

# Treatment of K562 cells with 1,25-dihydroxyvitamin D<sub>3</sub> induces distinct alterations in the expression of apoptosis-related genes BCL2, BAX, BCL<sub>XL</sub>, and p21

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**Abstract** Apoptosis, or programmed cell death, is a very important phenomenon in cytotoxicity induced by anticancer treatment. 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> (1,25-(OH)<sub>2</sub>D<sub>3</sub>), the active metabolite of vitamin D, inhibits the growth of multiple types of cancer cells including breast, colon, and prostate cancer cell lines. We studied alterations in the mRNA expression levels of BCL2, BAX, CYC, BCL-XL, and VDR genes in the K562 chronic myeloid leukemia cell line in response to treatment with 1,25-(OH)<sub>2</sub>D<sub>3</sub>. Morphological observation of K562 cells was evaluated by the staining with Wright's solution. Cell percentage at different phases of the cell cycle was measured, and apoptosis was measured by flow cytometry. The expression levels of the apoptosis-related genes were analyzed by real-time reverse transcription polymerase chain reaction. We found that treatment with 1,25-(OH)<sub>2</sub>D<sub>3</sub> down-regulates BCL2 and BCL-XL mRNA expressions, as well as up-regulates expressions of BAX and p21 mRNA. The expression pattern of CYC and VDR genes were not influenced. However, K562 cells treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> caused an

arrest of cell cycle progression in G1 phase resulting in a decreased number of cells in the S phase, complemented by an accumulation of cells in the G0–G1 phases. Our data show the modulatory effects of 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment in apoptosis-related genes in K562 cells.

**Keywords** 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> · K562 · Apoptosis

## Introduction

Vitamin D, especially its active physiological metabolite, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), plays a major role in calcium and phosphate homeostasis. During the past decade, emphasis has been placed on its role in cellular differentiation and proliferation, and it has been shown to have broad potential as an anticancer agent against different malignant cell lines derived from melanoma [1], myeloid leukemia [2, 3], breast cancer [4–6], prostate cancer, [7, 8], colon cancer [9], and retinoblastoma [10]. The 1,25-(OH)<sub>2</sub>D<sub>3</sub> analog binds with high affinity to a specific nuclear receptor (vitamin D receptor (VDR)) [11]. This binding induces a conformational change that activates the VDR and the dimerization with another nuclear receptor, the retinoic X receptor. The heterodimer then binds to specific DNA motifs called vitamin D response elements (VDREs) in the promoters of target genes and activates their transcription [12]. Target genes can be involved in phosphocalcic metabolism or in regulating cell division and cell death. Vitamin D treatment induces an arrest in the G1 phase of the cell cycle in numerous cancer cell lines, which is probably caused by the up-regulation of one or both of the cyclin-dependent kinase inhibitors (CDKIs) p21WAF1/cip1 or p27kip1. A VDRE has been found in the promoter of the p21 gene, indicating that vitamin D may directly

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activate its transcription [13–15]. No VDRE has been identified in the p27 gene, and its up-regulation seems to be more cell-type dependent [16].

Cell death by apoptosis occurs as a part of a natural regulatory process in the body, serving to establish and maintain a proper control of the cellular turnover. Apoptosis is closely linked to the cell cycle and is thus controlled in part by the same cell cycle regulatory machinery [17, 18]. Cancer is often associated with cells that fail to undergo apoptosis. This leads to the survival of aberrant cells, which would normally die, and, in turn, to malignant outgrowth. The fact that vitamin D is able to induce apoptosis in a number of different cancer cell types is therefore of immense interest and suggests that induction of apoptosis is likely to be one mechanism by which vitamin D exerts its anti-cancer effects. Induction of apoptotic features in response to vitamin D has been demonstrated in breast, colon, and prostate cancer cells as well as in melanoma, myeloma, and glioblastoma cells *in vitro*. However, the effect may vary between the different subtypes of these cells [19–21].

K562 is a chronic myeloid leukemia cell line established by Lozzio et al. in 1977 with rapid growth rate and poor differentiation [22]. It can be induced to erythroid differentiation by various compounds, but 1,25-(OH)<sub>2</sub>D<sub>3</sub> may inhibit the erythroid differentiation and shift the pathway of differentiation from the erythroid to the monocytic lineage [23].

