Arsenic trioxide-induced cell apoptosis and cell cycle arrest are potentiated by 1,25-dihydroxyvitamin D3 in human leukemia K562 cells

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Introduction

The vitamin D receptor (VDR), as a member of the nuclear receptor superfamily, is essential for initiating the intranuclear signaling pathways that are activated by the active metabolite of vitamin D. 1,25-dihydroxyvitamin [1,25(OH)2D3] is the biologically active form of vitamin D3, which directly or indirectly controls hundreds of genes in a cell- and tissue-specific manner (1,2). It is well known that arsenic trioxide (As2O3) has been successfully used to treat acute promyelocytic leukemia (APL) in traditional medicine (10). Recently, it has been approved by the US Food and Drug Administration for the treatment of relapsed/refractory APL (11). Studies have revealed that As2O3 exerts proapoptotic effects, not only in APL cells, but also in other hematopoietic malignancies and solid tumors (12,13). The underlying mechanisms of the antitumor activity of As2O3 have been associated with the induction of tumor apoptosis and inhibition of cell proliferation by promoting the production of reactive oxygen species (14). However, whether 1,25(OH)2D3 and As2O3 exert synergistic effects on the proliferation and differentiation of leukemia cells remains unknown.

Abstract. The interaction between 1,25-dihydroxyvitamin [1,25(OH)2D3] and vitamin D receptor (VDR) plays a critical role in regulating cell proliferation and programmed cell death. The present study aimed to investigate the effects of 1,25(OH)2D3 in combination with arsenic trioxide (As2O3) on the proliferation and cell cycle of a K562 leukemia cell line. K562 cells were treated with 100 nM 1,25(OH)2D3, 2.5 µM As2O3, and 100 nM 1,25(OH)2D3 combined with 2.5 µM As2O3. Cell proliferation was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt/phenazine ethosulfate method. Cell cycle progression and apoptosis were detected by flow cytometry. The expression levels of genes associated with the cell cycle and apoptosis were analyzed by reverse transcription-quantitative PCR and western blotting analyses. The present findings indicated that combined treatment of 1,25(OH)2D3 and As2O3 led to a significant increase in cytotoxicity, apoptotic cell death and G0 cell cycle arrest when compared to those treated with 1,25(OH)2D3 or As2O3 alone. The downregulation of the Bax/Bcl-2 ratio and decreased survivin expression may be involved in combined treatment-mediated apoptosis. G0/G1 cell cycle arrest induced by combined treatment was associated with the activation of p21 and p27. In addition, the increased expression of VDR was found to participate in the anticancer effect of combination treatment. The data suggested that the combination of 1,25-(OH)2D3 and As2O3 had clear synergistic effects on the inhibition of K562 cell proliferation, which could provide a novel therapeutic approach for the treatment of acute myeloid leukemia.

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In the present study, the antitumor effect of 1,25(OH)\(_2\)D3 combined with As\(_2\)O\(_3\) was investigated, and the underlying molecular mechanisms were determined using a K562 cell line established from human chronic myelogenous leukemia cells in blast crisis.

Materials and methods

Cell line, cell culture and reagents. The K562 cell line was purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Cytiva), 100 µg/ml penicillin, 10 µg/ml streptomycin, and 2 mmol/l L-glutamine. Cells were maintained in log phase growth at 37°C in a humidified atmosphere containing 5% CO\(_2\).

1,25(OH)\(_2\)D\(_3\) (Merck KGaA), also known as calcitriol, was dissolved in 100% ethanol at a concentration of 5x10\(^{-4}\) mol/l as a stock solution and stored at 20°C for use in the following experiments. For all experiments, dilutions of the stock solution were made in RPMI-1640 medium without FBS. The maximum concentration of ethanol in the culture did not exceed 0.1%. As\(_2\)O\(_3\) was purchased from Heilongjiang Harbin Yida Pharmaceutical Co., Ltd.

Optimal drug concentration screening and evaluation. Inhibition rate of the cell proliferation in K562 cells was measured using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt/phenaenzine ethosulfate (MTS/PES) assay. The cytotoxic effects of 1,25(OH)\(_2\)D\(_3\) on K562 cells were examined by treating 4,000 cells/well with 0.1% ethanol and 0, 10, 50, 100 or 200 nM 1,25(OH)\(_2\)D3 for 72 h (15), and then analyzed using a MTS/PES assay with the absorbance measured at a wavelength of 490 nm. For arsenic trioxide, no concentration gradient experiment was performed; the optimal concentration of 2.5 µM was obtained from the literature (16).

