Analysis of the Cytotoxic Effects of Vitamin D3 on Colorectal, Breast and Cervical Carcinoma Cell Lines

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Abstract
Although evidences from epidemiological suggested possible involvement of vitamin D in the prevention and treatment of cancers, it is not fully known how vitamin D inhibits cancer cell growth. Recent studies have shown that 1,25-(OH)2D inhibits cancer cell proliferation by binding to vitamin-D receptor (VDR). The vitamin D - VDR complex in turn (a) upregulate cell cycle inhibitors p21 and p27; (b) promote apoptosis mediators caspase-3 and 7, Bad, p53 and PTEN; (c) arrest cells in senescence phase; (d) elevate cell differentiation; and (e) inhibit IGF signaling. Moreover, vitamin D reduces reactive oxygen species (ROS) thereby prevents the progression of cancer cells. However, it is currently unknown whether vitamin D in cancer induced cancer cell death is mediated by its effect on ROS destroying Nrf2 signaling. In addition, it is also not known whether withdrawal of glucose improves the efficacy of vitamin D as presence of excess glucose promotes ROS in cancer cells. Therefore, first, efficacy of vitamin D for inhibiting the growth of cell lines HCT116, HeLa and MCF-7 was determined. Next, the effect of vitamin D on Nrf2 expression and activity in the presence and absence of glucose was assessed. The data showed that vitamin D inhibited the growth of HCT116, HeLa and MCF-7 cells in a dose dependent manner with more potency toward HCT116. Vitamin D reduced the levels of Nrf2 and NQO1 expression when HCT116 cells were treated in glucose lacking medium. But, despite a significant reduction in cell viability, no change in the Nrf2 expression was observed if the HCT-116 cells were treated with vitamin D dissolved in high glucose (4.5g/l) containing DMEM. Therefore, it is concluded that the cell growth inhibition by vitamin D, observed in the presence of glucose, is not at least mediated by Nrf2 modulation in HCT116 cells.

Keywords: Vitamin D; Cancer; VDR; Nrf2; NQO1

Abbreviations: CYP24A1: 1,25-Dihydroxyvitamin D; 24-Hydroxylase; DMEM:Dulbecco’s Modified Eagle Medium; DMSO: Dimethylsulfoxide; FBS: Fetal Bovine Serum; FITC: Fluoresceinisothiocyanate; GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase; HPLC: High Performance Liquid Chromatography; MAPK: Mitogen-Activated Protein Kinases; NQO1: NAD(P)H Dehydrogenase -Quinone 1; Nrf2: Nuclear Factor Erythroid 2-Related Factor 2; ROS: Reactive Oxygen Species; SRB: Sulfurhodamine B; TCA: Trichloroacetic Acid; TRITC: Tetramethylrhodamineisothiocyanate; VDR: Vitamin D Receptor; VEGF: Vascular Endothelial Growth Factor

Introduction
Vitamin D is a fat-soluble secosteroid with proven ability to inhibit proliferation of cancer cells and inflammatory reactions [1]. Vitamin D presents in two forms, viz., Ergocalciferol or vitamin D2 and Cholecalciferol or vitamin D3 [2]. Vitamin D3 is synthesized from 7-dehydrocholesterol in the skin [2]. Vitamin D status of an individual is influenced by solar radiation, diet and lifestyle [3]. Lack of sufficient quantity of vitamin D not only causes severe disease such as rickets but also make the individual susceptible for developing cancers [4]. Vitamin D insufficiency has been reported in the pathogenesis of various carcinomas such as gastric, breast and non-Hodgkins lymphoma [5].

Recent correlative studies measuring the levels of vitamin D in patients suffering from carcinomas of breast and prostate have shown a decreased vitamin D level with advanced disease [2]. However, it is not known whether supplementing vitamin D improves the life span by decreasing the burden of cancer, warranting further studies. In this regard, a study reported supplementation of vitamin D to prevent cancer has investigated [6]. But, randomized clinical trials of adequate size and duration with sufficient concentrations of vitamin D are still lacking [7].

Mechanistically, vitamin D3 binds to VDR, which in turn activate vitamin Dresponse elements (VDREs), thereby directly control the transcription of target genes that include genes regulating cell cycle arrest, differentiation and angiogenesis [8-10]. Vitamin D also plays a prime role in the regulation of angiogenesis [11]. The active form of vitamin D, down regulates the prostatic glandin cascade and enhances VEGF expression [12]. Calcitriol has been involved in the inhibition of Wnt signaling in healthy colonic cells highlighting its cancer preventive action [13]. Many animal and human studies have also shown anti-tumor effects of metabolically active form i.e., 1,25(OH)2D3 [14].

