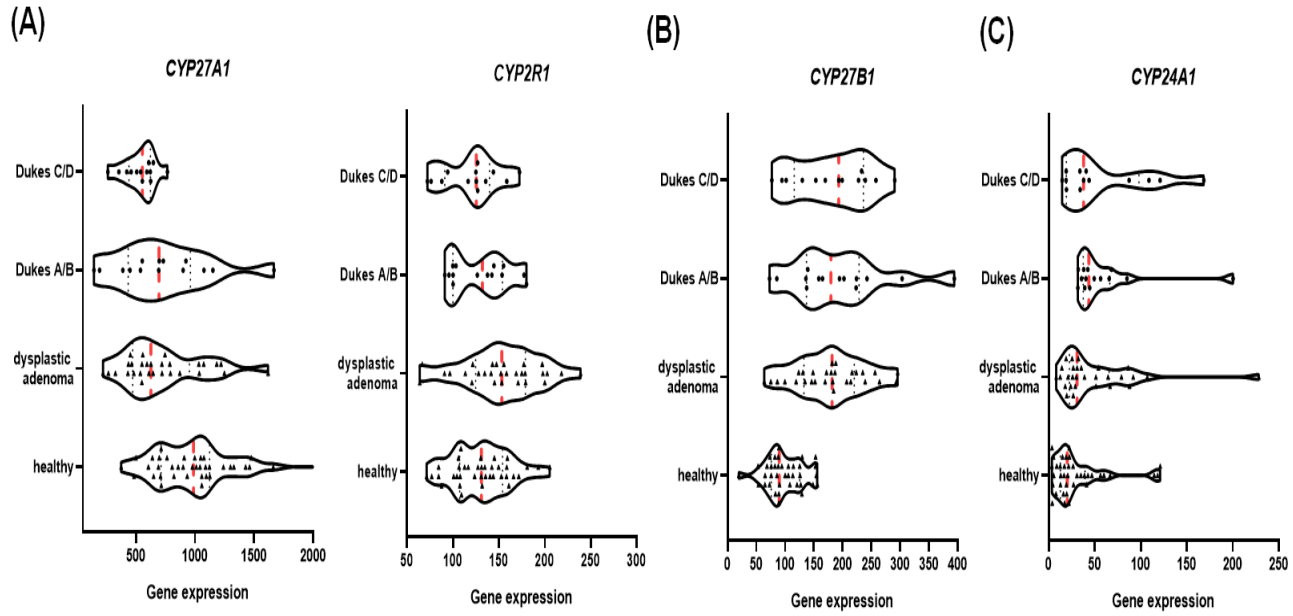

Figure 2. Stage-stratified expression of vitamin D metabolic genes in colorectal cancer. Expression levels of (A) *CYP27A1* and *CYP2R1*, (B) *CYP27B1*, and (C) *CYP24A1* are shown in healthy mucosa, dysplastic adenomas, and carcinomas stratified by Dukes stage (A/B vs. C/D) from the GSE37364 dataset. Individual sample values are overlaid on the plots

Stage-stratified analysis (Dukes A/B vs. Dukes C/D) refined this picture (Figure 2). *CYP27A1*, already reduced in adenomas, tended to decline further in Dukes C/D tumors. *CYP2R1*, after its initial adenoma-associated increase, returned to levels comparable to healthy mucosa in carcinomas. Notably, *CYP27B1* remained elevated across both adenomas and carcinomas. This likely reflects a decoupling between activation and signaling: despite persistent transcriptional upregulation of the 1α-hydroxylase, effective calcitriol action is limited by reduced VDR expression (Figure 3) and sustained CYP24A1-mediated catabolism. The human *CYP27B1* gene and its downregulation in the presence of $1\alpha,25(OH)_2D_3$ represent one of the best-characterized models of negative regulation by nuclear receptors. Negative feedback on *CYP27B1* largely relies on intact calcitriol-VDR signaling. In