Cell proliferation assay. Cell proliferation was determined in 96-well plates using an MTS/PES assay, according to the manufacturer's instructions. Briefly, following treatment with 100 nM 1,25(OH)\(_2\)D\(_3\) and 2.5 µM As\(_2\)O\(_3\), alone or combined, K562 cells were seeded in 96-well plates at a density of 4,000 cells/well. The same number of untreated cells were seeded as the control. Cultures were set up in triplicate and maintained at 37°C in a humidified atmosphere with 5% CO\(_2\). Following 24, 48, 72, 96 or 120 h of treatment, 10 µl MTS/PES (10 mg/ml; Promega Corporation) was added to each well for an additional 6 h incubation. A microplate reader was used to measure the absorbance value at 490 nm for each well, which represented K562 cell proliferation.

Assessment of apoptosis and cell cycle analysis. The apoptosis assay of K562 cells was performed using an Annexin V/propidium iodide (PI) apoptosis assay kit (BioLegend, Inc.), according to the manufacturer's instructions. Briefly, the cells were harvested and washed twice with cold phosphate-buffered saline (PBS). Cells were then resuspended in 1X binding buffer at a concentration of 1x10\(^4\) cells/ml. A total of 100 µl of the solution was transferred to a 5-ml culture tube with 5 µl Annexin V-FITC and 10 µl PI (50 mg/ml). Cells were gently vortexed and incubated for 15 min at room temperature in the dark. Following the addition of 400 µl of binding buffer, these cells were analyzed by flow cytometry (FCM). Annexin V and PI cells were considered viable cells, Annexin V+ and PI cells were considered early apoptotic cells and Annexin V+ and PI+ cells were considered late apoptotic cells.

Cell cycle distribution was determined by staining DNA with PI, as previously described (17). Briefly, ~5x10\(^5\) K562 cells were collected from cultures, washed twice with PBS and fixed in 70% pre-cold ethanol at 4°C overnight. The fixed cells were then collected and resuspended in PBS, containing 50 mg/ml PI and 100 µg/ml DNase-free RNase A. Cells were incubated for 1 h at room temperature and then analyzed by FCM.

Reverse transcription-quantitative PCR (RT-qPCR). K562 cells from each experimental group were collected and washed three times with cold PBS, and then total cell mRNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. cDNA was synthesized from 2 µg total RNA using a first-strand cDNA synthesis kit (Thermo Fisher Scientific, Inc.). The PCR amplification protocol was as follows: Denaturation at 94°C for 10 min, followed by 40 cycles of 94°C for 15 sec, 58°C for 30 sec and 72°C for 40 sec. The mRNA expression levels of VDR, Bcl-2, survivin, Bax, p21, p27 and β-actin were detected using ABI 7000 (Thermo Fisher Scientific, Inc.) and Talent qPCR PreMix (SYBR-Green) (Tiangen Biotech Co., Ltd.). The relative quantification based on the relative expression of target genes was calculated using the 2-ΔΔCT method (18). The PCR primers were designed based on the corresponding gene structure, and the sequences are listed in Table I. RT-qPCR was performed in triplicate.

Western blot analysis. Following various treatments with 100 nM 1,25(OH)\(_2\)D3 and 2.5 µM As2O3, alone or combined, K562 cells were collected and washed three times with pre-cooled PBS. The proteins were extracted using RIPA buffer (Thermo Fisher Scientific, Inc.) and quantified using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Proteins were separated via SDS-PAGE (15% gel) and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at 37°C for 2 h, and subsequently incubated with the primary antibodies mouse anti-VDR (1:1,000; cat. no. sc13133), mouse anti-Bcl-2 (1:1,000; cat. no. sc7382), mouse anti-survivin (1:1,000; cat. no. sc17779), mouse anti-Bax (1:1,000; cat. no. sc7480), mouse anti-Bcl-2 (1:1,000; cat. no. sc6246), mouse anti-p21 (1:1,000; cat. no. sc1641) and mouse anti-β-actin (1:1,000; cat. no. sc69879) (all from Santa Cruz Biotechnology, Inc.) at 4°C overnight. After five more washes with TBST, the blots were incubated with the horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:5,000; cat. no. ab6728; Abcam) for 1 h at room temperature. The immunoreactive bands were visualized by enhanced chemiluminescence (ECL) on a Kodak Image Station 4000 mm Pro system (Kodak Corporation). The quantitative data from bands were analyzed using the Image Tool system (version 3.0; www.bio-soft.net).

Statistical analysis. All statistical data are presented as the mean ± standard deviation. The treatment effects among different...
3 groups were compared using one-way analysis of variance and Bonferroni’s post hoc test. All analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

1,25(OH)2D3 exerts synergistic effects with As2O3 on the proliferation of K562 cells. The results of the MTS/PES assay revealed that 1,25(OH)2D3 suppressed the proliferation of K562 cells in a dose-dependent manner (Fig. 1). Since 100 nM 1,25(OH)2D3 displayed the most significant anticancer effect on K562 cells, that concentration was selected for subsequent experiments. The results showed that 100 nM 1,25(OH)2D3 or 2.5 µM As2O3 alone exerted inhibitory effects on K562 cells. Of note, the inhibition of cell proliferation was more effective with combined treatment than with treatment with As2O3 or 1,25(OH)2D3 alone (Fig. 2).