Vitamin D treatment also can induce apoptotic death of tumor cells [24–26]. The mechanisms involved in this induction are not well understood and appear to vary with the cell type. In this study, we investigated the apoptotic action of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in K562 erythroleukemia cell line. We report that the 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment is associated with an increase in apoptotic cell death in K562 cells.

## Materials and methods

### Materials

K562 leukemia cell line was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. Fetal calf serum (FCS), RPMI-1640 mediums were purchased from GIBCO/BRL. 1,25-(OH)<sub>2</sub>D<sub>3</sub> was the product of Roche Company. Light Cycler FastStart DNA MasterPLUS SYBR Green I kits were from Roche diagnostics. The other reagents were commercially available in Turkey.

### Cell culture and treatment of 1,25(OH)<sub>2</sub>D<sub>3</sub>

Cells were cultured in a humidified 5% CO<sub>2</sub> atmosphere at 37°C, in RPMI-1640 medium containing 10% FCS, anti-

biotics (penicillin and streptomycin), and 2 mM L-glutamine. The cells were synchronized with serum hunger method (2% FCS in RPMI-1640 medium) for 48 h, and then transferred to 10% FCS/RPMI-1640 medium. Cells were seeded at a constant density overnight, then treated with 50 nM 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 72 h. For all experiments, 1 mM 1,25-(OH)<sub>2</sub>D<sub>3</sub> stocks were prepared in 100% ethanol and diluted in cell culture medium to obtain a final concentration of 50 nM 1,25-(OH)<sub>2</sub>D<sub>3</sub> and 0.1% ethanol in culture. This concentration of 1,25-(OH)<sub>2</sub>D<sub>3</sub> was determined by related studies and by performing titration experiments to ascertain the minimum amount of 1,25-(OH)<sub>2</sub>D<sub>3</sub> which elicited clear and reproducible changes in our *in vitro* assays [7, 27, 28]. Controls received 0.1% ethanol vehicle (a concentration equal to 1,25-(OH)<sub>2</sub>D<sub>3</sub>-treated cells).

### Total RNA extraction and cDNA synthesis

Total RNA was extracted from cells using High Pure RNA isolation Kit according to the protocol provided by Roche Diagnostics. Complementary DNAs (cDNAs) were synthesized from 2 μg of the total RNA with SuperScript First-Strand Synthesis System for reverse transcription polymerase chain reaction (RT-PCR) according to the protocol provided by Invitrogen (CA, USA). The mixture was incubated at 42°C for 50 min, 72°C for 15 min. After the addition of 2 U RNase H, the PCR was performed in a volume of 20 μl containing 2 μl cDNA.

### Real-time RT-PCR

Real-time PCR was carried out using a Light Cycler<sup>®</sup> 2.0 (Roche Diagnostics) instrument and Light Cycler FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics) kit. Reactions were performed in a 20-μl volume with 5 pmol of each primer and 2 μl of cDNA template derived from reverse-transcribed RNA of untreated ethanol (control) and 72 h treated cells. Hypoxanthine phosphoribosyl-transferase (HPRT) housekeeping gene was used as endogenous control and reference gene for relative quantifications. Sequences of oligonucleotide primers were as follows: HPRT (F) 5'-GTGGAGATGATCTCTCAACT-3', HPRT (R) 5'-ACATGATTCAAATCCCTGAAG-3', BAX (F) 5'-AAGAAGCTGAGCGAGT-3', BAX (R) 5'-GCCCCATGATGTTTCTG-3', CYC (F) 5'-TGGGTGATGTTGAGA AAGG-3', CYC (R) 5'-TTTGTTCAGGGATGTACT-3', BCL XL (F) 5'-GCTGGTGGTTGACTTTC-3', BCL XL (R) 5'-GGATGGGTTGCCATTGA-3', VDR (F) 5'-AGCTG GCCCTGGCACTGACT-CTGCTCT-3', VDR(R) 5'-ATG GAAACACCTTGCTTCTTCTCCCTC-3' p21(F) 5'-GCA GACCAGCATGA CAGATTT-3' p21(R) 5'-GGATTA GGGCTTCTCTTGGA-3' The same thermal profile was optimized for all primers: a pre-incubation for 10 min at