GSE37364, however, VDR expression was reduced while *CYP24A1*, the enzyme responsible for calcitriol catabolism, was elevated. A positive correlation between VDR expression levels and calcitriol utilization in CRC has been identified (J. Sun 2017). Additionally, VDR is among the genes positively regulated by calcitriol (Gesmundo et al. 2020; Zhu et al. 2021); thus, reduced calcitriol synthesis in CRC may further contribute to decreased VDR expression. Together, these findings indicate that even if CYP27B1 supports calcitriol synthesis, signaling may not be effectively transduced and/or calcitriol may be rapidly catabolized. With feedback loops blunted, CYP27B1 can remain persistently high -an "ineffective" upregulation. However, this cannot be the sole explanation as *CYP27B1* regulation is complex. Turunen et al. identified both proximal negative VDREs and distal VDR-binding regions in the *CYP27B1* promoter that mediate context- and tissue-specific regulation (Turunen et al. 2007). Pathological contexts can also override these mechanisms. For example, Du et al. demonstrated that in colonic epithelial cells, TNF-α induces *CYP27B1* expression through NF-κB signaling, providing a non-canonical, inflammation-driven pathway that operates independently of VDR-mediated repression (Du et al. 2017). This may also explain why, even in Dukes C/D tumors, where VDR is reduced and *CYP24A1* is elevated, *CYP27B1* remains persistently upregulated, reflecting sustained inflammatory induction rather than functional feedback. In addition, SNP variants in CYP enzymes involved in vitD metabolism can alter enzymatic activity. Thus, increased gene expression may not always correlate with functional enzyme levels (Jacobs et al. 2013). SNP-directed variation in CYP functionality further highlights that vitD homeostasis is complex and can be shaped by genetic factors.

Overall, this imbalance strongly suggests that simply raising systemic vitamin D_3 levels through supplementation may not effectively restore intratumoral calcitriol signaling, as the pathway is impaired at multiple levels: substrate generation, hormone stability, and receptor signaling. It is also noteworthy that calcitriol exerts biological actions independent of VDR. For instance, it can inhibit the PI3K/AKT and MAPK signaling pathways, both critical for cancer development and progression, and can alter the activity of key proteins in the translational machinery (4E-BP1 and eIF-4E) (Gkotinakou et al. 2020).

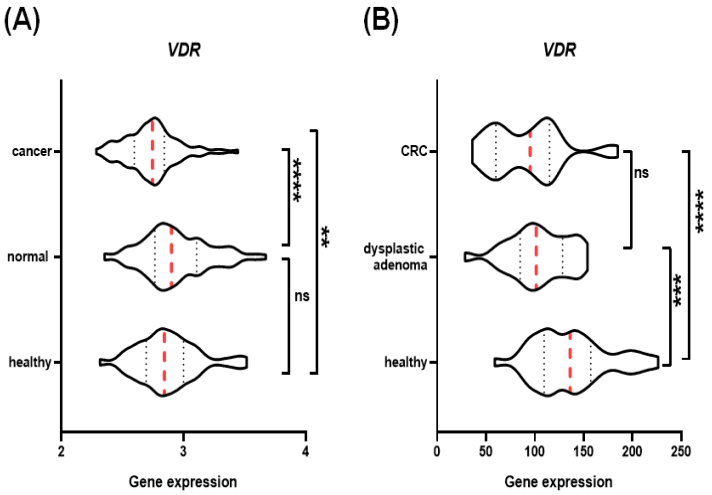

Figure 3. Expression of VDR in colorectal cancer. (A) Expression of VDR in healthy mucosa, adjacent noncancerous mucosa, and tumor tissues from the GSE44076 dataset (probe ID: 11722930_a_at). (B) Expression of VDR in healthy mucosa, dysplastic adenomas, and colorectal carcinomas from the GSE37364 dataset (probe ID: 213692_s_at). Ordinary one-way ANOVA is used for statistical analyses

