# 19-Nor-1,25(OH)$_2$D$_2$ (a Novel, Noncalcemic Vitamin D Analogue), Combined with Arsenic Trioxide, Has Potent Antitumor Activity against Myeloid Leukemia

Takashi Kumagai,[1,5] Lee-Yung Shih,[2,3] Susan V. Hughes,[4] Julian C. Desmond,[1] James O'Kelly,[1] Martin Hewison,[4] and H. Phillip Koeffler[1]

[1]Division of Hematology/Oncology, University of California at Los Angeles School of Medicine, Cedars-Sinai Medical Center, Los Angeles, California; [2]Department of Internal Medicine, Chang Gung Memorial Hospital, Taipei, Taiwan; [3]Chang Gung University, Taoyuan, Taiwan; [4]Department of Medical Sciences, University of Birmingham, Birmingham, United Kingdom; and [5]Department of Hematology, Ohme Municipal General Hospital, Tokyo, Japan

## Abstract

**Recently, we reported that a novel, noncalcemic vitamin D analogue (19-nor-1,25(OH)$_2$D$_2$; paricalcitol) had anticancer activity. In this study, we explored if paricalcitol enhanced anticancer effects of other clinically useful drugs *in vitro* against a large variety of cancer cells. Paricalcitol, when combined with As$_2$O$_3$, showed a markedly enhanced antiproliferative effect against acute myeloid leukemia (AML) cells. This combination induced monocytic differentiation of NB-4 acute promyelocytic leukemia (APL) cells and HL-60 AML cells and caused both to undergo apoptosis associated with down-regulation of Bcl-2 and Bcl-x$_L$. Paricalcitol induced monocytic differentiation of U937 AML cells, which was partially blocked by inducing expression of APL-related PML-retinoic acid receptor α (RARα) chimeric protein in the U937 cells containing a $Zn^{2+}$-inducible expression vector coding for this fusion protein (PR9 cells). Exposure to As$_2$O$_3$ decreased levels of PML-RARα in PR9 cells, and the combination of paricalcitol and As$_2$O$_3$ enhanced their monocytic differentiation in parallel with the As$_2$O$_3$-mediated decrease of PML-RARα. Furthermore, As$_2$O$_3$ increased the transcriptional activity of paricalcitol probably by increasing intracellular levels of paricalcitol by decreasing the function of the mitochondrial enzyme 25-hydroxyvitamin D$_3$-24-hydroxylase, which functions to metabolize the active vitamin D in cells. In summary, the combination of paricalcitol and As$_2$O$_3$ potently decreased growth and induced differentiation and apoptosis of AML cells. This probably occurred by As$_2$O$_3$ decreasing levels of both the repressive PML-RARα fusion protein and the vitamin D metabolizing protein, 25-hydroxyvitamin D$_3$-24-hydroxylase, resulting in increased activity of paricalcitol. The combination of both of these Food and Drug Administration–approved drugs should be considered for treatment of all-*trans* retinoic acid–resistant APL patients as well as those with other types of AML.** (Cancer Res 2005; 65(6): 2488-97)

## Introduction

Cancer therapies are usually toxic for normal cells. Induction of differentiation is sometimes a useful, less toxic cancer therapy that can supplement more aggressive approaches. For example, the use of all-*trans* retinoic acid for the treatment of acute promyelocytic leukemia (APL) often induces complete remissions as well as its use in the treatment of head and neck cancers and neuroblastomas (1–3).

1,25-Dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] is a member of the seco-steroid hormone family, which controls calcium homeostasis and bone metabolism. It can inhibit the growth of various types of malignant cells, including breast, prostate, colon, skin, and brain cancers as well as myeloid leukemia, by inducing differentiation, apoptosis, and cell cycle arrest. In a clinical study, orally administrated 1,25(OH)$_2$D$_3$ showed some activity for preleukemic patients (4). Because of the calcemic side effect of 1,25(OH)$_2$D$_3$, the dosage that could be safely given to these individuals was less than theoretically required for an anticancer effect as noted *in vitro* (5, 6). Therefore, novel, potent, but less calcemic analogues of vitamin D are being synthesized and tested (7–15).

19-Nor-1,25(OH)$_2$D$_2$ (paricalcitol) is a synthetic analogue of 1,25(OH)$_2$D$_2$ that is currently approved by the Food and Drug Administration for the clinical treatment of secondary hyperparathyroidism. This compound has very little calcemic potential as shown by several randomized clinical trials (16, 17). Antiproliferative effects of paricalcitol were reported recently against human prostate, leukemia, myeloma, and colon cancer cells *in vitro* and *in vivo* (18–20). These studies suggest that clinical trails of this analogue are reasonable; to this end, we are examining its effects in individuals with myelodysplastic syndrome.

In this study, we investigated the combinations of paricalcitol and either chemotherapeutic or related agents against human myeloid leukemia, myeloma, prostate, breast, and colon cancer *in vitro*. We found that the combination of paricalcitol and As$_2$O$_3$ had a strong antiproliferative effect against myeloid leukemia *in vitro*, and we studied the mechanism of action of this combination.

## Materials and Methods

**Cells and Samples.** Cell lines were obtained from American Type Culture Collection (Rockville, MD) and maintained according to their recommendations. Myeloid leukemia cell lines (HL-60, NB-4, and U937), myeloma cell lines (RPMI-8226, ARH-77, and NCI-H929), lung cancer cell lines (NCI-H125 and NCI-H520), and prostate cancer cell lines (LNCaP and PC-3) were grown in RPMI 1640 with 10% FCS. Breast cancer cell lines (MCF-7 and MDA-MB-231), colon cancer cell lines (HT-29 and SW837), a prostate cancer cell line (DU145), and endometrial cancer cell lines (Ishikawa, HEC59, and HEC1B) were maintained in DMEM with 10% FCS.

**Note:** H.P. Koeffler is Mark Goodson Endowed Chair in Oncology Research and is a member of the Jonsson Cancer Center and Molecular Biology Institute, University of California at Los Angeles.

**Requests for reprints:** Takashi Kumagai, Division of Hematology/Oncology, University of California at Los Angeles School of Medicine, Cedars-Sinai Medical Center, Davis Building 5068, 8700 Beverly Boulevard, Los Angeles, CA 90048. Phone: 310-423-7736; Fax: 310-423-0225; E-mail: kumamed1_2001@yahoo.co.jp.

The U937 cell line (PR9) that stably expressed PML-retinoic acid receptor α (RARα) in a $Zn^{2+}$-inducible fashion was described previously (21) and was kindly provided by Dr. Pelicci (Perugia University, Perugia, Italy). For induction of PML-RARα in PR9 cells, 0.1 mmol/L $ZnSO_4$ was added to the culture medium. Viable cell numbers were counted using trypan blue solution (Sigma-Aldrich, St. Louis, MO). The paricalcitol was generously provided by Scott Toner (Abbott Laboratories, Chicago, IL). PD98059 was obtained from LC Laboratories (Woburn, MA). Other compounds were obtained from Sigma-Aldrich.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assays.** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS, 5 mg/mL, Sigma-Aldrich) was used to measure cellular proliferation. Briefly, $10^3$ cells were incubated in culture medium containing test compounds for 96 hours followed the MTS assay as described before (19).

**Soft Agar Colony Assay.** Trypsinized and washed single-cell suspensions were enumerated and plated into 24-well flat-bottomed plates using a two-layer soft agar system with a total of $1 \times 10^3$ cells per well in a volume of 400 μL/well as described previously (11).

**Cell Cycle Analysis.** After treatment of $5 \times 10^4$ of cells with experimental compounds, cell cycle analysis was done as described previously (19).

**Western Blot Analysis.** Western blot was done as noted previously (19). Briefly, cells were washed and suspended in lysis buffer. After centrifugation, the suspension was collected. Protein (40 μg) was used for SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed sequentially with antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The blots were developed using the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL).

**Measurement of Apoptosis.** Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was done as described (19) using the *In situ* Cell Death Detection POD kit (Roche, Indianapolis, IN).

