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# Reversal effect of vitamin D on different multidrug-resistant cells

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**ABSTRACT.** We investigated the reversal effect of vitamin D on the multidrug-resistant leukemic Jurkat/ADR and K562/ADR cell lines and conducted a preliminary investigation of its reversal mechanism. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method was used to detect the reversal effect of vitamin D on multidrug-resistant cells. Real-time polymerase chain reaction was used to determine the effect of vitamin D on intracellular expression of mRNA of the multidrug-resistant gene (*MDRI*) and the multidrug-resistance-related gene (*MRP1*). A protein quantitative analysis method was used to determine the effect of vitamin D on intracellular glutathione content. After treatment of Jurkat/ADR and K562/ADR cells with vitamin D, multidrug resistance was reversed in a dose-dependent manner, which may have reduced mRNA expression of the *MDR1* and *MRP1* genes, the P-glycoprotein content on the cell surface, and the intracellular glutathione level. Different concentrations of vitamin D showed varying reversal effects on different multidrug-resistant cells. The resistance mechanism may be related to the inhibition of the expression of *MDR1* and *MRP1* genes.

**Key words:** Vitamin D; Jurkat/ADR; K562/ADR; Multidrug resistance; Reversal effect

 

## INTRODUCTION

Resistance of malignant tumors to chemotherapy is an important contributor to tumor chemotherapy failure (Xia and Smith, 2012). The ineffectiveness of traditional chemotherapy drugs leads to millions of deaths worldwide each year (Baguley, 2010). Over the past 20 years, scientists have extensively examined reversal drugs, of which 30 types of P-glycoprotein inhibitors have been investigated in hundreds of experiments. Because of their side effects, poor solubility, and ability to alter the pharmacokinetics of chemotherapy drugs, no P-glycoprotein inhibitor has yet been approved by the United States Food and Drug Administration (FDA) (van der Pol et al., 2003; Peer and Margalit, 2006). Therefore, it is very important to develop highly efficient multidrug-resistance (MDR) reversal agents with low toxicity. In addition to its effects on calcium and phosphorus metabolism, vitamin D also can inhibit cell proliferation and induce cell differentiation, it is a kind of effective and another promising tumor cells induced differentiation agent except the retinoic acid (Kennedy et al., 2004). The effects of vitamin D and its receptor in tumor therapy have received attention (Guy et al., 2004). A study conducted in China suggested that a combination of vitamin D and celecoxib effectively improved the effects of chemotherapy toward the breast cancer cell line MCF/ADR (Adriamycin), suggesting that vitamin D may reverse drug resistance. In this study, we examined the mechanism of this resistance reversal. By examining the effect of vitamin D on the acute lymphoblastic leukemia multidrug-resistant cell line Jurkat/ADR and the chronic myelogenous leukemia multidrug-resistant cell line K562/ADR, we determined that vitamin D could indeed reverse drug resistance in these cells. We provide a theoretical basis for the mechanism of the reversal of drug resistance in tumor cells and its clinical application.

## MATERIAL AND METHODS

### Cell culture

The human acute T lymphocyte leukemia multidrug-resistant cell line Jurkat/ADR, which was established in our laboratory, and the human chronic myeloid leukemia multidrug-resistant cell line K562/ADR, which was purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China), were cultured in RPMI-1640 medium (GIBCO Co., Ltd.; Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; GIBCO) at 37°C in a 5% $CO_2$ incubator (Heraeus Co., Ltd., Hanau, Germany), with the medium changed once every 2-3 days. According to the biological cultivation indicator, ADR was added to a final concentration of 0.5 mg/L to maintain cell resistance. Cells were cultured for 2 weeks before the experiment.

### Primers

The primer sequences used for amplification were as follows: the multidrug-resistance gene (*MDR1*) upstream primer: 5'-CCCATCATTGCAATAGCAGG-3' and the downstream primer 5'-GTTCAAACTTCTGCTCCT-CA-3', with a fragment length of 157 bp; the multidrug-resistance-related gene (*MRP1*) upstream primer: 5'-GGACCTGGACTTCGTTCTCA-

3', and downstream primer: 5'-CGTCCAGACTTCTTCATCCG-3', with a fragment length of 292 bp; and the *β-actin* upstream primer: 5'-TGCACAACGGCTCCGGCA-3' and downstream primer: 5'-AAGGTGTGGTGCCAGATTTTC-3', with a fragment length of 390 bp. Primers were designed by Sangon Biotech Co., Ltd. (Shanghai, China).

