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Vitamin D-Induced Cytotoxicity in HT-29 Colon Cancer Cells: Apoptotic Gene Expression Study

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Background: Colorectal cancer (CRC) remains a major cause of cancer deaths worldwide, emphasizing the need for new treatments. Although vitamin D is known to impact cell growth and apoptosis, its precise mechanisms in affecting CRC and regulating apoptosis-related genes are not fully understood. This study investigated the possible cytotoxicity of vitamin D on human colon adenocarcinoma HT-29 cells and examined changes in BAX and BCL-2 gene expression.

Methods: HT29 cells were cultured and exposed to vitamin D at concentrations ranging from 50 to 3750 µg/ml for 24 hours, with untreated cells as controls. Cell viability was assessed using an MTT assay, followed by RNA extraction. The RNA was reverse-transcribed into cDNA, allowing quantitative measurement of BAX and BCL-2 transcripts through real-time PCR, with data normalized to GAPDH. Statistical analysis of cell viability was performed using one-way ANOVA and Dunnett's post-hoc tests, while differences in gene expression were examined with t-tests.

Results: Vitamin D decreased HT-29 cell viability in a dose-dependent and nonlinear way ($p < 0.0001$), with the highest inhibition seen at concentrations between 250-500 µg/ml. However, there was no significant difference in BAX expression ($p = 0.1431$) or BCL-2 expression ($p = 0.5943$).

Conclusions: These data indicate that Vitamin D can cause cytotoxicity in HT-29 cells without altering the transcriptional targets BAX/BCL-2, and may also induce cell death through other pathways, such as inhibited proliferation. Overall, this suggests that Vitamin D has potential as a complementary therapy for CRC and warrants further mechanistic research, including studies with in vivo CRC models.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths worldwide, with approximately 1.9 million new cases each year (Siegel et al., 2023). The global burden is expected to increase to 3.2 million new cases by 2040 (Morgan et al., 2023). Most CRCs develop from adenomatous polyps through a series of genetic and epigenetic changes that ultimately allow cancer cells to evade apoptosis, the programmed cell death mechanism essential for removing damaged or unwanted cells (Druliner et al., 2016; Tian et al., 2024).

Apoptosis is tightly controlled by the BCL-2 family proteins, with pro-apoptotic BAX

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promoting mitochondrial outer membrane permeabilization and cell death, while anti-apoptotic BCL-2 inhibits this process (Qian et al., 2022; Jan, 2019). An increased BCL-2/BAX ratio is often seen in CRC and contributes to apoptosis resistance, tumor progression, and poor prognosis (Tian et al., 2024).

Beyond its classical role in calcium regulation, 1,25-dihydroxyvitamin D_3 (calcitriol), the active form of vitamin D, shows anti-proliferative, pro-differentiative, and pro-apoptotic effects in various cancer models (Fekete et al., 2025; Vuolo et al., 2012). Epidemiological and preclinical studies consistently associate higher vitamin D levels with decreased CRC risk and better survival (Klampfer, 2014; Ng, 2014). In vitro, calcitriol and its analogs inhibit proliferation and induce apoptosis in multiple CRC cell lines, including HT-29 (Chiang et al., 2015). However, the exact molecular mechanisms, especially the direct influence of vitamin D on the transcriptional regulation of key apoptotic genes BAX and BCL-2 in CRC cells, remain not fully understood. Although vitamin D affects apoptosis in other cancers and influences BCL-2 family members in non-colonic models (Dallavalasa et al., 2024), targeted studies in colorectal cancer cells are limited, and findings across studies are sometimes inconsistent regarding dose-dependency and the BAX/BCL-2 expression ratio.

This knowledge gap impedes the rational development of vitamin D-based or vitamin D-inspired treatments for CRC. Therefore, the present study aims to evaluate the cytotoxic effects of 1,25-dihydroxyvitamin D_3 (calcitriol) on the human colorectal adenocarcinoma cell line HT-29 and to assess whether these effects are associated with transcriptional changes in the pro-apoptotic gene BAX and the anti-apoptotic gene BCL-2. By clarifying these mechanisms, this work seeks to provide mechanistic insight into how vitamin D exerts anti-tumor effects in CRC and to support its potential as an adjuvant therapeutic agent.