**Measurement of Cell Surface CD14 Antigen on Acute Myeloid Leukemia Cells.** Myelocytic leukemia cell lines (HL-60, NB-4, and U937) were treated with paricalcitol and/or $As_2O_3$ and examined for CD14 expression by flow cytometry using CD14 antibody (DAKO, Carpinteria, CA) as described previously (15). Murine IgG1 antibody (DAKO) was used as a control.

**Nitroblue Tetrazolium Test.** Nitroblue tetrazolium (NBT) test was done as noted previously (19). Briefly, the cell suspension was mixed with an equal volume of NBT solution and incubated at 37°C for 30 minutes. The cells were cytocentrifuged onto glass slides, washed in cold methanol, and stained with safranin. The percentage NBT-positive cells were enumerated by light microscopy.

**Real-time Quantitative PCR.** RNA was prepared using a RNeasy kit obtained from Qiagen (Valencia, CA). Gene expression was quantified using real-time quantitative PCR (TaqMan, iCycler, Bio-Rad, Hercules, CA). The sequences of the primer sets used for this analysis were as follows: PCR primers used to amplify 25-hydroxyvitamin $D_3$-24-hydroxylase (CYP-24) were 5′-AGGCCACGTTGAAGACTTGT-3′ (forward) and 5′-TTCTTCTGGAGAAGCCCAAA-3′ (reverse), CCAAT/enhancer binding protein β (C/EBPβ): 5′-GACAAGCACAGCGACGAGTA-3′ (forward) and 5′-GTGCTGCGTCTCCAGGTT-3′ (reverse), and those used to amplify 18S rRNA were 5′-AAACGGCTACCACATCCAAG-3′ (forward) and 5′-CCTCCAATGGATCCTCGTTA-3′ (reverse). Primers were synthesized by Invitrogen (Carlsbad, CA).

Probes were purchased from Applied Biosystems (Foster City, CA) and were labeled with the reporter dye FAM in the 5′ end and the quencher dye TAMRA in the 3′ end. Amplification reactions were done with the Universal TaqMan PCR Master Mix (Applied Biosystems) in triplicates in an iCycler iQ system. The thermal cycling conditions using TaqMan method were as follows: 2 minutes at 50°C and 10 minutes at 95°C followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. To determine the relative expression level of each sample, additional reactions with four serial 5-fold dilutions of cDNA from HL-60 cells treated with paricalcitol were done to generate a standard curve, which related the threshold cycle to the log input amount of template.

In case of using SYBR Green method, a melting curve analysis was done following PCR to identify the correct product by its specific melting temperature, because SYBR Green intercalates nonspecifically into PCR products. Melting curve analysis included 95°C for 5 seconds, 65°C for 15 seconds, and heating to 95°C at a rate of 0.1°C/2 seconds with continuous reading of fluorescence. The signal in this study was generated by the binding of the fluorophore SYBR Green (Biozym, Hess. Oldendorf, Germany) to dsDNA. Baseline and threshold calculations were done with the iCycler software. The chemical composition of the PCR assays was according to the descriptions of the Taq polymerases. The amplification followed a three-step PCR with denaturation at 94°C for 20 seconds, annealing at 60°C for 10 seconds, elongation at 65°C for 25 seconds, and 20 seconds at melting temperature. PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and photographed.

**Determination of Mitochondrial Membrane Potential.** Mitochondrial membrane potential was analyzed with a lipophilic cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1, Sigma-Aldrich) using a method described previously (22). JC-1 selectively incorporates into mitochondria. Intact mitochondrial membranes display a negative charge, which attracts positively charged JC-1 molecules. JC-1 aggregates form as a result of accumulation in the mitochondria. The monomeric form emits green fluorescence at 527 nm, whereas aggregated JC-1 molecules emit red fluorescence at 590 nm. Therefore, red fluorescence emission, assayed by fluorescence-activated cell sorting, is interpreted as a function of mitochondrial membrane potential. Cells (HL-60 and NB-4) that had been incubated either with or without paricalcitol and/or $As_2O_3$ were washed with PBS and incubated with 10 μg/mL JC-1 for 10 minutes at 37°C. Cells were transferred on ice for fluorescence-activated cell sorting analysis. Forward and side light scatters were used to gate and exclude cellular debris. Cells were excited at 488 nm, and JC-1 emission was collected on FL-1 and FL-2 channels at 530 and 590 nm, respectively. Ten thousand cells were analyzed per sample.

**Measurement of CYP-24 Activity.** Cells treated with paricalcitol (0.1 μmol/L for NB-4 and 0.01 μmol/L for HL-60) and/or $As_2O_3$ (0.6 μmol/L for NB-4 and 0.8 μmol/L for HL-60) for 4 days were pelleted and washed twice in serum-free RPMI 1640. Cells ($1 \times 10^6$) per sample were added to 1.5 pmol [$^3$H]25-hydroxyvitamin $D_3$ in a glass tube and incubated for 4 hours at 37°C. All assays were done in triplicate for each treatment condition. Following incubation, methanol 500 (μL) was added to the cell suspension, and the mixture was mixed thoroughly. Chloroform (5 mL) was added to each tube, and the mixture was vortexed for 10 seconds. Samples were centrifuged at 1,800 rpm for 10 minutes, and the chloroform layer was isolated and evaporated under $N_2$ at room temperature. Sterols were resuspended in dichloromethane (60 μL) by vortexing and separated on silica TLC plates in dichloromethane/isopropanol (9:1, v/v). Conversion of [$^3$H]25-hydroxyvitamin $D_3$ to [$^3$H]24,25-dihydroxyvitamin $D_3$ was measured on a Bioscan System 200 Imaging TLC plate scanner (Edmonds, WA) and expressed as fmol converted $hour^{-1}$ $1 \times 10^6$ $cells^{-1}$.

## Results

**Paricalcitol, in Combination with $As_2O_3$, Has a Prominent Antiproliferative Activity against Human Myeloid Leukemia Cells.** The antiproliferative effects of paricalcitol in combination with other clinically useful therapeutic agents were examined against various cancer cell lines *in vitro*. Initial screening was done using the rapid MTS assay with a relative short exposure of 4 days to the various agents. These experiments for each agent were done to identify a concentration that produced modest growth inhibition to test subsequently its combination with paricalcitol to identify those pairs that produced enhanced activity (data not shown). Paricalcitol slightly enhanced both daunorubicin's inhibition of growth when tested against myeloid leukemia cells (HL-60 and U937) and Adriamycin's effect on proliferation of MCF-7 breast cancer cells (Fig. 1*A*). Paricalcitol also slightly augmented the antiproliferative effects of Adriamycin and taxol against breast (MCF-7 and MDA-MB-231) and prostate

(PC-3 and DU145) cancer cells. The combination of paricalcitol and dexamethasone had prominent antiproliferative activity against multiple myeloma cells (NCI-H929 and RPMI-8226; Fig. 1*A*). Among the various treatment combinations, paricalcitol and $As_2O_3$ had the most notable antiproliferative effects against myeloid leukemia cells (HL-60 and NB-4) compared with either drug alone. Each drug suppressed the clonal growth of both cell lines in a dose-dependent manner, but the two drugs together had markedly enhanced antiproliferative effects on these cells (Fig. 1*B*).