## Vitamin D nontoxic dose screening by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The number of living cells was detected each day in order to determine vitamin D nontoxic doses using the MTT method. Because vitamin D is cytotoxic to cells, when combined with other chemotherapy drugs, a vitamin D dosage <10% of the inhibition rate should be used. Logarithmic growth phase cells were collected and inoculated on 96-well plates, with each well containing 90 μL 1 x $10^4$ K562/ADR, Jurkat/ADR cells. After 48 h, 10 μL 5 g/L MTT was added to each well. Cells were incubated at 37°C for 4 h, and the reaction was terminated by adding 100 μL 10% sodium dodecyl sulfate to each well. Plates were shaken in a tabletop centrifuge for 10 min to fully dissolve the crystals. Absorbance of each well was measured using an Microplate Reader at a wavelength of 490 nm.

## Reversal of resistance and drug concentration-dependence experiments

A single cell suspension of 1 x $10^4$ cells/mL was prepared in RPMI-1640 medium containing 10% FBS, which was inoculated on 96-well plates. Each well contained a 200 μL volume. Following cell adhesion to the plate, chemotherapeutic agents were added to each well. Based on the above-described experimental results and vitamin D (BD Biosciences, Franklin Lakes, NJ, USA) nontoxic doses, the cells were divided into 2 groups. Group 1 was the vitamin D + ADR, cytarabine, cyclophosphamide, daunorubicin, or vincristine group; doxorubicin hydrochloride for injection, daunorubicin for injection, vincristine sulfate for injection, and cytarabine and cyclophosphamide were obtained from Zhejiang Haizheng Co., Ltd. (Zhejiang, China). Ten microliters vitamin D was added to reach concentrations of $10^{-6}$, $10^{-7}$, $10^{-8}$ and $10^{-9}$ mg/mL, for a total of 4 groups. Next, 5 μL ADR, cytarabine, cyclophosphamide, daunorubicin, or vincristine was added to a final concentration of 0.05 μg/mL in each group. Group 2 was the chemotherapy group (ADR or Ara-C, cyclophosphamide, daunorubicin, or vincristine); each well contained 75 μL chemotherapy medicine at a final concentration of 0.05 μg/mL.

The drug concentration-dependence experiments were carried out using the MTT method as described above.

## Real-time polymerase chain reaction (PCR)

Total RNA in cells was extracted using one-step TRIzol and a total RNA extraction kit (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse-transcribed into cDNA at 37°C for 60 min. The dye method (SYBR Green RealMasterMix; 5 PRIME, Hamburg, Deutschland) for real-time PCR analysis was carried out to examine the mRNA expression of the *MDR1* and *MRP1* genes in different samples, with *β-actin* used as an internal standard. For each reaction, 10 μL PCR amplification system (SYBR Green RealMasterMix), 7 μL $ddH_2O$, 1 μL

forward and reverse primers, and 1 μL cDNA were added, for a total reaction volume of 20 μL. The *MDR1*, *MRP1*, and *β-actin* amplification reaction conditions were 95°C for 2 min, 94°C denaturing for 15 s, 56°C annealing for 15 s, and 72°C extension for 30 s for 30 cycles. A solubility curve was generated. After several preliminary experiments, optimum reaction conditions were determined based on the fluorescence cycle threshold value for each sample. Each experiment was repeated 3 times and average values were calculated to determine variations in the mRNA expression of each gene.