Material and Methods

Cell Culture and Cytotoxicity Evaluation

HT-29 cells (Royan Research Center, Isfahan, Iran) were maintained in DMEM with 10% FBS, 2 mM L-glutamine, penicillin (80 mg/L), streptomycin (50 mg/L), and Na_2HCO_3 (2 g/L) at 37°C, 5% CO_2. Vitamin D3 (cholecalciferol) was dissolved in absolute ethanol (≤0.1% final concentration). HT-29 cells were treated with vitamin D_3 at concentrations of 130, 650, 1300, 2600, 3900, 6500, and 9760 µM (equivalent to 50, 250, 500, 1000, 1500, 2500, and 3750 µg/mL, respectively) for 24 h. Doses were selected based on prior in vitro studies using high cholecalciferol concentrations to mimic supplementation effects (Fekete et al., 2025; Dallavalasa et al., 2024).

Isolation of Total RNA and Synthesis of cDNA

Specific flasks were used to trypsinize the cultured cells, and then they were collected by centrifugation at 1,320 g for 3 minutes at 37°C. The total RNA was subsequently extracted using a commercial kit (Sinaclon, Iran), which employed the Trizol method, for both treatment and control cell lines. Nanodrop analysis was used to evaluate the efficiency and effectiveness of the RNA extraction. Additionally, a spectrophotometer (Termoscientific, Wilmington, DE, USA) was utilized to assess the purity of the extracted RNA at 260–280 nm, and agarose gel electrophoresis at a 0.5% concentration was performed to verify the quality of the RNA. Once the pure RNA was prepared, cDNA was synthesized from it using oligo dT primers following the Sinaclon reverse transcriptase kit protocol. The synthesized cDNA was immediately prepared for real-time PCR, and the remaining cDNA was stored at -70°C.

Quantitative Real-Time PCR Analysis

The real-time PCR technique was used in this study to measure the expression levels of BAX and BCL-2 genes, as well as variations between treated and control samples, using the GAPDH gene as an internal control for data normalization. The BAX and BCL-2 gene expression levels were quantified using the qRT-PCR method, following the manufacturer's instructions, with triplicate employing Syber Green-I (Sinaclon, Iran) on a Mic Real-Time PCR Cycler (BMS, South Korea). For the real-time PCR, primers for BAX, BCL-2, and GAPDH (Kandhavelu et al., 2024) were used. A 25 µl reaction mixture included 5 pmol of each forward and reverse primer for BAX (5' TTCATCTCAGTCCCCTGCCC 3', 5' GGAGACAGGGACATCAGTCG 3'), BCL-2 (5' CCTGTGGATGACTGAGTACC 3', 5' GAGACAGCCAGGAGAAATCA 3'), and GAPDH (5' GGAGCGAGATCCCTCCAAAAT 3', 5' GGCTGTTGTCATACTTCTCATGG 3'), 2X PCR Master Mix with Sybr Green I, and 2 µl of

cDNA. The PCR conditions and protocols were validated through analysis of standard samples in triplicate. A five-fold serial dilution of cDNA derived from the HT-29 cell line was used to evaluate the expression of BAX and BCL-2. The PCR protocol included an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 45 seconds, annealing at 56°C for 45 seconds, and extension at 72°C for 60 seconds. Melting curve analysis was performed from 72°C to 95°C. Differences in BAX and BCL-2 expression between control and vitamin D-treated HT-29 cells, normalized to GAPDH mRNA levels, were quantified using the 2-ΔΔCT method.

Statistical analysis

The desktop version of GraphPad Prism 10.6.0 was used for statistical analysis and to evaluate the effect of Vitamin D on colon cancer cell (HT-29) viability as well as on BAX and BCL-2 transcript levels. Cell viability was assessed using a one-way ANOVA to compare different concentrations of Vitamin D, followed by Dunnett's post-hoc test to determine if there were any significant differences from the control. Homogeneity of variances was confirmed with Brown-Forsythe and Bartlett's tests. An unpaired t-test with Welch's correction was performed to compare treatment and control groups for BAX and BCL-2 transcript levels, with an F-test conducted to verify equal variances between groups.

Results

Effect of Vitamin D on the Viability of HT-29 Cells

HT-29 cells were treated with vitamin D at concentrations of 50, 250, 500, 1000, 1500, 2500, and 3750 µg/ml (n = 5 per group). Cell viability was measured and expressed as a percentage of the control (control = 100%) (Figure 1). One-way ANOVA revealed a significant difference among groups ($F(7,32) = 37.41$, $p < 0.0001$, $R^2 = 0.8911$). Homogeneity of variances was confirmed by Brown-Forsythe ($F(7,32) = 1.078$, $p = 0.3999$) and Bartlett's tests ($p > 0.05$). Dunnett's multiple comparisons test versus control showed significant reductions in cell viability at all tested concentrations (adjusted $p < 0.0001$ for all comparisons; Table 1).