We focused our attention on the paricalcitol and $As_2O_3$ combination and its antiproliferative activity against myeloid

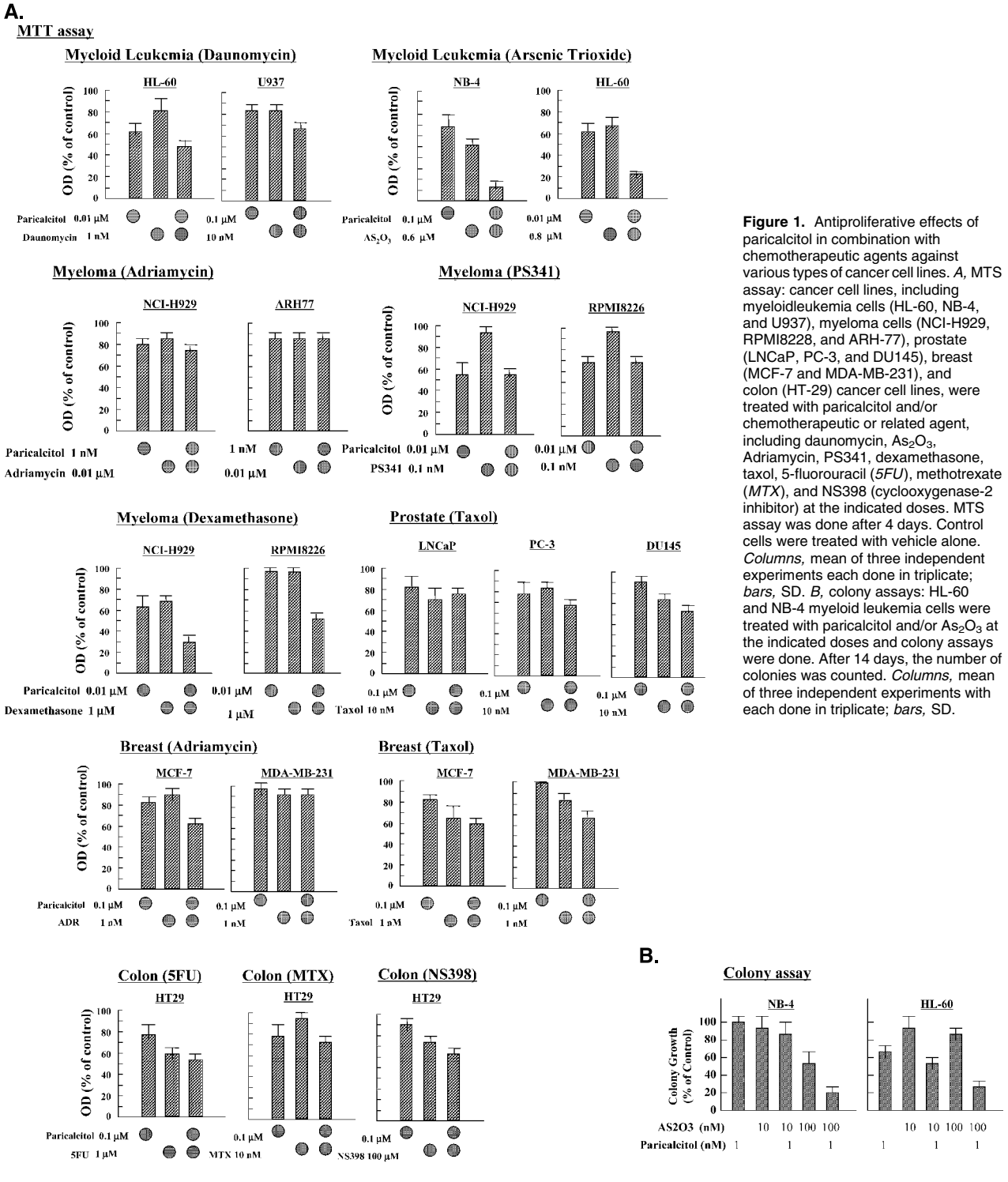


**Figure 1.** Antiproliferative effects of paricalcitol in combination with chemotherapeutic agents against various types of cancer cell lines. *A,* MTS assay: cancer cell lines, including myeloidleukemia cells (HL-60, NB-4, and U937), myeloma cells (NCI-H929, RPMI8228, and ARH-77), prostate (LNCaP, PC-3, and DU145), breast (MCF-7 and MDA-MB-231), and colon (HT-29) cancer cell lines, were treated with paricalcitol and/or chemotherapeutic or related agent, including daunomycin, $As_2O_3$, Adriamycin, PS341, dexamethasone, taxol, 5-fluorouracil (*5FU*), methotrexate (*MTX*), and NS398 (cyclooxygenase-2 inhibitor) at the indicated doses. MTS assay was done after 4 days. Control cells were treated with vehicle alone. *Columns,* mean of three independent experiments each done in triplicate; *bars,* SD. *B,* colony assays: HL-60 and NB-4 myeloid leukemia cells were treated with paricalcitol and/or $As_2O_3$ at the indicated doses and colony assays were done. After 14 days, the number of colonies was counted. *Columns,* mean of three independent experiments with each done in triplicate; *bars,* SD.

leukemia cells. Time course assays of viable cell numbers counted by trypan blue assays were done during which the cells were treated with paricalcitol (0.1 μmol/L for NB-4 and 0.01 μmol/L for HL-60) and $As_2O_3$ (0.6 μmol/L for NB-4 and 0.8 μmol/L for HL-60; Fig. 1*C*). Results showed that the numbers of viable cells in the population treated with both drugs was significantly less ($P < 0.05$) compared with diluant controls or the leukemic cells exposed to each drug alone for 5 (NB-4) or 6 (HL-60) days. We cultured prostate (LNCaP, PC-3, and DU145), breast (MCF-7), colon (HT-29), endometrial (Ishikawa, HEC59, and HEC1B), and lung (NCI-H125 and NCI-H520) cancer cell lines with paricalcitol (0.1 μmol/L) and $As_2O_3$ (1 μmol/L) and measured cell growth (MTS assay) on day 4 of culture. This combination showed some additive antiproliferative effects on PC-3 and slight effects on DU145 prostate cancer cells (Fig. 1*D*).

**Paricalcitol, Combined with $As_2O_3$, Markedly Enhanced Monocytic Differentiation of HL-60 and NB-4 Myeloid Leukemia Cells with Subsequent Increased Apoptosis.** We have shown previously that paricalcitol induces monocytic differentiation and apoptosis of human myeloid leukemia cells (19). Here, we explored the effect of paricalcitol in combination with $As_2O_3$ on differentiation and apoptosis of NB-4 and HL-60 cells (Fig. 2*A*). After treatment with paricalcitol (0.1 μmol/L for NB-4 and 0.01 μmol/L for HL-60) and $As_2O_3$ (0.6 μmol/L for NB-4 and 0.8 μmol/L for HL-60) for 3 days, CD14, a marker of monocytic differentiation, was measured by flow cytometry in NB-4 and HL-60 cells. Neither paricalcitol nor $As_2O_3$ alone induced CD14 expression in NB-4 cells (Fig. 2*A*). In HL-60 cells, paricalcitol alone, but not $As_2O_3$, increased levels of CD14. The combination of both compounds markedly induced CD14 expression on both NB-4 and HL-60 cells compared with either drug alone (Fig. 2*A*). Differentiation was also measured by induction of superoxide production as monitored by percentage of cells able to reduce NBT. Cells were treated with either paricalcitol (0.1 μmol/L for NB-4 and 0.01 μmol/L for HL-60) and/or $As_2O_3$ (0.6 μmol/L for NB-4 and 0.8 μmol/L for HL-60; Fig. 2*B*). For NB-4, paricalcitol combined with $As_2O_3$ markedly increased the NBT reduction (38% positive cells), whereas each compound alone had no effect. For HL-60 cells, NBT reduction was increased by paricalcitol (14% positive cells), and $As_2O_3$ enhanced the activity of paricalcitol (48% positive cells).