Calcitriol Reduces Cell Proliferation in Human CRC Cells

Figure 4 presents the results of an MTT assay conducted to assess changes in cell proliferation in the human CRC cell line HCT-116 treated with 100 nM calcitriol for 48 hours (Gesmundo et al. 2020; Vaughan-Shaw et al. 2022). In line with the previous studies (Padi et al. 2013) calcitriol exhibited an

anti-proliferative effect on CRC cells. When the *in silico* analysis results and the *in vitro* findings demonstrating its anti-proliferative effect are evaluated together, calcitriol can be suggested as a potential target for anti-tumor effects in CRC. An additional advantage of calcitriol's anti-tumor efficacy in CRC is its reported anti-proliferative effect specifically related to tumorigenesis (Carlberg and Muñoz 2022). In an organoid model derived from healthy individuals, calcitriol treatment led to an increased expression of stem cell marker genes such as LRIG1, MSI1, LGR5, PTK7, SMOC2, and MEX3A in undifferentiated stem cells while maintaining basal proliferation levels, without inducing transformation into cancer stem cells. However, calcitriol supported the tumorigenic differentiation of colon cancer stem cells (Fernández-Barral et al. 2020).

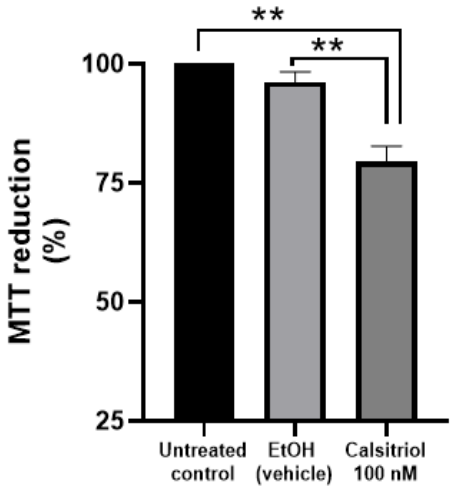

Figure 4. Calcitriol treatment in CRC cell line HCT-116. MTT reduction in cells treated with 100 nM calcitriol or vehicle (EtOH) is presented as a percentage relative to the untreated control group. Statistical significance was determined using a *t*-test

The literature and the *in silico* data presented in this study indicate that calcitriol, due to its anti-tumor effects, may be considered for use in CRC, particularly in newly diagnosed and early-stage CRC patients. As presented in Table 1, the phase 3 SOLARIS trial (NCT04094688) evaluated the combination of high-dose vitD with standard chemotherapy plus bevacizumab in previously untreated mCRC patients. The study found that high-dose vitD (8000 IU/day; equal to 200 µg/day) did not introduce additional toxicity or adverse effects. However, progression-free survival, overall survival, and overall response rate showed no significant differences compared to the standard-dose VitD (400 IU/day). Notably, subgroup analysis revealed a potential benefit in patients with left-sided primary tumors, where high-dose vitD appeared to prolong progression-free survival. Although not statistically significant, this finding suggests a possible role for vitD in specific patient populations (Ng K et al. 2024). Identifying the characteristics of subgroups that may benefit from high-dose vitD is crucial for future research, as the data indicate a promising avenue for targeted treatment approaches. Further studies are needed to explore this potential benefit and refine patient selection criteria. On the other hand, considering that cancer-related metabolic changes, as well as individual differences (mutations and/or SNPs), affect vitD bioavailability, supplementing with the active form, calcitriol, rather than vitD itself, may be proposed as a more effective anti-cancer approach in CRC research.