95°C, followed by 40 amplification cycles of denaturation at 95°C for 10 s, primer annealing at 59°C for 5 s, and primer extension at 72°C for 10 s. H<sub>2</sub>O was included as a no-template control. Melting curves were derived after 40 cycles by a denaturation step at 95°C for 10 s, followed by annealing at 65°C for 15 s, and a temperature rise to 95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement. Final cooling was performed at 40°C for 30 s. Melting curve analyses of each sample were done using LightCycler Software version 4.0.0.23 (Roche Diagnostics). The analysis step of relative quantification is a fully automated process done by the software, with the efficiency set at 2 and the cDNA of untreated cells defined as the calibrator. All experiments were done in triplicates.

#### Morphological observation of K562 cells during 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced differentiation

The cells treated with 50 nM concentrations of 1,25-(OH)<sub>2</sub>D<sub>3</sub> were smeared on microscope glass plates and stained with Wright's solution for 5 min. After naturally drying, the morphological changes of the cells were observed under the light microscope.

#### Flow cytometry for annexin V/propidium iodide

Apoptosis was assessed by staining cells with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). In brief, cells were washed with phosphate-buffered saline (PBS) and suspended in serum-free, binding buffer including CaCl<sub>2</sub>. The level of annexin V binding was determined by using a commercially available annexin V apoptosis detection kit (Annexin V-FITC Kit, Beckman Coulter, PN IM3546), according to the manufacturer's instructions. The cells were subsequently analyzed by a Coulter Epics XL.MCL flow cytometer (Beckman Coulter, Miami, FL). Approximately 10,000 events were collected for each sample. The percentage distributions were calculated by Expo32 ADC software (Beckman Coulter). Cells were classified as apoptotic (positive annexin V and negative PI), late apoptotic/necrotic (positive annexin V and positive PI), or viable (negative annexin V and PI). Unstained samples were used as negative fluorescence controls.

#### Flow cytometry for cell-cycle determination

Cells were washed with phosphate-buffered saline and suspended in PBS containing 20 µg/ml RNase A, 0.2% Triton X-100, 0.2 mM EDTA, and 20 µg/ml propidium iodide. Cell-cycle determination from cells was done using Coulter DNA PREP Reagents Kit according to the protocol provided by Beckman Coulter. After incubation at +4°C for 20 min, the cells were subjected to Coulter Epics XL.MCL

flow cytometer for determination of DNA contents. The cell percentage at each phase of cell cycle and the appearance of apoptosis (sub-G1 hypoploid cell fraction) were analyzed, and the flow-cytometric histogram was drawn automatically by Expo32 ADC software (Beckman Coulter). The experiment was performed at least three times, and a representative experiment is shown.

#### Statistical analysis

*p* values were determined by Epi Info StatCalc v6.0 software developed by the Centers for Disease Control and Prevention in Atlanta, GA (USA).