We examined the effect of the combination of both agents on the induction of apoptosis using two measurements: (*a*) percentage of cells in the sub-$G_1$ population of the cell cycle and (*b*) TUNEL assay. NB-4 and HL-60 cells were cultured with paricalcitol (0.1 μmol/L for NB-4 and 0.01 μmol/L for HL-60) and/or $As_2O_3$ (0.6 μmol/L for NB-4 and 0.8 μmol/L for HL-60) for 4 days. For NB-4 cells, $As_2O_3$ alone induced apoptosis as shown by an increased percentage of cells in the sub-$G_1$ population (33%), whereas 4% and 5% of control and paricalcitol-treated cells, respectively, were in the sub-$G_1$ population. The combination of

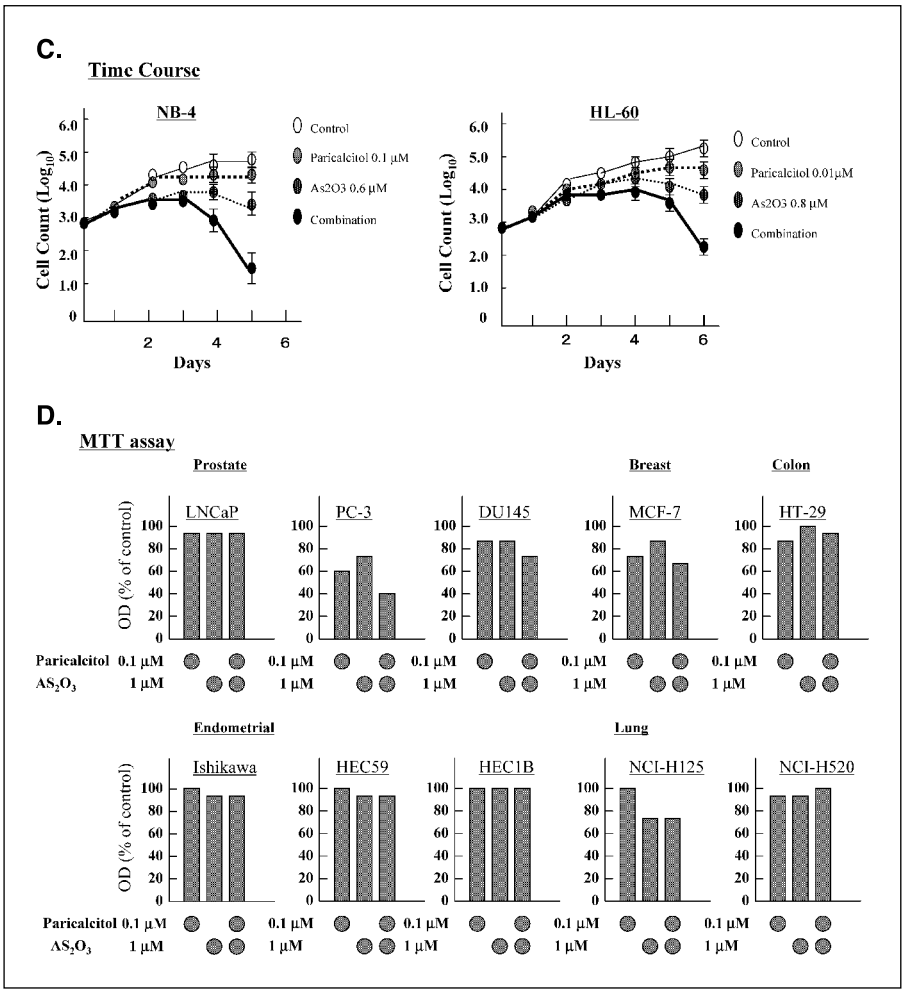


**Figure 1.** *Continued. C,* time course assays: HL-60 and NB-4 myeloid leukemia cell lines were treated with paricalcitol ($10^{-8}$ mol/L for HL-60 and $10^{-7}$ mol/L for NB-4) and/or $As_2O_3$ ($8 \times 10^8$ mol/L for HL-60 and $6 \times 10^{-7}$ mol/L for NB-4). Control cells were treated with vehicle alone. Cell numbers were counted daily by trypan blue assay. *Points,* mean of three independent experiments with triplicate dishes; *bars,* SD. *D,* effect of paricalcitol and $As_2O_3$ on growth of cancer cells. Prostate (LNCaP, PC-3, and DU145), breast (MCF-7), colon (HT-29), endometrial (Ishikawa, HEC59, and HEC1B), and lung (NCI-H125 and NCI-H520) cancer cell lines were treated with paricalcitol (0.1 μmol/L) and $As_2O_3$ (1 μmol/L). MTS assay was done after 4 days. *Columns,* mean percentage of control of three independent experiments with triplicate dishes per experimental point; *bars,* SD.

both compounds dramatically increased apoptotic cell death, with 97% of NB-4 cells in the sub-$G_1$ population (Fig. 2*C*). We also determined the percentage of apoptotic NB-4 cells by TUNEL assay after treatment. The combination increased apoptosis (68% of the cells) compared with either agent alone (paricalcitol, 3%; $As_2O_3$, 14%; Fig. 2*D*). Apoptosis was also prominent in HL-60 cells exposed to both compounds as measured by the percentage of cells in the sub-$G_1$ population (46%) compared with those treated with either paricalcitol (12%) or $As_2O_3$ (20%) alone and by TUNEL assay (46% apoptotic cells in the presence of both agents compared with 7% and 20% in the presence of paricalcitol or $As_2O_3$ alone, respectively; Fig. 2*C* and *D*).