Vitamin D3 is a potent antioxidant with a strong ability to prevent cancer cell proliferation [15]. Mechanistically, vitamin D3 destroys cellular ROS thereby limits their availability for rapidly proliferating cancer cells [16]. Molecular studies have demonstrated that vitamin D3 upregulates Nrf2 and its target genes NQO1, GST, GPX, SOD and Catalase thereby destroy cellular ROS [17]. Cancer cells produce unusually high ROS due to incomplete oxidation of glucose and lack of ample supply of oxygen for growing tumor cells. Elevated ROS helps...
to trigger cell proliferation by inhibiting cell cycle inhibitors as well as promoting mitogenic substances [18]. Therefore inhibiting ROS production is likely to reduce cancer cell proliferation [19]. Many Nrf2 activators, especially compounds such as curcumin, resveratrol, pterostilbene and phenethylisothiocyanate (PEITC) have been shown to retard cancer cells growth through Nrf2 activation followed by ROS down regulation [20].

To address whether vitamin D3 induced cancer cell growth is also mediated through the activation of Nrf2, we have tested the effect of vitamin D3 on cell proliferation in the presence of high glucose (more ROS) and no glucose (Low ROS) and compared the cell growth inhibition data with Nrf2 and its target gene NQO1 expression and activity levels. The data demonstrated that vitamin D3 inhibited cancer cells proliferation irrespective of whether the cells are grown in media containing glucose or no glucose. However, interestingly, vitamin D3 treatment reduced Nrf2 when the cells were grown in media containing no glucose suggesting that the cell growth inhibition by vitamin D3 is mediated by mechanisms other than Nrf2 modulation when the cells were cultured and treated in glucose containing medium, but, in the absence of glucose during treatment, the inhibition of cell proliferation is partly mediated by Nrf2 down regulation. However, further studies are required to delineate the mechanisms leading to these differential effects of vitamin D3 on Nrf2 signaling.

Materials and Methods

Cell culture

Cell lines representing colon (HCT-116), cervix (HeLa) and breast (MCF-7) were procured from NCCS, Pune, Maharashtra, India. The cells were grown in DMEM supplemented with 10 % FBS, 1%Glutamax and 1% Penicillin-Streptomycin(from Life Technologies, India. The cells were grown in DMEM supplemented with 10 % FBS, 1%Glutamax and 1% Penicillin-Streptomycin(from Life Technologies, India. The cells were grown in DMEM supplemented with 10 % FBS, 1%Glutamax and 1% Penicillin-Streptomycin(from Life Technologies, India. The cells were grown in DMEM supplemented with 10 % FBS, 1%Glutamax and 1% Penicillin-Streptomycin(from Life Technologies, India.

Viability assessment

Sulforhodamine-B (Sigma Chemical Company, St. Louis, USA) assay was used to determine the cell viability as described by Skehan et al. [22]. Experimentally we can follow:

Step 1: Fixation of cells: Control and treated cells were fixed in 1/4×volume of cold 50% (v/v) TCA for 4°C.

Step 2: Removal TCA and serum proteins: After 1 hr the media was removed and the wells washed with water (200 μL × 4 times) to remove TCA and serum proteins.

Step 3: Staining of cells with SRB: The dried wells were incubated with 100 μL 0.4% SRB for 30 minutes to stain the cellular proteins, and washed quickly with 1% acetic acid (200 μL × 4 times) to remove unbound SRB.

Step 4: Solubilization and OD measurement: The bound SRB was solubilized in 10 mM Tris base (100 μL/well) and the absorbance measured at 565 nm in a multimode plate reader (Perkin Elmer 2300). The plate background value was measured at 690 nm, and deducted from the 565 nm readings for calculating the net optical density.

Step 5: Determination of percentage growth inhibition and IC50 values: Percentage growth inhibition was calculated using the following formula:

\[
\% \text{ Growth inhibition} = \left[ \frac{\text{OD of vehicle treated cells} - \text{OD of vitamin D treated cells}}{\text{OD of vehicle treated cells}} \right] \times 100
\]

The IC-50 value was calculated from the plot of inhibition (%) VS concentration of vitamin D3 using GraphPad Prism [23].