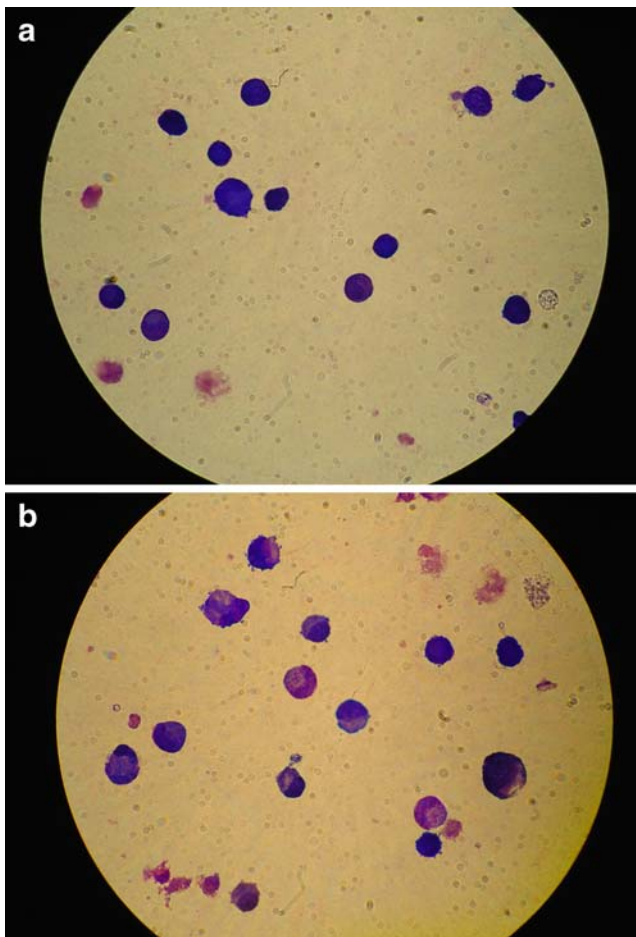
## Results

#### Morphological changes of K562 cells after 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment

Wright stains of K562 cells were performed on cytospin preparations from untreated cells, 1,25-(OH)<sub>2</sub>D<sub>3</sub> induced cells, and ethanol control group (Fig. 1). After the treatment of 1,25-(OH)<sub>2</sub>D<sub>3</sub>, K562 cells were differentiated toward monocytic lineage. This was evidenced by the increased vacuoles in the cytoplasm and condensed nuclear chromatin (Fig. 1).

#### Flow cytometry data for annexin V/PI staining

The Annexin V assay uses FITC-conjugate Annexin V to detect phosphatidylserine (PS) exposure by flow cytometry, while simultaneously, PI dye is used to distinguish viable, necrotic or apoptotic cells in the late terminal stages, as PI is internalized only in cells whose membranes have become permeable. The results of the Annexin/PI study are shown in Fig. 2. K562 cells were untreated (A) or treated for 72 h with 50 nM 1,25-(OH)<sub>2</sub>D<sub>3</sub> (B), and controls received 0.1% ethanol (C). Cells were incubated with Annexin V-FITC in a buffer containing PI and analyzed by flow cytometry. Untreated cells were primarily Annexin V-FITC and PI negative, indicating that they were viable and not undergoing apoptosis. After a 72-h treatment (B), there were primarily three populations of cells: cells that were viable and not undergoing apoptosis (Annexin V-FITC and PI negative), cells undergoing apoptosis (Annexin V-FITC positive and PI negative), and cells observed to be Annexin V-FITC and PI positive, indicating that they were in end-stage apoptosis or already dead. A decrease of approximately 10% in viability is observed in untreated K562 cells, most probably due to the incubation period in serum-free media. After 72 h of 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment, cell viability decreases statistically significantly to 84.4% (*p*<0.001),



**Fig. 1** Morphological changes of K562 cells after being treated with 50 nM 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentration of 1,25-(OH)<sub>2</sub>D<sub>3</sub>. **a** K562 cells without 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment (×400). **b** K562 cells treated with 50 nM 1,25-(OH)<sub>2</sub>D<sub>3</sub> (×400)

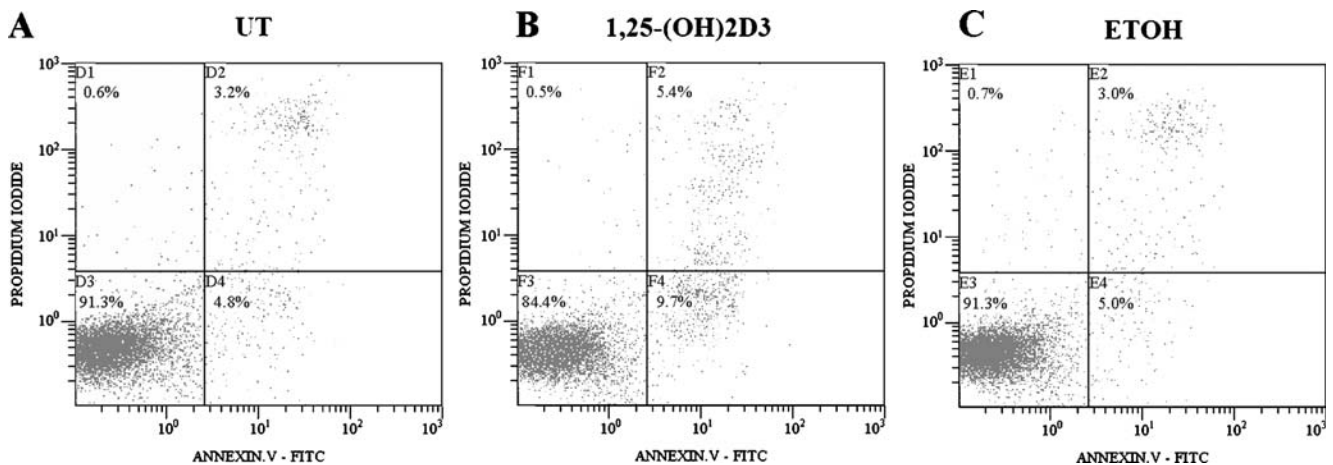
cells undergoing apoptosis increased statistically significantly 9.7% ( $p < 0.001$ ), and end-stage apoptosis or already dead cells increased statistically significantly 5.4% ( $p < 0.001$ ) in K562 cell populations.