However, the limitations of calcitriol use in clinical settings, especially in anti-cancer applications, must also be considered. The primary concern is the risk of hypercalcemia (Almouazen et al. 2013). Fakih et al. investigated the intravenous administration of calcitriol (Calcijex, Roche Pharmaceutical Corporation) in combination with gefitinib on a weekly schedule and found that very high doses of calcitriol were well tolerated in prostate cancer patients. Grade 3 hypercalcemia at a dose of 98 µg per week was identified as the dose-limiting toxicity for weekly intravenous calcitriol combined with

gefitinib. In this regimen, the recommended phase II dose was set at 77 µg per week when used alone and 98 µg per week when co-administered with high-dose dexamethasone. At 98 µg calcitriol, systemic exposure reached around 30 ng/h/24 h, a level comparable to that seen in murine models where calcitriol exhibited strong antitumor activity. Beer et al. also assessed high-dose oral calcitriol (as Rocaltrol) and found that a dose of 0.5 µg/kg per week was very safe. Additionally, a regimen starting with 165 µg of DN-101 (high-dose, oral formulation of calcitriol) in the first week, followed by 45 µg weekly, was well tolerated without any notable toxicity. A linear correlation between DN-101 dose and area under the curve (AUC) was observed up to 165 µg. Intermittent dosing schedules, whether weekly or for three consecutive days per week, led to hypercalcemia only at doses of approximately 100 µg following intravenous administration. Serum calcium levels temporarily increased (11-13 mg/dL) within one to three days after completing either a single-dose or three-day regimen. However, dose-limiting hypercalcemia was only observed at concentrations exceeding ~30 ng/h/mL (El-Sharkawy and Malki 2020).

Phase I and II trials investigating calcitriol with cytotoxic agents have encountered challenges related to determining the optimal biological dose and the maximum tolerated dose (MTD) of calcitriol. A phase II trial, in which combined calcitriol with docetaxel in patients with advanced prostate cancer, showed no significant toxicity, with 81% of patients experiencing a prostate-specific antigen (PSA) response, suggesting a potential benefit of calcitriol. This led to the development of DN-101 which was tested in ASCENT I, a large randomized trial comparing docetaxel with or without DN-101. Although DN-101 showed a trend toward improved survival and reduced docetaxel toxicity, statistical significance was not reached. Encouraged by these preliminary results, ASCENT II, a larger phase III trial, was conducted to assess survival benefits. However, the study suffered from design flaws, including an asymmetric comparison between treatment arms and uncertainty regarding the optimal calcitriol dose. The trial was terminated due to a higher mortality rate in the investigational arm, though later analysis indicated that deaths were due to disease progression rather than calcitriol-related toxicity. Despite the failure of ASCENT II, substantial evidence suggests calcitriol's potential in cancer therapy. Several trials combining calcitriol with chemotherapeutic agents have reported antitumor activity without unexpected toxicity. However, most of these studies did not reach the MTD. While preclinical data support further exploration of calcitriol in combination with various chemotherapy agents, additional well-designed clinical trials are needed to determine its optimal use in cancer treatment (El-Sharkawy and Malki 2020).

These studies have shown that calcitriol could be used at high doses in cancer patients, where anti-cancer effects were observed. These doses caused mild to moderate, yet temporary, hypercalcemia, without cytotoxicity or unexpected side effects. However, several factors have prevented definitive conclusions regarding calcitriol's use in cancer treatment: the small number of patients in these clinical studies, the heterogeneity of the study populations (e.g., inclusion of patients at different cancer stages and lack of consideration for baseline calcidiol levels), the use of additional drugs and/or supplements (such as calcium and ω-3 fatty acids), and the variation in administration methods (oral or intravenous). Additionally, differences in treatment regimens (daily, weekly, continuous, or intermittent administration) have led to inconsistencies in the findings regarding calcitriol's role in cancer therapy (Trump et al. 2004; Trump 2018).