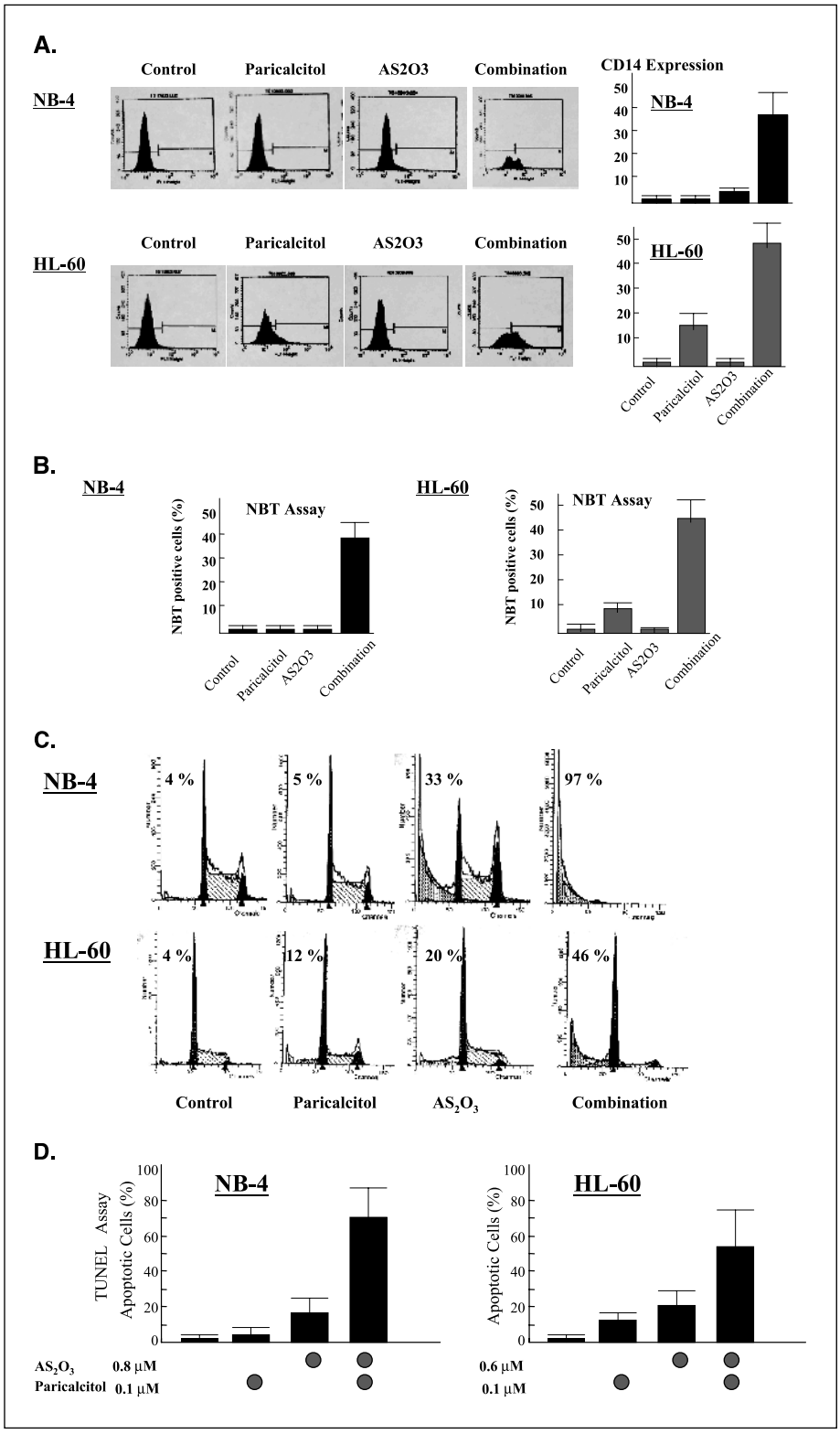


**Figure 2.** Paricalcitol combined with $As_2O_3$ markedly enhanced monocytic differentiation of HL-60 and NB-4 myeloid leukemia cells with subsequent apoptosis. NB-4 and HL-60 myeloid leukemia cell lines were treated with paricalcitol ($10^{-7}$ mol/L for NB-4 and $10^{-8}$ mol/L for HL-60) and/or $As_2O_3$ ($6 \times 10^{-7}$ mol/L for NB-4 and $8 \times 10^{-8}$ mol/L for HL-60). Control cells were treated with vehicle alone. *A,* after 3 days, CD14 cell surface marker of monocytic/macrophage differentiation was measured by flow cytometry. Mean of three independent experiments with triplicate dishes; *bars,* SD. *B,* NBT reduction as a measure of myeloid or monocytic differentiation. *Columns,* mean of three independent experiments in triplicate dishes analyzed on day 3 of culture; *bars,* SD. *C,* cell cycle analysis of NB-4 cells done by flow cytometry after 4 days culture. Percentage of cells in sub-$G_1$ population. *D,* TUNEL assay. Quantitative analysis of apoptosis of NB-4 cell line on day 4 of culture. *Columns,* mean percentage of TUNEL-positive cells of three independent experiments; *bars,* SD.

**Modulation of Gene Expression by Paricalcitol and $As_2O_3$ in Myeloid Leukemia Cells.** The enzyme CYP-24 catalyzes the first step in the catabolism of $1,25(OH)_2D_3$ (23). Expression of CYP-24 is activated by the binding of its ligand $1,25(OH)_2D_3$ to vitamin D receptor (VDR). The VDR-ligand complex (VDR-retinoid X receptor) binds to the vitamin D response element in the CYP-24 promoter and initiates transcription (24, 25). Because we have reported recently that the activity of paricalcitol is mediated through VDR (19), we examined the expression of CYP-24 as an early vitamin D target gene by real-time PCR. The levels of CYP-24 mRNA increased $2 \times 10^4$-fold after NB-4 cells were cultured with paricalcitol (0.1 μmol/L, 24 hours) compared with control NB-4 cells (Fig. 3*A*). When the cells were exposed to both paricalcitol (0.1 μmol/L) and $As_2O_3$ (0.6 μmol/L), the transcriptional level of CYP-24 increased even more ($4 \times 10^4$-fold compared with control), suggesting that $As_2O_3$ enhanced transcriptional activation through the VDR (Fig. 3*A*). The level of expression of VDR and retinoid X receptor was not altered by exposure of NB-4 cells to either paricalcitol (0.1 μmol/L) and/or $As_2O_3$ (0.6 μmol/L; Fig. 3*B*; data not shown).

A recent study showed that *C/EBPβ* is a target gene of VDR and has an important role in CYP-24 transcription (26, 27). We examined the mRNA and protein expression of C/EBPβ by real-time reverse transcription-PCR (Fig. 3*A*) and Western blot analysis (Fig. 3*B*). In NB-4 cells, expression of C/EBPβ was low. Exposure to either paricalcitol or $As_2O_3$ for 24 hours increased the level of C/EBPβ mRNA by 4.3- and 2.5-fold compared with control cells, respectively. In contrast, the combination of both drugs increased expression by 90-fold compared with control (Fig. 3*A*). These results suggest that $As_2O_3$ increased the transcriptional activity of some *VDR* target genes. Expression of C/EBPβ protein also increased after exposure of NB-4 to both drugs (Fig. 3*B*). In addition, C/EBPβ protein levels increased when HL-60 cells were exposed to paricalcitol and these levels increased even more in the presence of both paricalcitol and $As_2O_3$ (Fig. 3*C*). Western blot analysis showed that $As_2O_3$ either alone or with paricalcitol resulted in the dramatic loss of the PML-RARα fusion protein (Fig. 3*B*).

As shown above, in association with enhancement of differentiation by the combination of paricalcitol and $As_2O_3$, HL-60 and NB-4 cells underwent apoptosis (Fig. 2). Expression of the antiapoptotic proteins Bcl-2 and Bcl-$x_L$ markedly decreased (48% and 38%, respectively) after combined treatment of HL-60 cells with paricalcitol and $As_2O_3$; however, the level of expression of the proapoptotic protein Bax was unchanged by either drug alone or in combination (Fig. 3*C*).

Vitamin D has been suggested to act through genomic and nongenomic pathways (28). Activated extracellular signal-regulated