### Effects of vitamin D on glutathione (GSH) content in K562/ADR and HL60/ADR cells

Logarithmic growth phase cells at a 1 x $10^9$ cells/L concentration were divided into no drug and drug treatment groups. Cells were collected after culturing for 4 h and suspended in 1 mL PBS after washing. This sample was frozen in liquid nitrogen 3 times followed by additional lysis by sonication. The cell supernatant was obtained by centrifuging the sample at 1500 rpm for 10 min. Absorbance was measured after the chromogenic reaction, and then the optical density (OD) in each well was measured at a 490-nm wavelength. A standard curve was generated according to the sample concentration and its corresponding OD, and then the corresponding sample concentration was calculated based on the sample OD value. This enabled quantification of the GSH content in experimental cells. The experiment was repeated 3 times.

### Statistical analysis

Data are reported as means ± SD, and differences among groups were compared using the Student *t*-test and analysis of variance with the SPSS 17 analysis software (SPSS, Inc., Chicago, IL, USA).

## RESULTS

### Screening the nontoxic doses of vitamin D

For screening using the MTT assay, the effective nontoxic vitamin D doses were $10^{-6}$ mg/mL for K562/ADR cells and $10^{-5}$ mg/mL for Jurkat/ADR cells. For subsequent concentration-dependent experiments and statistical analyses, the vitamin D group was divided into the following 4 concentration groups: $10^{-6}$, $10^{-7}$, $10^{-8}$, and $10^{-9}$ mg/mL groups.

### Vitamin D3-induced reversal of drug resistance in K562, K562/ADR, Jurkat, and Jurkat/ADR cells, and reversal concentration dependence

Analysis of the reversal effects after combined application of vitamin D and other drugs on K562/ADR and Jurkat/ADR cells showed that vitamin D reversed the drug resistance of the K562/ADR of ADR, DOX, VCR, and Arc-a ($P < 0.05$) (Tables 1 and 2). In addition, there were reversal effects for all drugs; vitamin D had the strongest reversal effect on Arc-a resistance, and the lowest effect on ADR resistance.

**Table 1.** Drug resistance reversal effect of vitamin D-combined application of ADR, DOX, Ara-C, VCR on K562/ADR cells after 48 h.

| Concentration | K562/ADR cell line $IC_{50}$ | Reversal fold |
|---|---|---|
| ADR control group | 23.1 | - |
| ADR+Vit D ($10^{-6}$ mg/mL) | 1.12 ± 0.74 | 22.502 |
| ADR+Vit D ($10^{-7}$ mg/mL) | 2.23 ± 3.1 | 11.106 |
| ADR+Vit D ($10^{-8}$ mg/mL) | 7.8 ± 0.07 | 3.01 |
| ADR+Vit D ($10^{-9}$ mg/mL) | 11.2 ± 0.45 | 1.915 |
| *t* value | 3.16 | |
| P value | 0.042 | |
| DOX control group | 37.7 | - |
| DOX+Vit D ($10^{-6}$ mg/mL) | 0.12 ± 0.74 | 314.87 |
| DOX+Vit D ($10^{-7}$ mg/mL) | 1.03 ± 3.1 | 36.708 |
| DOX+Vit D ($10^{-8}$ mg/mL) | 10.8 ± 0.07 | 3.18 |
| DOX+Vit D ($10^{-9}$ mg/mL) | 26.2 ± 0.45 | 1.145 |
| *t* value | 4.13 | |
| P value | 0.036 | |
| Ara-C control group | 43.4 | - |
| Ara-C+Vit D ($10^{-6}$ mg/mL) | 0.12 ± 0.64 | 361.4 |
| Ara-C+Vit D ($10^{-7}$ mg/mL) | 12.03 ± 0.23 | 3.658 |
| Ara-C+Vit D ($10^{-8}$ mg/mL) | 20.8 ± 0.32 | 2.56 |
| Ara-C+Vit D ($10^{-9}$ mg/mL) | 40.2 ± 0.54 | 1.0255 |
| *t* value | 3.15 | |
| P value | 0.047 | |
| VCR control group | 46.8 | - |
| VCR+Vit D ($10^{-6}$ mg/mL) | 2.12 ± 2.54 | 23.231 |
| VCR+Vit D ($10^{-7}$ mg/mL) | 21.2 ± 0.1 | 2.956 |
| VCR+Vit D ($10^{-8}$ mg/mL) | 47.8 ± 0.03 | 0.96 |
| VCR+Vit D ($10^{-9}$ mg/mL) | 43.2 ± 0.24 | 1.0625 |
| *t* value | 6.84 | |
| P value | 0.001 | |

Data are reported as means ± SD. ADR = Adriamycin; Vit D = vitamin D; DOX = doxorubicin; Ara-C = cytarabine; VCR = vincristine.