1,25(OH)2D3 enhances the As2O3-induced apoptosis of K562 cells. K562 cells were treated with 100 nM 1,25(OH)2D3 and/or 2.5 µM As2O3 for 72 h. Fig. 3A shows a representative example of apoptotic cells in untreated K562 cells and cells treated with 1,25(OH)2D3, As2O3 or a combination of both. The results showed that the percentage ratio of early and late apoptotic cells in the combination group was significantly higher than that in the blank control and 1,25(OH)2D3 or As2O3 treatment groups (P<0.01; Fig. 3B).

Table I. Reverse transcription-quantitative PCR primers.

| Gene   | Direction | Sequence                        |
|--------|-----------|---------------------------------|
| VDR    | Sense     | 5'-AGCTGGCCCTGGCAGCTCTGCTCTC-3' |
|        | Antisense | 5'-ATGGAAACACCTTGTCTTCTCCCT-3' |
| Bcl-2  | Sense     | 5'-ATCGGCGTGGAGCTGACGTCG-3'     |
|        | Antisense | 5'-CACGCCAGGAAATCAACAGAG-3'     |
| Survivin| Sense     | 5'-CCCTTGCCTGAGCCCTTTTC-3'      |
|        | Antisense | 5'-CTGGCTCCAGCCTTCCA-3'         |
| Bax    | Sense     | 5'-GGACGAACGTGACATGAAATGG-3'    |
|        | Antisense | 5'-GCAAAGTAGAAGGAGGCAAC-3'      |
| p21    | Sense     | 5'-GCAAGACCAGCATGACAGTT-3'      |
|        | Antisense | 5'-GGATTAGGGCTCTCCTTGG-3'       |
| p27    | Sense     | 5'-CCCTTGGGCGGGGTGGAC-3'        |
|        | Antisense | 5'-TTTGGAGACCCTTGGAAAC-3'       |
| β-actin| Sense     | 5'-TCTGGGACCACACCCTTCTACAATG-3'|
|        | Antisense | 5'-AGCAGGCCTGGATAGCAACG-3'      |

Figure 1. Proliferation of K562 cells is suppressed by 1,25(OH)2D3 in a dose-dependent manner. K562 cells at an initial density of 4,000 cells/well were treated with different concentrations of 1,25(OH)2D3 (10, 50, 100 or 200 nM) for 72 h, and then analyzed by MTS/PES assay absorbance measured at a wavelength of 490 nm. Untreated, without 1,25(OH)2D3 treatment; ethanol, with 0.1% ethanol treatment. Data are presented as the mean ± SD of three independent experiments. **P<0.01 and ***P<0.001 vs. untreated group.

1,25(OH)2D3, 1,25-dihydroxyvitamin; ns, not significant.

Figure 2. Synergistic effect of As2O3 and 1,25(OH)2D3 in the treatment of K562 cells. MTS/PES assay results showed that 2.5 µM As2O3 and 100 nM 1,25(OH)2D3 exerted a synergistic inhibitory effect on K562 cells. Data are presented as the mean ± SD of three independent experiments. *P<0.05 and **P<0.01 vs. K562 cells treated with a combination of As2O3 and 1,25(OH)2D3. As2O3, arsenic trioxide; 1,25(OH)2D3, 1,25-dihydroxyvitamin.
1,25(OH)2D3 and As2O3 induce G0/G1 cell cycle arrest. Cell cycle distribution was analyzed via FCM. Fig. 4A shows a representative example of cell cycle distribution in untreated K562 cells and cells treated with 1,25(OH)2D3, As2O3, or a combination of both. The results revealed that the combined treatment markedly increased the population of cells in the G0/G1 phase and significantly decreased the population of cells in the S-phase compared with cells treated with 1,25(OH)2D3 or As2O3 alone. By contrast, the percentage of cells in the G2/M phase was relatively unaffected (Fig. 4B). These data demonstrated that the combined treatment induced significant arrest of cell cycle progression in the G0/G1 phase.