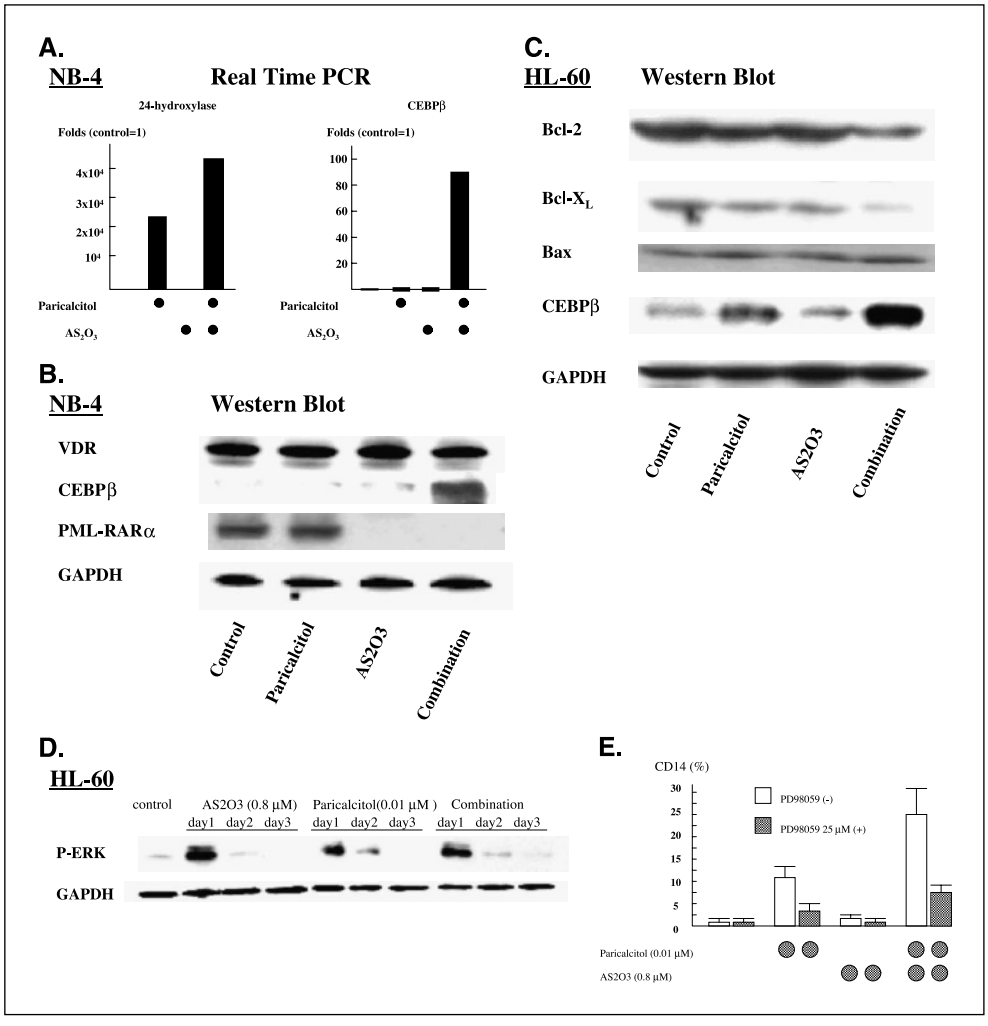


**Figure 3.** Modulation of expression of target genes in myeloid leukemia cells cultured with paricalcitol and $As_2O_3$. NB-4 and HL-60 myeloid leukemia cell lines were treated with paricalcitol (0.1 μmol/L for NB-4 and 0.01 μmol/L for HL-60) and/or $As_2O_3$ (0.6 μmol/L for NB-4 and 0.8 μmol/L for HL-60). Control cells were treated with vehicle alone. *A,* Western blot from cell lysate of NB-4 cells (3 days) probed sequentially with antibodies to VDR, C/EBPβ, and RARα (to detect the fusion protein PML-RARα) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Amount of protein in each lane was normalized by glyceraldehyde-3-phosphate dehydrogenase. *B,* after treatment of NB-4 cells for 3 days, mRNA was extracted and expression of CYP-24 mRNA was measured by real-time PCR. *C,* Western blot of cell lysate of HL-60 cells after 3 days of culture with the above compounds probed sequentially with antibodies to Bcl-2, Bcl-$x_L$, Bax, C/EBPβ, and glyceraldehyde-3-phosphate dehydrogenase. *D,* cell lysates of HL-60 cells after 1, 2, or 3 days of culture with the above compounds and used for Western blot, which was probed sequentially with antibodies to phosphorylated ERK (*P-ERK*) and glyceraldehyde-3-phosphate dehydrogenase. *E,* paricalcitol-induced differentiation is blocked by PD98059. After pretreatment either with or without PD98059 (25 nmol/L) for 1 hour, HL-60 cells were cultured with paricalcitol (0.01 μmol/L) and/or $As_2O_3$ (0.8 μmol/L) for 2 days. Control cells were treated with vehicle alone. CD14 expression was measured by flow cytometry.

kinase (ERK) seems to have an important role in monocytic differentiation induced by vitamin $D_3$ in HL-60 cells (29). We found that the levels of phosphorylated ERK increased to a similar degree after exposure of HL-60 to either paricalcitol and/or $As_2O_3$. Highest levels of phosphorylation expression occurred on the first day of exposure to either of the compounds (Fig. 3*D*). Because the expression of phosphorylated ERK might be important for differentiation, we treated HL-60 cells with the selective mitogen-activated protein kinase/ERK inhibitor, PD98059 (25 μmol/L), in combination with paricalcitol and/or $As_2O_3$; CD14 expression, a marker of monocytic differentiation, was measured by flow cytometry (Fig. 3*E*). When HL-60 cells were treated with paricalcitol and/or $As_2O_3$ in the presence of PD98059, the differentiation induced by paricalcitol decreased by 23% compared with paricalcitol alone, and the magnitude of inhibition was not significantly changed by the combination of both compounds. Therefore, monocytic differentiation induced by paricalcitol, and the enhanced differentiation by the addition of $As_2O_3$, could be blocked by inhibiting the ERK pathway.

**Paricalcitol, in Combination with $As_2O_3$, Overcomes the Block of Differentiation Mediated by the PML-RARα Fusion Protein Present in APL Cells.** NB-4 promyelocytic leukemia cells express the *PML-RARα* fusion gene, which has an important role in the pathogenesis of APL. $As_2O_3$, which is used as a therapeutic agent for this disease, is reported to enhance the degradation of this fusion protein (30). We found that $As_2O_3$ caused a profound decrease in the protein level of PML-RARα in NB-4 cells after 3 days such that we could not detect its expression by Western blot (Fig. 4*A*).

To examine the association between levels of the fusion protein and the effect of the combination treatment, we used an engineered U937 monocytic leukemia cell line (U937-PR9) that had a stably integrated PML-RARα cDNA expression vector under the control of the $Zn^{2+}$-inducible murine metallothionein 1 promoter (21). U937 cells transfected with the empty vector (U937-PMT) were used as a control. When we cultured wild-type U937 with paricalcitol and/or $As_2O_3$, paricalcitol increased monocytic differentiation, and the combination of both agents further increased the percentage of CD14-expressing cells. $As_2O_3$ alone did not induce CD14 expression. This suggested that $As_2O_3$ enhanced paricalcitol-induced monocytic differentiation of U937 cells. Similarly, the combination also enhanced paricalcitol-induced monocytic differentiation of THP-1 monocytic leukemia cells (data not shown).

Next, we cultured either U937-PR9 or U937-PMT cells with paricalcitol and $As_2O_3$. Treatment of U937-PMT control cells with paricalcitol induced monocytic differentiation, and paricalcitol in combination with $As_2O_3$ enhanced their monocytic differentiation in either the presence or the absence of $Zn^{2+}$ (Fig. 4*B*). Next, we cultured U937-PR9 experimental cells with $Zn^{2+}$, resulting in these cells expressing PML-RARα (Fig. 4*A*), and the differentiation induced by paricalcitol in these cells was reduced by 80% compared with PR9 without $Zn^{2+}$ (Fig. 4*B*). Addition of $As_2O_3$ to the medium decreased in a dose-dependent manner the protein levels of PML-RARα in the $Zn^{2+}$-stimulated U937-PR9 cells. At 0.4 μmol/L $As_2O_3$, PML-RARα was rarely detected in U937-PR9 cells (Fig. 4*A*). The combination of paricalcitol and $As_2O_3$ enhanced the differentiation of U937-PR9 cells in parallel with the $As_2O_3$-induced decrease of PML-RARα levels (Fig. 4*B*).

**$As_2O_3$, Induced the Damage of Mitochondria and Decreased CYP-24 Activity in Acute Myeloid Leukemia Cells.** The mitochondrial enzyme *CYP-24* is a transcriptional target gene of vitamin D. We showed that CYP-24 mRNA was markedly increased in the presence of paricalcitol (19) and that this was prominently enhanced by $As_2O_3$ in NB-4 cells (Fig. 3*B*). This enzyme catalyzes the initial step in the conversion of the active molecule $1,25(OH)_2D_3$ into its less active metabolite $1,24,25(OH)_2D_3$. Therefore, induction of this enzyme by $1,25(OH)_2D_3$ and other active vitamin D compounds has a negative feedback on levels of the active vitamin D (31–34). On the other hand, $As_2O_3$ induces apoptosis of many cancers through mitochondrial membrane collapse (35–37). This raises the possibility that the $As_2O_3$ inactivates the mitochondrial enzyme CYP-24 leading to an increase in intracellular levels of the active vitamin D compound and thus explaining the enhanced activity of paricalcitol in the presence of $As_2O_3$.

To explore this hypothesis, we first examined if the mitochondria were damaged by either $As_2O_3$ and/or paricalcitol in NB-4 cells. Mitochondrial potential was measured using the JC-1 dye in conjunction with flow cytometry (Fig. 5*A*). The geometric mean of red fluorescence intensity was recorded as a function of mitochondrial potential. The control cells showed a geometric mean of 133.9 for red fluorescence intensity. Cells cultured with paricalcitol alone ($10^{-8}$ mol/L) and $As_2O_3$ (0.6 μmol/L) alone displayed similar mitochondrial potential levels, with geometric means of red fluorescence of 147.4 and 140, respectively. However, when treated with both compounds together, the level of red fluorescence decreased to a geometric mean of 69.2, indicating a marked reduction in mitochondrial membrane potential of these NB-4 APL cells compared with control and either agent alone.

The CYP-24 is a mitochondrial enzyme. Because $As_2O_3$ either alone or in combination with paricalcitol induces mitochondria damage, we hypothesized that $As_2O_3$ would decrease the activity of this enzyme. Activity of CYP-24 in leukemia cell lines was monitored by measuring the conversion of $25(OH)_2D_3$ to $1,24,25(OH)_2D_3$ using TLC analysis. HL-60 and NB-4 cells were cultured with paricalcitol and/or $As_2O_3$ for 3 days. Control cells were treated with vehicle alone. As expected, paricalcitol alone increased the activity of CYP-24 in HL-60 and NB-4 cells, whereas $As_2O_3$ alone decreased activity compared with control cells (Fig. 5*B*). When cells were treated with paricalcitol and $As_2O_3$, CYP-24 activity was significantly less in cells cultured with paricalcitol alone (HL-60, $P < 0.05$; NB-4, $P < 0.05$; Fig. 5*B*). These results indicated that $As_2O_3$ decreased the enzyme activity of CYP-24. This would be expected to result in higher intracellular levels of biologically active paricalcitol in leukemia cells. Mitochondrial injury induced by $As_2O_3$ may be the cause of this CYP-24 inactivation.