**Table 2.** Drug resistance reversal effect of vitamin D-combined application of ADR, DOX, Ara-C, VCR on Jurcut/ADR cells after 48 h.

| Concentration | Jurkat/ADR cell line $IC_{50}$ | Reversal fold |
|---|---|---|
| ADR control group | 38.1 | - |
| ADR+Vit D ($10^{-6}$ mg/mL) | 0.34 ± 0.74 | 112.02 |
| ADR+Vit D ($10^{-7}$ mg/mL) | 1.23 ± 3.1 | 37.26 |
| ADR+Vit D ($10^{-8}$ mg/mL) | 17.9 ± 0.07 | 2.41 |
| ADR+Vit D ($10^{-9}$ mg/mL) | 24.2 ± 0.45 | 1.969 |
| *t* value | 3.13 | |
| P value | 0.046 | |
| DOX control group | 32.5 | - |
| DOX+Vit D ($10^{-6}$ mg/mL) | 0.62 ± 0.71 | 212.37 |
| DOX+Vit D ($10^{-7}$ mg/mL) | 5.03 ± 3.1 | 6.21 |
| DOX+Vit D ($10^{-8}$ mg/mL) | 17.8 ± 0.03 | 1.98 |
| DOX+Vit D ($10^{-9}$ mg/mL) | 29.2 ± 0.15 | 1.015 |
| *t* value | 4.24 | |
| P value | 0.0189 | |
| Ara-C control group | 40.1 | - |
| Ara-C+Vit D ($10^{-6}$ mg/mL) | 0.22 ± 0.64 | 182.3 |
| Ara-C+Vit D ($10^{-7}$ mg/mL) | 16.03 ± 0.23 | 2.68 |
| Ara-C+Vit D ($10^{-8}$ mg/mL) | 29.8 ± 0.32 | 1.516 |
| Ara-C+Vit D ($10^{-9}$ mg/mL) | 36.2 ± 0.54 | 1.020 |
| *t* value | 5.56 | |
| P value | 0.005 | |
| VCR control group | 46.8 | - |
| VCR+Vit D ($10^{-6}$ mg/mL) | 1.12 ± 2.54 | 45.13 |
| VCR+Vit D ($10^{-7}$ mg/mL) | 15.2 ± 0.14 | 3.456 |
| VCR+Vit D ($10^{-8}$ mg/mL) | 27.3 ± 0.23 | 2.96 |
| VCR+Vit D ($10^{-9}$ mg/mL) | 43.2 ± 0.24 | 1.9625 |
| *t* value | 7.02 | |
| P value | 0.001 | |

Data are reported as means ± SD. For abbreviation, see legend to Table 1.

## Expression determination of *MDR1* and *MRP1* mRNA

Nontoxic doses of vitamin D were applied to the K562/ADR and Jurkat/ADR cells for 24 h. Amplification by real-time PCR showed that compared with the control group, mRNA expression of *MDR1* and *MRP1* decreased with increasing vitamin D concentrations in a dose-dependent manner (Tables 3 and 4).

**Table 3.** Effect of vitamin D (Vit D) on mRNA expression of K562/ADR (MDR1 and MRP1 genes).

| Group | MDR1 mRNA | MRP1 mRNA |
|---|---|---|
| Control group | 0.87 ± 0.04 | 0.30 ± 0.13 |
| Vit D ($10^{-6}$ mg/mL) | 0.19 ± 0.34 | 0.25 ± 0.3 |
| Vit D ($10^{-7}$ mg/mL) | 0.26 ± 0.25 | 0.22 ± 0.07 |
| Vit D ($10^{-8}$ mg/mL) | 0.41 ± 0.31 | 0.33 ± 0.21 |
| Vit D ($10^{-9}$ mg/mL) | 0.66 ± 0.32 | 0.29 ± 0.11 |
| *t* value | 13.21 | 26.3 |
| P value | <0.05 | <0.05 |

Data are reported as means ± SD.