Effects of 1,25(OH)2D3 and As2O3 on the expression of apoptosis-related and cycle-regulated genes and proteins. To further investigate the underlying molecular mechanisms of cell proliferation suppression, apoptosis induction and G0/G1 cell cycle arrest, the mRNA and protein expression levels of VDR, Bcl-2, Bax, survivin, p21 and p27 were analyzed in K562 cells using RT-qPCR and western blot analysis. Fig. 5 presents the relative mRNA quantification of these genes in untreated K562 cells and cells treated with 1,25(OH)2D3, As2O3, or a combination of both. The results revealed that the combined treatment with 1,25(OH)2D3 and As2O3 resulted in a marked reduction in the levels of Bcl-2 and survivin when compared with untreated cells and 1,25(OH)2D3 or As2O3 alone, while the levels of the VDR, Bax, p21 and p27 were significantly increased following combined treatment. Representative immunoblots are presented in Fig. 6A. Similar to the observed mRNA expression levels, the protein expression levels of VDR, Bax, p21 and p27 were found to be increased, while the expression
levels of Bcl-2 and survivin were found to be significantly decreased (Fig. 6B).

**Discussion**

The ability of 1,25(OH)2D3 to induce apoptosis has been demonstrated in various tumor cells. Although, to the best of our knowledge, the mechanisms underlying the apoptotic effects have not yet been fully elucidated, 1,25(OH)2D3 may induce cell death by triggering the intrinsic, mitochondria-dependent pathway (6). According to cell type, 1,25(OH)2D3 can increase the expression levels of proapoptotic factors Bax and Bcl-2 homologous antagonist killer (Bak) and/or decrease their anti-apoptotic equivalents Bcl-2 and B-cell lymphoma-extra large, thus directing the cells towards apoptosis rather than towards survival (19,20). Elucidating the precise molecular mechanism underlying the antiproliferative action of 1,25(OH)2D3 could help identify new biomarkers for targeted treatment with novel vitamin D analogs.

The results of the present study showed that both 1,25(OH)2D3 and As2O3 are reagents that can effectively inhibit the proliferation of K562 leukemia cells. FCM showed that As2O3 significantly increased the rate of late apoptotic cells, but had no significant effect on the rate of early apoptotic cells. By contrast,
1,25(OH)2D3 only increased the rate of early apoptotic cells, but did not affect the rate of late apoptotic cells. When As2O3 and 1,25(OH)2D3 were combined for the treatment of K562 cells, a synergistic effect was observed. It is well known that apoptosis is regulated by a series of genes. Survivin is a member of the inhibitor of apoptosis family of proteins, which are involved in the inhibition of apoptosis and regulation of the cell cycle (21). Upregulation of survivin promotes tumor progression by inhibiting both the intrinsic and extrinsic pathways of apoptosis, altering sensitivity to antitumor drugs or prolonging the survival of cancer stem cells (22). It has been hypothesized that survivin can also serve as a universal tumor antigen, since it is expressed in the majority of human hematological malignancies and has the potential to trigger immune effector responses (23). The results of the present study indicated that the combined treatment of 1,25(OH)2D3 and As2O3 may significantly decrease the expression of survivin, while 1,25(OH)2D3 and As2O3 alone cannot affect its expression. Therefore, blocking the function of survivin through the use of various drugs or molecular approaches is a promising therapeutic strategy for leukemia.

Previous studies have demonstrated that both 1,25(OH)2D3 and As2O3 can arrest the cell cycle at the G1 phase, probably through the upregulation of one or both of the cyclin-dependent kinase inhibitors (p21 and p27), so as to inhibit the malignant proliferation of K562 cells (15,16,24). In accordance with these results, the data generated in the present study indicated that treatment with As2O3 and 1,25(OH)2D3 alone caused clear G1/S arrest, while the number of cells in the G2/M phase was relatively unaffected. Further research showed that the G1/S blockade induced by combined treatment with 1,25(OH)2D3 and As2O3 could upregulate the protein expression of p21 and p27, while treatment with 1,25(OH)2D3 only upregulated the expression of p21. In addition, 1,25(OH)2D3 could increase the expression of VDR, while As2O3 did not affect the expression.

Due to the small amount of experimental conditions in the present study, there are still some limitations to the present study and some aspects that were not investigated. The present study focused on the changes observed from traditional apoptosis and cell cycle-related genes in regard to the underlying anticancer molecular mechanisms. However, the effects of combined treatment on mitochondrial dysfunctions, oxidative stress and regulation of calcium influx are not involved. Future studies could use a reactive oxygen species assay kit to quantify the levels of intracellular reactive oxygen species and apply fluorescent rhodamine derivatives.

Overall, the results of the present study provided evidence that the addition of 1,25(OH)2D3 may increase the therapeutic efficacy of As2O3, which may in turn decrease adverse effects and initiate a more comprehensive antitumor pathway. This potential method urgently requires further investigation to elucidate the potential therapeutic benefits of a variety of non-APL hematological malignancies.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions
YLZ, JHR, XNG and JNZ performed the research, analysis and interpretation of the data. YLZ and XNG drafted the manuscript and gave final approval of the version to be published. SKQ designed the study and supervised preparation of the manuscript, and provided general support. YLZ and SKQ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.
Competing interests

The authors declare that they have no competing interests.

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