## Discussion

In this study, we show that treatment with paricalcitol, when combined with clinically achievable concentrations of $As_2O_3$ (0.6-0.8 μmol/L; ref. 38), results in strong antiproliferative effects on the human myeloid leukemia cell lines HL-60 and NB-4. NB-4 cells were established from a patient with APL and HL-60 was derived from a patient with acute myeloid leukemia M2 with promyelocytic features (39). This combination also produced effects against PC-3 and DU145 prostate cells. Paricalcitol combined with dexamethasone also significantly decreased the growth of myeloma cells (NCI-H929 and RPMI-8226). This deserves further investigations.

We reported previously that the induction of macrophage differentiation by paricalcitol may be mediated through VDR (19). The CYP-24 is the enzyme responsible for the first step in the catabolism of $1,25(OH)_2D_3$ (23). Transcriptional induction of expression of CYP-24 is dependent on ligand activation of VDR

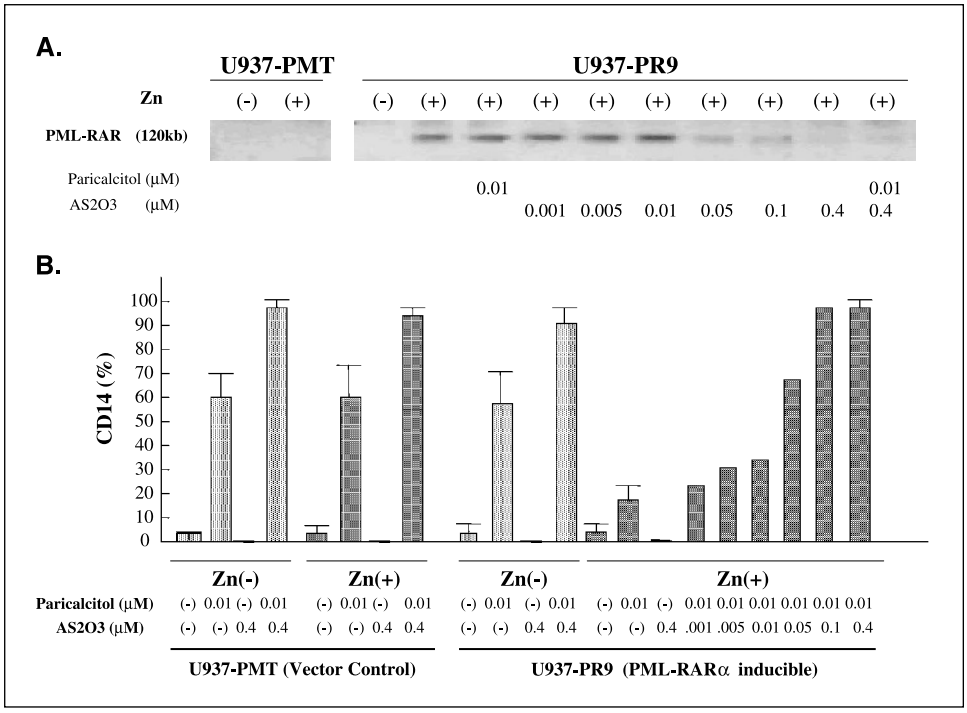


**Figure 4.** Paricalcitol in combination with $As_2O_3$ overcomes the block of differentiation by the PML-RARα fusion protein. *A,* U937 cells stably transfected with either the control empty vector (U937-PMT) or the PML-RARα cDNA under the control of the $Zn^{2+}$-inducible murine metallothionein 1 promoter (U937-PR9) were treated either with (+) or without (-) $Zn^{2+}$ for 2 days. Paricalcitol and/or $As_2O_3$ at the indicated concentrations were added to the cells. Cell lysates were harvested and used for Western blot, which was probed with antibodies to RARα to detect the PML-RARα fusion protein (120 kb). *B,* U937-PMT and PR9 cells were cultured either with or without $Zn^{2+}$ as well as with either paricalcitol and/or $As_2O_3$ as indicated for 3 days, and CD14 expression was measured by flow cytometry.

and binding of the complex to the vitamin D response element of the promoter of the *CYP-24* gene (24, 25).

Transcription of CYP-24 was activated by paricalcitol and enhanced by $As_2O_3$, although $As_2O_3$ alone did not induce expression of the enzyme in NB-4 cells (Fig. 3*A*). In addition, the mRNA and protein expression of C/EBPβ was slightly increased by paricalcitol, and the combination of both drugs also enhanced the mRNA and protein expression of this transcriptional factor in NB-4 cells (Fig. 3*A* and *B*). In addition, in HL-60 cells, paricalcitol increased the protein expression of C/EBPβ, and the combination of both drugs enhanced its expression (Fig. 3*C*). These result suggested that $As_2O_3$ enhance the transcriptional activity of paricalcitol-activated VDR. In contrast, the activity of CYP-24 was decreased by $As_2O_3$ in both HL-60 and NB-4 cells. This mitochondrial enzyme catalyzes the initial step in the conversion of the active molecule $1,25(OH)_2D_3$ into the less active metabolite $1,24,25(OH)_2D_3$, resulting in the inhibition of antiproliferative effects of vitamin D (31–34). In fact, CYP-24 has been suggested to be an oncogene (34). We believe that $As_2O_3$ inhibits intracellular CYP-24 activity by

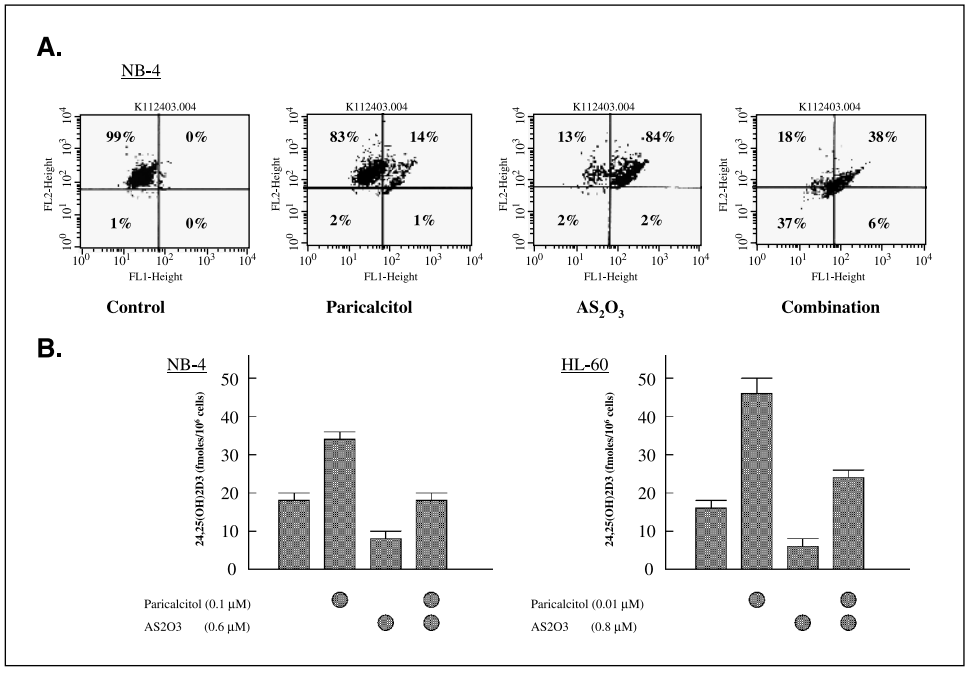


**Figure 5.** $As_2O_3$ in combination with paricalcitol induced mitochondrial injury and suppressed the activity of the CYP-24 enzyme in leukemia cells. *A,* mitochondrial potential was measured using the JC-1 dye in conjunction with flow cytometry in NB-4 cells treated with paricalcitol and/or $As_2O_3$ for 3 days. Red fluorescence, mitochondrial potential. *B,* myeloid leukemia cell lines were treated with paricalcitol (0.1 μmol/L for NB-4 and 0.01 μmol/L for HL-60) and/or $As_2O_3$ (0.6 μmol/L for NB-4 and 0.8 μmol/L for HL-60) for 3 days. Control cells were treated with vehicle alone. *B,* activity of CYP-24 was determined by measuring the conversion of [$^3$H]25-hydroxyvitamin $D_3$ to [$^3$H]24,25-dihydroxyvitamin $D_3$ by NB-4 and HL-60 using TLC analysis. Cells were cultured (4 days) with either $As_2O_3$, paricalcitol, both (at indicated concentrations), or diluant.

disrupting mitochondrial integrity, resulting in higher intracellular levels of the vitamin D analogue. Therefore, although higher intracellular levels of the ligand can stimulate transcription of CYP-24, $As_2O_3$ blocks its activity. The combination of vitamin D and inhibitors of CYP-24, such as genistein and isoflavone, have been suggested to be a useful approach to cancer therapy (40, 41). Our studies extend these observations to two clinically relevant compounds.