**Table 4.** Effect of vitamin D (Vit D) on mRNA expression of Jurkat/ADR (MDR1 and MRP1 genes).

| Group | MDR1 mRNA | MRP1 mRNA |
|---|---|---|
| Control group | 0.22 ± 0.12 | 0.59 ± 0.03 |
| Vit D ($10^{-6}$ mg/mL) | 0.18 ± 0.03 | 0.21 ± 0.13 |
| Vit D ($10^{-7}$ mg/mL) | 0.21 ± 0.05 | 0.39 ± 0.08 |
| Vit D ($10^{-8}$ mg/mL) | 0.17 ± 0.01 | 0.42 ± 0.05 |
| Vit D ($10^{-9}$ mg/mL) | 0.20 ± 0.32 | 0.50 ± 0.11 |
| *t* value | 32.21 | 17.3 |
| P value | <0.05 | <0.05 |

Data are reported as means ± SD.

Figure 1 shows *MDR1* and *MRP1* gene expression after electrophoresis. With increasing vitamin D concentrations, *MDR1* and *MRP1* gene expression decreased relative to *β-actin* expression in a dose-dependent manner (Figure 1).

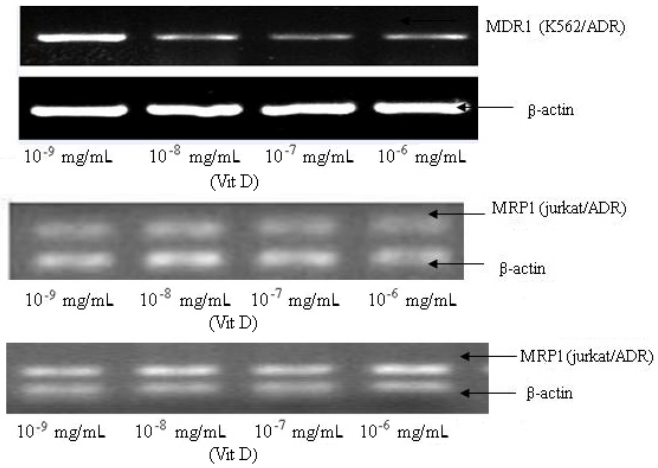


**Figure 1.** Expression situation of MDR1 and MRP1 genes after electrophoresis.

## Effect of vitamin D on GSH content in K562/ADR and Jurkat/ADR cells

Increasing concentrations of vitamin D affected the content of GSH in Jurkat/ADR and K562/ADR cells, showing that the drug-resistance reversal mechanism of K562/ADR and Jurkat/ADR cells could involve the inhibition of MRP-mediated GSH drug efflux pump function (Table 5).

**Table 5.** Effect of vitamin D (Vit D) on GSH content in K562 /ADR and Jurkat/ADR cells detected by human glutathione kit.

| | K562/ADR (GSH concentration·$U^{-1}$·$L^{-1}$) | Jurkat/ADR (GSH concentration·$U^{-1}$·$L^{-1}$) |
|---|---|---|
| Control group | 189.4 ± 21.3 | 332.1 ± 34.1 |
| Vit D ($10^{-6}$ mg/mL) | 156.3 ± 17.3 | 184.9 ± 15.6 |
| Vit D ($10^{-7}$ mg/mL) | 163.6 ± 25.0 | 203.3 ± 12.8 |
| Vit D ($10^{-8}$ mg/mL) | 174.9 ± 30.2 | 267.2 ± 17.7 |
| Vit D ($10^{-9}$ mg/mL) | 177.5 ± 11.3 | 295.6 ± 32.1 |
| F value | 11.28 | 14.32 |
| P value | 0.0007 | 0.0018 |

Data are reported as means ± SD.