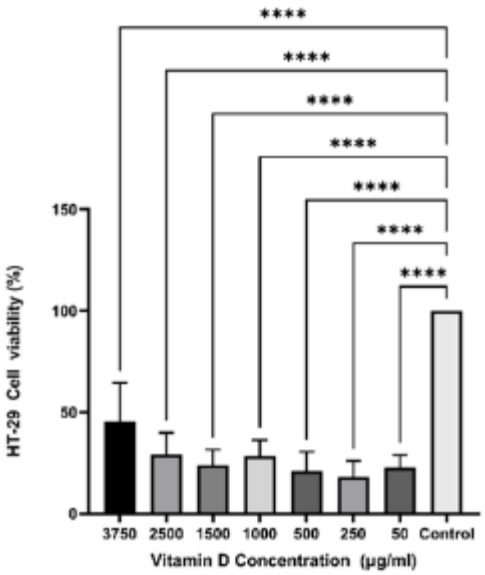

Fig. 1- Bar graph depicting HT-29 cell viability (%) across vitamin D concentrations (3750–50 µg/ml) and control, with asterisks indicating significance (**** $p < 0.0001$).

Table 1- Dunnett's multiple comparisons test results.

Comparison	Mean Difference	95% CI	Adjusted p-value
Control vs. 3750	54.59	37.26 to 71.92	<0.0001
Control vs. 2500	70.80	53.47 to 88.13	<0.0001
Control vs. 1500	76.26	58.93 to 93.59	<0.0001
Control vs. 1000	71.51	54.19 to 88.84	<0.0001
Control vs. 500	78.96	61.63 to 96.29	<0.0001
Control vs. 250	81.81	64.48 to 99.14	<0.0001
Control vs. 50	77.27	59.94 to 94.59	<0.0001

Effects of Vitamin D on BAX and BCL-2 Expression in HT-29 Cells

HT-29 cells were treated with vitamin D at the concentration that produces the maximum antiproliferative effect (n = 4–5 per group). The relative mRNA expression of BAX and BCL-2 was measured by qPCR and normalized to GAPDH. An unpaired t-test with Welch's correction showed no significant difference in BAX expression (control 1.060 ± 0.382, treated 1.993 ± 0.832; difference 0.9323 ± 0.4818, t =

1.935, df = 3.186, p = 0.1431) or BCL-2 expression (control 1.127 ± 0.594, treated 0.8440 ± 0.312; difference -0.2835 ± 0.4854, t = 0.5841, df = 3.541, p = 0.5943). Variances were equal according to F-tests (BAX p = 0.4945; BCL-2 p = 0.6399). Data are presented in Figure 2 and summarized in Table 2.