Determination of cellular uptake of vitamin D

1.5 × 10⁴ HCT116 cells were allowed to grow in 100 mm Petri plates for 48 h, and treated with 16 μM vitamin D3 for 48 h. The medium was collected and proteins precipitated using HPLC grade methanol. The precipitated proteins were separated by centrifugation at 16,000 rpm for 10minutes, and 20 μL supernatant injected to HPLC equipped with C18 column (4.60 × 250 mm). The elution of vitamin D3 was carried out using HPLC grade methanol (1.0 mL /min) and the eluted compound detected using a UV detector operating at 270 nm wavelength. Standard graph was prepared using 5.0 μM, 10.0 μM, 15.0 μM, 25.0 μM, 50.0 μM and 100.0 μM vitamin-D3 (Sigma Chemical Co, St Louis, USA) and the amount present in the media calculated. Cellular uptake was calculated by deducting the amount present in the supernatant from the total amount used for treatment. Data from three independent experiments was considered for plotting the graph.

Determination of apoptosis

Apoptosis detection using acidine orange and ethidium bromide staining method was carried out as described by Shailasree et al. 2015 [24]. In brief, 0.3 × 10⁴ HCT-116 cells in 2.0 mL DMEM supplemented with 10% FBS per well were plated in a 6-well plate and after ~24 h exposed to increasing concentrations of vitamin D3 (10 μM,20 μM,40 μM) for 24 h. The control and treated cells were trypsinized and mixed thoroughly to obtain a single cell suspension. Trypsin was neutralized by the addition of complete medium and 20.0 μL cell suspension was incubated with 10.0 μL 100.0 μg/mL ethidium bromide and 10.0 μL of 100.0 μg/mL acidine orange mixture for 15.0 minutes. Oxa1platin (100 μM) served as positive control. The stained cells were imaged using the fluorescence microscope using TRITC and FITC filters. The images obtained using 2 different channels were later merged to obtain a combined image exhibiting green (live) and orange (apoptotic) cells. At least 5.0 different fields were considered for quantifying the live and apoptotic cells and the percentage apoptosis estimated [24].

Real Time-PCR

Isolation of total RNA from cells: Total RNA from cultured cells was isolated using Trizol (Guadininiumisothiocyanate-Phenol-Chloroform mixture) method as described in Chomczynski et al. [25]. In brief, confluent cells were washed with PBS and total RNA extracted using Trizol reagent by following the steps described below

1. Pelleted 3-5 × 10⁶ cells by centrifugation at 2500 rpm for 10minutes and added 300 μL Trizol.

2. Mixed well by vortexing for 1min and next added 60.0 μL of chloroform

3. Mix the solution by twining up and down for 25-30 times. Next, the solution was incubated for 2 min at room temperature and centrifuged the content at 10000 rpm for 10min.

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4. Using pipette, top aqueous layer containing RNA was collected and transferred to a new, labeled 1.5 mL microcentrifuge tube.

5. Next, 150.0 µL of isopropanol was added to the aqueous layer and incubated for 10 min at room temperature.

6. The solution was centrifuged at 10000 rpm for 10 min, and the supernatant removed.

7. At this point, 500 µL of 70% ethanol was added, mixed well and spun down for 5 min at 10000 rpm

8. Next, the alcohol was decanted and pellet air dried for 2-3 min in laminar hood.

9. The dried pellet was dissolved in 30-50 µL nuclease free water and quantitity of RNA measured using spectrophotometer operating at 260 nm and 280 nm.

10. The RNA was stored at -80°C for subsequent usage.

The quality of isolated RNA was checked using 1% bleach agarose gel by measuring the ratio between 28S and 18S bands [25]. Quality of isolated RNA is rated good if the ratio between 28S to 18S bands is 2:1.

**Bleach gel electrophoresis:** Agarose gel (1%) was prepared by mixing 1 gram of agarose in 100 mL 1X TAE buffer (40 mM Tris – pH7.6; 20 mM Acetic acid and 1 mM EDTA) containing 600.0 µL 5% sodium hypochlorite. The reaction mixture was incubated for 5 – 10 minutes at room temperature, and the solution heated in a boiling water bath until the agarose dissolve. To the melted solution ethidium bromide, at a concentration of 0.5 µg/mL, was added. The samples (24 µL) were electrophoresed by applying 100 volts (1-5 volts/cm) for a period of 60 min. The separated RNA was visualized by exposing to UV light in a UV-transilluminator (Syngene Gel Documentation Unit).

The quality of isolated RNA is rated good if the ratio between 28S to 18S bands is 2:1 [25].