Cell percentages at different phases of cell cycle and cell apoptosis after 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment

As shown in Fig. 3, representative cell percentages at different phases of cell cycle: untreated control group, K562 cells treated with 50 nM 1,25-(OH)<sub>2</sub>D<sub>3</sub>, and the ethanol control group. At the 50-nM concentrations of 1,25-(OH)<sub>2</sub>D<sub>3</sub>, the percentage of K562 cells in S phase decreased statistically significantly ( $p < 0.001$ ) as compared with the untreated controls. The number of cells in the G<sub>2</sub>-M compartment was relatively unaffected ( $p < 0.87$ ) by treatment with 1,25-(OH)<sub>2</sub>D<sub>3</sub>. In addition, the percentage of K562 cells in sub-G<sub>1</sub> (apoptotic cell) fraction was increased statistically significantly ( $p < 0.001$ ) when compared with the untreated controls. Also, the percentage of K562 cells in G<sub>0</sub>-G<sub>1</sub> phases increased statistically significantly ( $p < 0.008$ ) when compared with the untreated controls. These data show that 1,25-(OH)<sub>2</sub>D<sub>3</sub> caused an arrest of cell cycle progression in G<sub>1</sub> phase, resulting in a decreased number of cells in the S phase complemented by an accumulation of cells in the G<sub>0</sub>-G<sub>1</sub> phases.

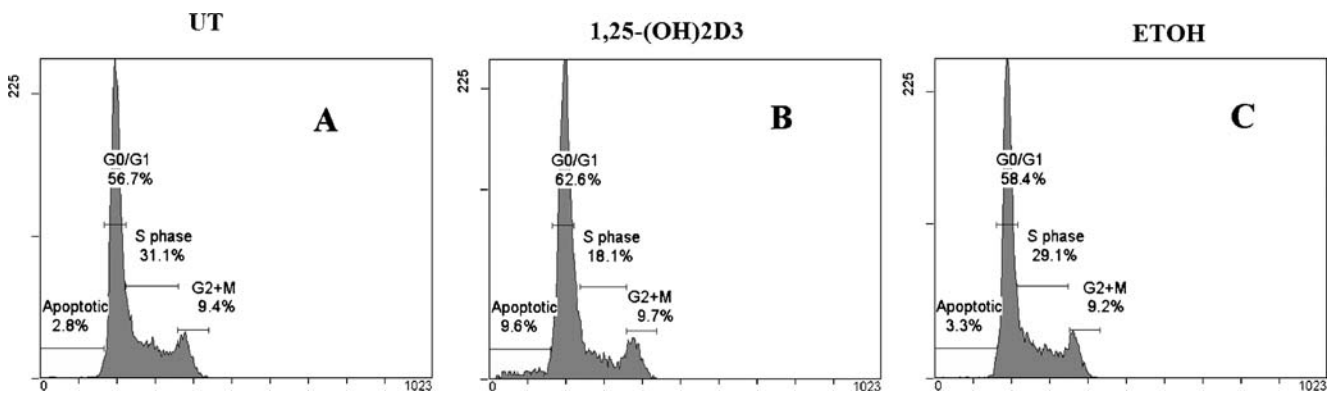
Real-time analysis for BCL2, CYC, BAX, BCL-XL, VDR, and p21

RNA was isolated from untreated, 1,25-(OH)<sub>2</sub>D<sub>3</sub>-treated, and ethanol group in K562 cells at the end of 72 h and subjected to real-time PCR assay as described in “Materials and methods.” For each time point, three individual