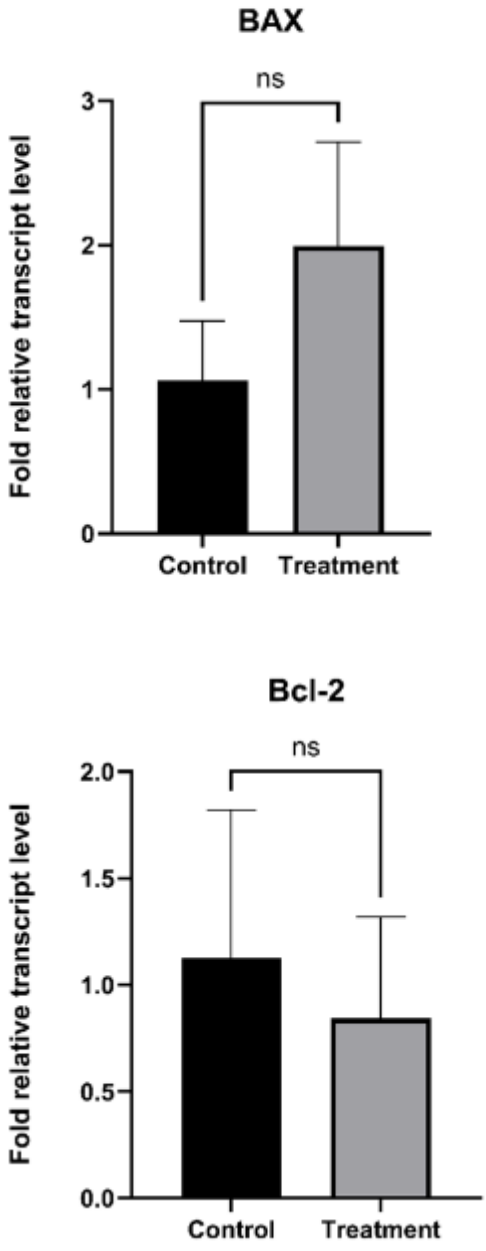

Fig. 2- Bar graphs depicting relative transcript levels of BAX and Bcl-2 in control and treatment groups of HT-29 cells, with error bars indicating standard errors (ns = not significant, p > 0.05).

Table 2- Statistical summary of unpaired t-tests with Welch's correction.

Gene	Mean Control	Mean Treatment	Mean Difference ± SEM	t-value (df)	p-value	R squared
BAX	1.060	1.993	0.9323 ± 0.4818	1.935 (3.186)	0.1431	0.5402
BCL-2	1.127	0.8440	-0.2835 ± 0.4854	0.5841 (3.541)	0.5943	0.08788

Discussion

The findings indicate that vitamin D treatment leads to a significant reduction in the survival of the colorectal cancer cell line HT-29 in a manner proportional to the doses used but not linear, with the most pronounced effects on cell growth observed at the middle range of 250–500 µg/ml concentrations. This study has answered the key question by confirming the role of vitamin D as a cell survival inhibitor (Fekete et al., 2025; Garland et al., 2006), likely through disrupting cellular proliferation pathways, as evidenced by the substantial variance explained by the treatment in the ANOVA analysis. However, the absence of significant changes in BAX and BCL-2 transcript levels does not support the idea that vitamin D induces apoptosis by altering these major regulators. Instead, it suggests that the cytotoxic effect may involve other pathways, such as cell cycle arrest or differentiation, which do not directly trigger apoptosis (Vuolo et al., 2012; Dallavalasa et al., 2024). These insights highlight the potential of vitamin D as a treatment for colorectal cancer, indicating that the optimal dosage might be specific enough to maximize its anti-oncogenic effects without necessarily engaging the traditional apoptotic machinery. This understanding broadens the application of the drug, emphasizing that its anticancer actions are multifaceted.

The results demonstrate a clear concentration-dependent reduction in HT-29 colon cancer cell viability following 48–72 hours of exposure to 1,25-dihydroxyvitamin D3, aligning with many previous reports on the antiproliferative and pro-differentiation effects of the active vitamin D metabolite in colorectal cancer cell lines (Zhang et

al., 2023). Cell viability gradually declined from 3750 µg/mL to about 250–100 µg/mL, reaching the lowest levels (~30–40% of control) in this mid-to-high concentration range. However, at the highest concentrations tested (500–3750 µg/mL), viability partially increased again, producing a bell-shaped (non-monotonic) dose–response curve. Because cell viability did not drop below approximately 30% even at the highest doses and then increased again, it was not possible to accurately calculate a traditional IC50 value from these data. The loss of antiproliferative effectiveness at super-micromolar concentrations likely results from nonspecific cytotoxicity rather than targeted, receptor-based growth inhibition or apoptosis. Similar bell-shaped responses have been observed for 1,25-dihydroxyvitamin D3 and its analogs in different cell types. These responses are generally linked to off-target membrane effects, calcium overload, or activation of cellular stress pathways that override VDR-mediated antiproliferative signals at very high doses (He et al., 2022).

Thus, the growth-inhibitory effect appears strongest in the 50–250 µg/mL range, with signs of saturation or reversal at higher concentrations caused by nonspecific toxic mechanisms rather than saturation of the vitamin D receptor (VDR) pathway alone. These findings support the antiproliferative potential of 1,25-dihydroxyvitamin D3 in HT-29 cells within a therapeutically relevant concentration range but also highlight the importance of avoiding supra-physiological doses that could cause nonspecific cytotoxicity.

In contrast, the significant viability reductions in our study exceed those reported in some literature where vitamin D's effects are boosted by combinations, such as with thymoquinone (TQ) or 5-fluorouracil (5-FU) (Milczarek et al., 2014), resulting in increased cytotoxicity through PI3K/AKT/mTOR inhibition. While monotherapy in our experiments caused significant inhibition at all tested concentrations, these combination approaches in existing research boost antiproliferative effects, suggesting that our findings could be further improved in synergistic settings. This reveals a possible discrepancy in efficacy thresholds, as standalone vitamin D in HT-29 cells has been reported to have moderate effects in less differentiated states, whereas our data show strong independent activity, possibly due to differences in experimental durations or cell passage numbers.