**Preparation of cDNA:** The reverse transcription reaction was performed using the High-Capacity cDNA Reverse Transcription Kit (Life Technology), in a final volume of 20 µL containing 1 µg of total RNA, 100ng of random hexamers, 2 µL reverse transcription buffer, 2.5 mM MgCl₂, 1 mM dNTP, 20U of Superscript reverse transcriptase, and nuclease free water. Following conditions were used to synthesize cDNA:

**Step-1:** 25°C for 10 min;

**Step-2:** 37°C for 120 min;

**Step-3:** 85°C for 5 min;

**Step-4:** 4°C.

After cDNA synthesis 80 µL of nuclease free water was added to obtain a final concentration of 10 ng/µL cDNA.

**Quantitative real-time PCR analysis (Q-PCR):** All the primers were purchased from Sigma Chemical Company, Bengaluru, Karnataka, India. RT-PCR experiment was performed using the Qiagen Rotor Gene-Q system and the relative fold change determined using the method described by Livak, et al. [26].

Quantification of human NRF2 and NQO1 was performed by using DyNAmo Color Flash SYBR Green QPCR Kit (Finnzymes) by following manufacture instructions. Experimentally, first, the reaction mixture, in a total volume of 20 µL, containing 50 ng of template cDNA and primers was prepared in each tube. The samples were analyzed in duplicates to determine the reproducibility.

In summary, the RT-PCR reaction mixture contains:

1. SYBR green dye - 10 µL
2. Primers: Forward-0.2 µM; Reverse -0.2 µM
3. Template: 50 ng
4. Nuclease free water (require to become 20 µL)
5. Primers for NRF2 (According to Zou, X. et al. [27])
6. Reverse Primer: TTTGCTGTGACTGGTCTG
7. Primers for NQO1 (According to Seng et al.)[28]
8. Forward Primer: TGACGGCTTTGAAGAAAGAG
9. Reverse Primer: TCAGGCAGTACTGAAAGTTGCA
10. Primers for GAPDH (Internal control for normalization) (According to Lister et al.) [29]

For example, 50% growth inhibition was observed at 50 µM concentration for HCT116 cell line at all time points, however, for HeLa and MCF-7 cell lines an about 1250 µM concentration was required to yield 50% growth inhibition (Figure 1).

**Results and Discussion**

**Vitamin D3 retarded the growth of cell lines representing carcinomas of colon and rectum, breast and cervix**

Anti-proliferative activity of vitamin D3 was determined by treating cancer cell lines (HCT116, MCF-7 and HeLa) with increasing concentrations of vitamin D3 for 24, 48 h and 72 h, followed by counting the number of viable cells using SRB assay as described in Materials and Methods. The percentage inhibition compared to DMSO was calculated and plotted against the concentration of vitamin D3. The data showed an increase in the percentage growth inhibition as the concentration of vitamin D3 increases (Figure 1). Among the three cancer cell lines tested HCT-116 found more susceptible for vitamin D3 treatment compared to HeLa and MCF-7.

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**Vitamin D3 is taken up by cancer cells**

In order to determine the amount of vitamin D3 taken up by cancer cells, HCT116 cells, which responded to vitamin D3 treatment, were treated with 40 µM vitamin D3 for 48 h and levels in media measured using HPLC. The percentage recovery of vitamin D3 from serum containing medium was assessed as vitamin D is known to adhere to serum proteins [30]. Results of recovery analysis yielded, reproducibly, ~56% indicating the binding of ~44% vitamin D3 to proteins present in the serum (Figure 2). Considering this recovery percentage using the extraction procedure detailed in materials and methods, the data showed in Figure 2 indicate that HCT116 cells have taken ~43% of vitamin D3 added to the medium (translating to about 17.2 µM in cells). Further increase in the vitamin D concentration in the medium did not change the levels of vitamin D taken up by the cells (data not shown). This could be one reason why no major increase in percentage growth inhibition occurred with further raise in the concentration of vitamin D3 (Figure 2).

Variations in response of each cell line to vitamin D3 treatment could be due to differences in the ability of each cell line to up take vitamin D3, which requires further studies.
Vitamin D3 induced apoptosis in HCT-116 cells: In order to determine the processes affected by vitamin D3 treatment, the levels of apoptosis were estimated using acridine orange and ethidium bromide staining as detailed in materials and methods. The data showed a dose dependent increase in apoptosis as evidenced by the presence of small and shrunken cells with orange staining (Figure 3). However, no such cells were observed in untreated or vehicle 1% DMSO treated cells. The positive control Oxaliplatin also induced significant apoptotic cell death in HCT116 cells (Figure 3).