**Fig. 2** Flow cytometry percentage distributions after annexin V/propidium iodide staining in 50 nM 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment in K562 cells. **a** K562 control cells without 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment. **b** K562

cells treated with 50 nM 1,25-(OH)<sub>2</sub>D<sub>3</sub>. **c** Ethanol control cells without 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment

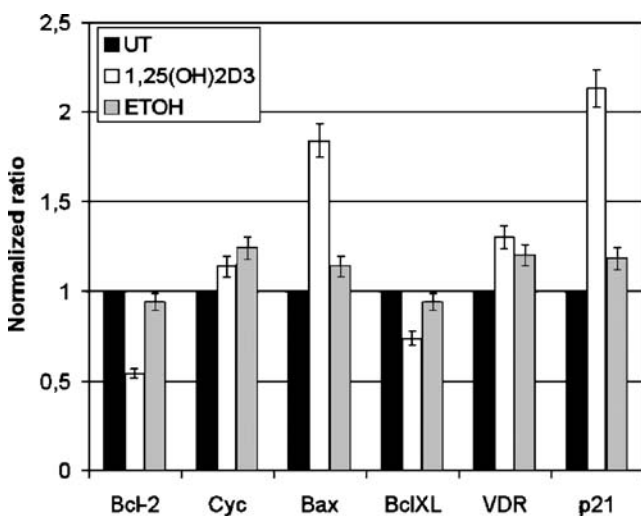


**Fig. 3** Flow cytometric analysis of cell apoptosis. **a** K562 control cells without 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment. **b** K562 cells treated with 50 nM 1,25-(OH)<sub>2</sub>D<sub>3</sub>. **c** Ethanol control cells without 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment. (*X* axis relative content of cell DNA; *Y* axis cell number)

preparations were independently subjected to mRNA expression. Figure 4 represents the real-time PCR assay relative quantification suits in the untreated control group, K562 cell treated with 50 nM 1,25-(OH)<sub>2</sub>D<sub>3</sub>, and the ethanol control group. At 50 nM concentrations of 1,25-(OH)<sub>2</sub>D<sub>3</sub>, BCL2 expression showed a 0.54-fold and BCL-XL expression showed a 0.74-fold decrease when compared with the untreated controls. The expression pattern of CYC and VDR gene expression seemed similar in all three conditions. A 1.84-fold increase for BAX mRNA expression and 2.13-fold increase for p21 mRNA expression were observed at 50 nM concentrations of 1,25-(OH)<sub>2</sub>D<sub>3</sub> at 72 h.

## Discussion

Resistance to apoptosis is a documented feature of BCR-ABL positive cells in chronic myelogenous leukemia. The



**Fig. 4** mRNA levels after 50 nM 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment in K562 cells. Gene expression is first normalized to the reference gene HPRT, followed by normalization of the sample ratio to the calibrator ratio (UT untreated cells and ETOH ethanol control)

K562 erythroleukemia cell line harbors the t(9;22)(q34;q11) translocation. Numerous studies have established that 1,25-(OH)<sub>2</sub>D<sub>3</sub> modulates cell-cycle progression, differentiation, invasion, and apoptosis of many cell types in vitro [29]. In K562 cells that are partially responsive to 1,25-(OH)<sub>2</sub>D<sub>3</sub>, the pathways of 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced apoptotic signaling are not well documented.

In a morphological study, it was observed that K562 cells were differentiated toward monocyte after the treatment of 1,25-(OH)<sub>2</sub>D<sub>3</sub>. This was further evidenced by the increased expression of CD11b epitope, an adhesion marker on monocyte surface, detected by antibody and flow cytometry [30]. Figure 1 shows that untreated K562 cells have large round nuclei, a small rim of blue-gray cytoplasm, and a high nuclear-to-cytoplasmic ratio. Occasional nucleoli are evident, and the nuclear chromatin has a somewhat granular open pattern. Exposure to 1,25-(OH)<sub>2</sub>D<sub>3</sub>, for 72 h produced slightly smaller cells with a decreased nuclear-to-cytoplasmic ratio. The cytoplasm was more abundant than that of the untreated cells, and the nuclei had a more coarse condensed appearance with folding and indentations of their membranes, reminiscent of mature cells of the myeloid monocytic lineage.