## DISCUSSION

More than 90% of child leukemia cases are acute leukemia and acute lymphoblastic leukemia (ALL) accounts for 70% of all leukemias in children (Moghrabi et al., 2007). Childhood ALL remission rates continue to improve with combined chemotherapy, but because of the multidrug resistance of leukemia cells, most traditional chemotherapy drugs can become ineffective over time, resulting in clinical recurrence in some patients following combined chemotherapy, even after allogeneic hematopoietic stem cell transplantation (Gratwohl et al., 2009; Dickinson et al., 2010). Therefore, screening for safe and effective resistance reversal agents is becoming increasingly important.

In addition to regulation of calcium and phosphorus metabolism, vitamin D functions to induce cell differentiation, inhibit cell proliferation, and regulate oncogene expression and immunity (Tse et al., 2010). Low water [$1,25(OH)_2D_3$] in serum can increase the risk and mortality rate of colon cancer and breast cancer patients (Chen et al., 2013). All-trans retinoic acid (ATRA) and vitamin D play very important roles in antitumor pathways (Tse et al., 2010; Welsh, 2012) by regulating the cell cycle of tumor cells and inducing cell apoptosis. *Ex vitro* and animal experiments have shown that vitamin D can induce mouse myeloid leukemia cell differentiation and prolong the survival time of leukemia mice. Additional studies (Seo et al., 2005; Long et al., 2009) have confirmed that vitamin D can promote the transition of tumor cells into mature cells. This indicates that at a dose that induces differentiation, vitamin D can induce tumor cells to differentiate into mature cells, thereby inhibiting tumor cell proliferation.

Recent studies have found that ATRA and vitamin D can significantly improve the sensitivity of multidrug resistance of tumor cells to chemotherapy drugs. Studies have also confirmed that ATRA and vitamin D, combined with chemotherapy, have a synergistic effect on the enhancement of lung cancer susceptibility. Following combination use of these drugs, low-concentration chemotherapy drugs are effective and have reduced side effects (Peer and Margalit, 2006; Chow et al., 2011). After chemotherapy for breast cancer was combined with vitamin D, the drug resistance of cells to doxorubicin was significantly reduced and cell apop-

tosis was promoted (Roberti et al., 2006; Taghizadeh et al., 2007; Sun et al., 2008). However, whether vitamin D has a reversal effect on drug resistance in acute, chronic leukemia and its mechanism remains unknown.

We found that vitamin D reversed the drug resistance to Adriamycin, cyclophosphamide, cytarabine, and daunorubicin in K562/ADR and Jurkat/ADR cells. In K562/ADR cells, this effect was strongest on cytarabine and weakest on Adriamycin. In the Jurkat/ADR cell line, the drug-resistance reversal effect of vitamin D on Adriamycin was strongest, and was weakest for cyclophosphamide, vincristine, and other chemotherapy drugs. The reversal effect of vitamin D may become stronger with increasing concentrations.

Examination of the relationship between mRNA expression and drug resistance showed that mRNA expression of the *MDRl* gene in the K562/ADR cell line decreased with increasing vitamin D concentration, but had no effect on Jurkat/ADR cells. Similarly, mRNA expression of the *MDRl* gene in Jurkat/ADR cells decreased with increasing vitamin D concentration, but showed no obvious expression change in the K562/ADR cell line.

Increasing concentrations of vitamin D affected the GSH content in Jurkat/ADR cells, which also occurred in a drug concentration-dependent manner. This indicates that the reversal of drug resistance in Jurkat/ADR cells occurs through the inhibition of GSH drug efflux pump function. The effect was also observed in K562/ADR cells.

In summary, nontoxic vitamin D doses and K562/ADR and Jurkat/ADR could reduce mRNA expression of the *MRD1* gene in cells and glutathione contents. These results indicate that vitamin D can reverse multidrug resistance. However, the particular cytokines, signaling pathways, up- and downstream gene regulation mechanisms, gene interactions, and whether this method could be used simultaneously with chemotherapy drugs remain unclear.

Vitamin D was shown to reverse chemotherapy drug resistance, making it a candidate drug for further analysis of late-stage resistance reversal drugs and chemotherapy synergists.

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