Another major factor limiting the clinical use of calcitriol is the challenge of designing suitable dosage forms (Trump 2018). In patients with advanced prostate cancer, oral administration of calcitriol (28 µg per day for three consecutive days each week) combined with dexamethasone (4 mg daily for four days) is well tolerated and considered safe. Muindi JR et al. investigated dose-escalating regimens of calcitriol (three consecutive days per week) with paclitaxel (80 mg/kg weekly for four weeks) and

calcitriol (three consecutive days per month) with carboplatin (320 mg/m² monthly). In both studies, calcitriol was safely administered at doses of 38 μg per day for three days per week alongside paclitaxel and 28 μg per day for three days per month with carboplatin. However, these trials revealed that high-dose calcitriol (as Rocaltrol) was impractical, as the 38 μg dose required 76 caplets, and its pharmacokinetics were unfavorable (El-Sharkawy and Malki 2020). Thus, more advantageous formulations need to be developed to evaluate calcitriol's anti-cancer efficacy, including in CRC (El-Sharkawy and Malki 2020). Its low water solubility, instability when exposed to oxygen, heat, and light, and challenges in ensuring content uniformity pose significant pharmaceutical limitations [55]. Various commercial formulations have been developed to address these issues, including an injectable form (CALCIJEX, AbbVie Inc., Illinois, USA), an oral solution (Rocaltrol, Roche Ltd., Basel, Switzerland), and soft capsules (Rocaltrol, Roche Ltd., Basel, Switzerland) (Ramalho et al. 2015; Yuan et al. 2013; Yue et al. 2020). The half-life of oral calcitriol formulations is 10 hours, while for intravenous formulations, it is 5 hours (Bailie and Johnson 2002). Due to this short half-life, current systemic formulations fail to maintain the required active levels of calcitriol (Almouazen et al. 2013); in other words, neither sustained release at high doses nor prolonged release can be achieved. Additionally, calcitriol's high protein-binding capacity further limits its efficacy. For these reasons, existing pharmaceutical formulations are considered unsuitable for anti-cancer applications (Almouazen et al. 2013; Nicolas et al. 2018). Furthermore, existing liquid formulations have unavoidable and well-documented disadvantages: administration requires clinical supervision, injections are painful, and repeated long-term injections are uncomfortable. The solvents used to enhance calcitriol's solubility in injectable formulations may cause pain and irritation. Oral oil-based solutions designed to improve solubility are often unpalatable and induce nausea, making them less desirable. In soft capsule formulations, the residual water inside the capsule shell promotes calcitriol oxidation, compromising stability. Additionally, oral soft capsules have long disintegration times and slow dissolution rates. The challenges of improving solubility and preventing oxidation in current calcitriol formulations (as seen in soft capsules) represent major obstacles to its development for anti-cancer applications. To overcome these limitations, new delivery models and dosage forms must be developed (Ramalho et al. 2015; Yuan et al. 2013; Yue et al. 2020). Solid dosage forms, in contrast to liquid formulations, provide enhanced physicochemical stability, reduced production costs, and improved patient compliance, making them the ideal option for developing a calcitriol dosage model (Yue et al. 2020).

In addition to formulation challenges, another critical limitation for calcitriol therapy can be the enhanced catabolism mediated by CYP24A1 (H. Sun et al. 2016). Elevated CYP24A1 rapidly degrades calcitriol into inactive metabolites, thereby diminishing its therapeutic potential despite adequate synthesis or supplementation. One promising strategy to overcome this limitation is the co-administration of calcitriol with CYP24A1 inhibitors. Preclinical studies have shown that selective inhibition of CYP24A1 enhances the antiproliferative and pro-apoptotic effects of calcitriol by prolonging its half-life and sustaining its biological activity (Jeon and Shin 2018; Kósa 2013). Such combination approaches could restore or amplify calcitriol's biological activity in the tumor microenvironment.

CONCLUSION

Phase I and II trials so far have demonstrated limited antitumor activity, constrained by factors such as the MTD, biological dose, optimal scheduling, and available pharmaceutical formulations of calcitriol. As an affordable, natural agent with broad anticancer effects, calcitriol offers several advantages for clinical evaluation, as highlighted in this study. However, the need for appropriate dosage

forms for both vitD deficiency treatment and anticancer applications remains evident. Specifically for cancer, tumor-targeted calcitriol treatments should also be considered.

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Conflict of Interest

The author declares no conflict of interest.

Author's Contributions

Sinem Tunçer Çağlayan conducted the content analysis, research, material supply, and article writing.

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