Although there was a moderate trend toward increased BAX, the expression levels of BAX and BCL-2 did not significantly change, leading to the conclusion that vitamin D might not act through the BCL-2 family of proteins in HT-29 cells (Qian et al., 2022) or that the conditions under which the treatment was applied were not adequate to reveal the changes. This suggests the possibility of alternative apoptosis pathways, such as caspase-independent or ROS-mediated mechanisms (Carneiro and El-Deiry, 2020), which require further study to understand their role in cancer therapy overall.

Our observations on gene expression differ from several studies where vitamin D or its analogs significantly upregulate BAX while downregulating BCL-2 in colorectal cancer cells, leading to increased BAX/BCL-2 ratios and higher levels of apoptosis. For example, calcitriol-loaded nanoparticles increased the BAX/BCL-2 ratio in HT-29 cells by elevating ROS, contrasting with our non-significant results, which may be due to differences in delivery methods or exposure times—12 hours in the cited studies versus potentially longer in our protocol. This discrepancy adds to the literature by suggesting that regulation may be context-specific, with vitamin D's pro-apoptotic effects possibly relying on factors like oxidative stress inducers, indicating that monotherapy may primarily target proliferation rather than apoptosis in this cell line.

This study has several limitations. First, gene expression analysis was conducted with a small sample size (n=3 experiments), which likely reduced statistical power and explains the wide confidence intervals and moderate effect sizes observed for BAX and BCL-2. Second, cell viability and apoptosis were only assessed using the MTT assay. Due to financial constraints and limited access to flow cytometry during the study, confirmatory tests like annexin V/PI staining via flow cytometry or other methods for detecting apoptosis could not be performed. Although the MTT assay was done in triplicate with strict positive and negative controls, providing reproducible results, the lack of an independent apoptosis assay is an important limitation for future research. Moreover, the in vitro setup limits

the applicability to in vivo conditions, where immune interactions, tumor microenvironment, and pharmacokinetics may influence vitamin D effects. The absence of time-course experiments further prevents understanding temporal changes in gene regulation and apoptosis. Recent large-scale trials, including the SOLARIS study (Morelli et al., 2022; Kuznia et al., 2022), indicate that high-dose vitamin D3 supplementation may not offer substantial benefits in metastatic colorectal cancer overall; however, potential subgroup benefits in patients with left-sided tumors warrant further study (Morelli et al., 2022; Ng et al., 2019).

To overcome these limitations, future studies should incorporate larger biological replicates, include additional apoptosis assays such as flow cytometry with annexin V / propidium iodide, caspase-3/7 activity measurement, or Western blotting for cleaved PARP, and conduct time-course analyses. Exploring clinically relevant models, like patient-derived organoids or xenograft systems, is also recommended. Utilizing multi-omics approaches and testing drug combinations that act synergistically could further enhance understanding of vitamin D-induced apoptosis's therapeutic potential in colorectal cancer.

Conclusion

In summary, 1,25-dihydroxyvitamin D3 exerted a significant concentration-dependent antiproliferative effect on HT-29 colorectal cancer cells, with maximal growth inhibition (~60–70% reduction relative to control) observed at 50 –250 µg/ml. Higher concentrations resulted in partial recovery of viability, producing a bell-shaped dose–response curve. Although substantial cytotoxicity was achieved, no significant changes in *BAX* or *Bcl*-2 mRNA levels were detected, suggesting that the observed reduction in cell viability is mediated primarily through cytostatic or differentiation-inducing mechanisms rather than classical apoptosis in this model. These findings indicate that 1,25-dihydroxyvitamin D3 possesses antiproliferative activity against HT-29 cells within a defined concentration range, supporting its potential utility as an adjunct therapeutic agent in VDR-expressing colorectal tumors. However, the bell-shaped response and the apparent contribution of nonspecific cytotoxicity at higher doses highlight the need for careful dose optimization. Further studies are required to (i) confirm these mechanisms in additional cell lines and in vivo models, (ii) explore synergistic combinations with standard chemotherapeutic or pro-apoptotic agents, and (iii) establish safe and effective dosing regimens in clinical settings.

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