Treatment of most cell types with 1,25-(OH)<sub>2</sub>D<sub>3</sub> or analogs has been found to cause an arrest of cell cycle progression in G1 phase resulting in a decreased number of cells in the S phase complemented by an accumulation of cells in the G0–G1 phases [14, 31]. The number of cells in the G2–M compartment is relatively unaffected by treatment with vitamin D. However, in some cell types such as in the human leukemia cell line HL-60, block of cells in the G2 phase has been observed in response to treatment with vitamin D [32, 33]. The result shown in Fig. 2 indicates that the effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 72 h treatment of K562 cells induced apoptosis and arrest of cell cycle progression in G0–G1 phases, resulting in a decreased number of cells in S phase when compared with untreated and ethanol controls.

Apoptosis is accompanied by a loss of membrane phospholipid integrity, resulting in the externalization of PS to the cell surface. The fluorochrome-conjugated protein Annexin V binds to PS on the cell surface, which can be detected by flow cytometry, rendering this assay a sensitive method to analyze apoptosis. Flow cytometry data for annexin V/PI staining confirms to apoptosis.

Recent *in vitro* studies have indicated that in breast cancer cells, vitamin D-induced apoptosis is not dependent on the activation of any known caspase from the cellular proteolytic machinery [26, 34, 35]. In addition, induction of apoptosis by vitamin D in these cells appeared to be independent of the p53 tumor suppressor status, as both cells containing wild-type p53 and cells carrying a mutated form of p53 have been found to undergo apoptosis in response to treatment with vitamin D [34, 35]. In this study, we investigated the roles of apoptosis-related genes in the erythroleukemia cell line K562, which is p53-null. We were interested in this cell line because K562 cells have very low levels of endogenous BCL2 and abundant expression of endogenous BAX protein, despite the cells' noted resistance to many chemotherapeutic agents and radiation. Although the lack of p53 and presence of BCR-ABL may contribute to the highly resistant nature of K562 cells, it is also possible that Bax and Bcl-2 play nontraditional roles in apoptosis regulation in these cells.

The p53 protein is well known as the master regulator of the p21 gene. Recent studies show p21 to characterize the critical p53 and VDR binding sites. The cyclin-dependent kinase inhibitors p21 play an important role in progression through the cell cycle by inhibiting cyclin-dependent kinase activity and causing G0–G1 arrest. We show here that p21 is up-regulated in K562 cell line by 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment.

In myeloma cells, breast, colon, and prostate cancer cell lines, a down-regulation of the two anti-apoptotic members of this family, BCL2 and BCL-XL, and an up-regulation of the pro-apoptotic BAX and BAK proteins have been demonstrated after treatment with 1,25-(OH)<sub>2</sub>D<sub>3</sub> [25, 36]. Especially, down-regulation of BCL2 in conjunction with translocation of BAX to the mitochondria has recently been shown to be of particular importance for the induction of vitamin D-mediated apoptosis in the MCF-7 cells [35]. Moreover, overexpression of BCL2 has been shown to block 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced apoptosis in both MCF-7 breast cancer cells and in human LNCaP prostate cancer cells, further supporting the key role of the BCL2 family of proteins in 1,25-(OH)<sub>2</sub>D<sub>3</sub>-mediated apoptosis [26, 37]. This latter effect may be due to BCL2 preventing the efflux of CYC from the mitochondria, which is assumed to be a necessary step in the initiation of the apoptotic process [38]. CYC release with a concomitant decrease in mitochondrial membrane potential has recently been shown to take place in response to 1,25-(OH)<sub>2</sub>D<sub>3</sub>-mediated apoptosis [36], but

the exact link to the BCL2 family of proteins is still poorly characterized.

In conclusion, our data show the modulatory effects of 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment in apoptosis-related genes in K562 cells. While 72 h 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment does not influence the expression of proapoptotic CYC and VDR gene expressions, it influences the expression of pro-apoptotic BAX, anti-apoptotic BCL2, and BCL-XL gene expressions. It is important to note that these observations are valid for the K562 cell line that harbors the BCR-ABL chimeric gene. The application of these observations to other cell lines or cell populations requires further studies.

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