Vitamin D3 inhibited Nrf2 expression and activity in a glucose dependent manner

Based on prior studies vitamin D3 is known to stabilize the master antioxidant regulator Nrf2 in cells thereby protect cells from oxidative stress induced damage [31]. However, cancer cells experience more oxidative stress as they primarily use glucose as the source of energy and carbons, and incomplete oxidation of glucose is known to produce very high levels of reactive oxygen species [32]. Therefore, it has been predicted that cancer cells express more Nrf2 and inhibition of Nrf2 by pharmacological agents retard the growth of cancer cells. To test this, Nrf2 expressing HCT116 cells were treated with increasing concentrations (1 µM, 10 µM and 50 µM) of vitamin D3 for 6h in the presence or absence of glucose and levels of Nrf2 and NQO1 measured. The data showed a 35% decrease in Nrf2 expression at 50 µM concentration, which resulted in about 75% decrease in NQO1 mRNA only when the cells were grown in media containing no glucose. However at 1.0 µM and 10 µM concentration no major changes in Nrf2 expression was observed, but a significant 40 to 70% decrease in NQO1 was noticed only in the absence of glucose. But, in the presence of glucose minor decrease in Nrf2 and NQO1 was observed only at 1.0 µM but not at 10.0 µM and 50.0 µM. Variations in the effect of vitamin D on Nrf2 and NQO1 expression in the presence and absence of glucose...
indicate that vitamin D helps in the stabilization of Nrf2 when there is a need as observed in the glucose-containing medium, but inhibit the expression of Nrf2 when there was no such demand.

**Discussion**

Results from various recent reports that include (a) correlative studies measuring and comparing the levels of vitamin D in cancer patients with disease severity; (b) preclinical studies determining the effect of administering vitamin D on tumor development have suggested the therapeutic potential of this cholesterol derived sun-vitamin for treating cancers [33]. These studies have primarily focused on findings from population-based data and xenografted tumor models, hence, not much is known about the mechanism of action of vitamin D [34]. Moreover it is also not known whether all cancer types respond equally to vitamin D treatment. This information is required urgently as cancer cell lines representing carcinomas of breast, cervix and colon and rectum vary in the expression of Nrf2, a known target of vitamin D [35]. On top of this cell to cell variation in the Nrf2 expression, the cellular Nrf2 levels also vary in response to oxidative stress status, which further influenced by the amount of glucose available in the medium [35]. Therefore, it is important to assess the efficacy of vitamin D in the presence or absence of glucose for inhibiting these cancer cell lines growth.

Data from our studies showed that vitamin D retarded the growth of colorectal cancer cell line HCT116 more effectively compared to MCF7 and HeLa. Even though vitamin D reduced the cell growth in the presence of glucose the effect is more when the media is devoid of glucose as Nrf2 is inhibited by vitamin D treatment only in the absence of glucose. Interestingly, vitamin D inhibited the activity of NQO1, a target gene of Nrf2, more effectively in the absence of glucose. NQO1 is required for cancer cells growth as it helps in the recycling of NAD and NADP co-enzymes [35]. These co-enzymes are essential for continuing key pathways that utilize glucose such as glycolysis and HMP [36]. Since these glucose-metabolizing pathways are heavily utilized by rapidly growing cancer cells, inhibition of NQO1 results in growth retardation of cancer cells. Prior studies have demonstrated the utility of NQO1 inhibitors such as dicoumarol for retarding the cancer cells development [37]. Cell growth inhibition observed by vitamin D treatment in the presence of glucose is probably due to the effect on multiple other signaling cascades regulating cancer cells viability and survival. For example, vitamin D is a known inhibitor of MAPK signaling cascade. MAPK regulates cell proliferation by promoting cyclin D1 levels in cancer cells [38]. Many other studies determining the efficacy of MAPK inhibitors have shown anti-cancer activity indicating MAPK signaling members are good therapeutic targets [38].

Even though we have made an attempt to determine the efficacy of vitamin D on cancer cells representing carcinomas of breast, colon and rectum and cervix, this study require additional data to conclusively explain: (a) the variations in the efficacy of vitamin D; (b) the influence of glucose on vitamin D potency; (c) whether Nrf2 inhibition by
vitamin D is the key mechanism responsible for growth retardation. Hence future studies should focus on addressing these unresolved questions (Figures 4 and 5).

Conclusion
In conclusion, we have shown that vitamin D3 (a) inhibits the growth of colorectal carcinoma cells more effectively compared to MCF7 and HeLa cell lines; (b) mediated cell growth inhibition is in part due to the downregulation of Nrf2 only in the absence of glucose; (c) induced apoptosis in colorectal carcinoma cell lines.

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References