Recently, studies related to the identification of coactivators of VDR-mediated transcription have been a major focus of vitamin D research (26, 27). C/EBPβ is induced by $1,25(OH)_2D_3$ in renal and osteoblastic cells and can enhance the transcription of CYP-24 induced by $1,25(OH)_2D_3$. Thus, C/EBPβ seems to be transcriptionally stimulated by vitamin D compounds and to have a cooperative effects with VDR in enhancing the $1,25(OH)_2D_3$-induced transcription (26, 27).

During the last decade, the efficiency of $As_2O_3$ has been established for both newly diagnosed and relapsed patients with APL. It can be used as a single agent and induces complete remissions with only minimal myelosuppression (42). The NB-4 promyelocytic leukemia cell line is a good model for APL having the prerequisite t(15;17) chromosomal translocation specific for APL. In this study, paricalcitol when combined with $As_2O_3$ greatly suppressed the growth of NB-4 cells by inducing monocytic differentiation and subsequent apoptosis, whereas paricalcitol alone was unable to induce monocytic differentiation of these cells.

The PML-RARα fusion protein impairs differentiation in response to vitamin D *in vitro* and *in vivo* (43–45). This block of differentiation is thought to occur by the aberrant recruitment of corepressor proteins and histone deacetylases and perhaps by direct sequestering the vitamin $D_3$ receptor (45, 46).

$As_2O_3$ may be efficacious in APL by several mechanisms. Arsenic rapidly inhibits the interaction of the SMRT corepressor with PML-RARα leading to the reduction of the repression of retinoid target genes (47). Secondly, arsenic enhances the degradation of the PML-RARα by promoting the binding of SUMO to the fusion protein (48, 49). A recent study suggests that arsenic increases acetylation of histones H3 and H4 leading to gene activation (38).

Our study showed that the $As_2O_3$-enhanced differentiation mediated by paricalcitol was associated with a decreased protein expression of PML-RARα in NB-4 and U937-PR9 cells containing a $Zn^{2+}$-inducible PML-RARα expression vector (Figs. 3*A* and 4), suggesting that the degradation of this fusion protein by $As_2O_3$ has an important role for the enhanced response to paricalcitol. Therefore, targeting of the fusion protein might be important in allowing vitamin D compounds to induce differentiation. This is further supported by the fact that arsenic does not lead to degradation of the PLZF-RARα fusion protein and is not effective in this subtype of APL (50, 51).

In addition to its VDR-mediated genomic actions, $1,25(OH)_2D_3$ can activate other signal pathways, including ERKs, in a nongenomic manner, which may effect diverse processes, including cell proliferation and differentiation (52). A rapid but transient activation of ERK1/2 has been reported following induction of monocytic differentiation of HL-60 cells by high concentrations ($10^{-6}$ mol/L) of $1,25(OH)_2D_3$ (29, 53). On the other hand, rapid inactivation of ERK1/2 has been observed when U937 leukemia cells were treated with $As_2O_3$, which was associated with apoptosis (54). We treated HL-60 cells with a moderate concentration ($10^{-7}$ mol/L) of paricalcitol and/or $As_2O_3$ ($4 \times 10^{-7}$ mol/L). Paricalcitol induced maximal expression of phosphorylated ERK after 1 day (Fig. 3*D*). $As_2O_3$ also activated ERK1/2, which was also maximal at 1 day of exposure (Fig. 3*D*). The selective mitogen-activated protein kinase/ERK kinase inhibitor PD98059 inhibited paricalcitol-induced differentiation of HL-60 cells; the combination of paricalcitol and $As_2O_3$ could not overcome this inhibition. These data suggest that the activation of mitogen-activated protein kinase/ERK kinase is necessary for the differentiation induced by paricalcitol as well as the combination of paricalcitol and $As_2O_3$.

Higher concentrations of arsenic have been shown to induce apoptosis of a variety of human hematopoietic malignancies and solid tumors (55, 56). Concerning the pathways whereby arsenic compounds induce apoptosis, attention has focused on their potential effects on the mitochondria. A leading mechanism involves alteration of transmembrane potential by modulating the permeability of the transition pore cortex leading to the release of cytochrome C, culminating in the cleaving of caspases, including caspase-3 and caspase-9 (38). In our study, $As_2O_3$ combined with paricalcitol decreased mitochondrial potential. This mitochondrial damage induced by the combination of both agents impaired the activity of the mitochondrial enzyme CYP-24 leading to a higher sensitivity to paricalcitol.

In this study, we have found that paricalcitol and $As_2O_3$ potently slowed growth and induced differentiation of myeloid leukemia cells, and this probably occurred by $As_2O_3$ decreasing levels of the PML-RARα fusion protein in APL and inactivating CYP-24 activity in mitochondria resulting in the increased activity of paricalcitol. The combination of both of these Food and Drug Administration–approved drugs should be considered for all-*trans* retinoic acid–resistant APL patients and as maintenance therapy for some types of acute myeloid leukemia. Furthermore, the combination of dexamethasone and paricalcitol deserves further exploration for the therapy of multiple myeloma.

## Acknowledgments

Received 8/4/2004; revised 11/15/2004; accepted 1/6/2005.

**Grant support:** NIH, Parker Hughes Fund, and Abbott Laboratories.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Scott Toner (Abbott Laboratories) for helpful suggestions.

## References

1. Huang ME, Ye YC, Chen SR, et al. Use of all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia. Blood 1988;72:567–72.
2. Castaigne S, Chomienne C, Daniel MT, et al. All-*trans* retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results. Blood 1990;76:1704–9.
3. Warrell RP Jr, Frankel SR, Miller WH Jr, et al. Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-*trans*-retinoic acid). N Engl J Med 1991;324:1385–93.
4. Koeffler HP, Hirji K, Itri L. 1,25-Dihydroxyvitamin $D_3$: *in vivo* and *in vitro* effects on human preleukemic and leukemic cells. Cancer Treat Rep 1985;69:1399–407.
5. Staquet MJ, Byar DP, Green SB, Rozencweig M. Clinical predictivity of transplantable tumor systems in the selection of new drugs for solid tumors: reply to a commentary. Cancer Treat Rep 1985;69:1339–40.
6. Koeffler HP, Amatruda T, Ikekawa N, Kobayashi Y, DeLuca HF. Induction of macrophage differentiation of human normal and leukemic myeloid stem cells by 1,25-dihydroxyvitamin $D_3$ and its fluorinated analogues. Cancer Res 1984;44:5624–8.
7. Abe J, Nakano T, Nishii Y, Matsumoto T, Ogata E, Ikeda K. A novel vitamin $D_3$ analog, 22-oxa-1,25-dihydroxyvitamin $D_3$, inhibits the growth of human

breast cancer *in vitro* and *in vivo* without causing hypercalcemia. Endocrinology 1991;129:832–7.
8. Zhou JY, Norman AW, Akashi M, et al. Development of a novel 1,25(OH)2-vitamin $D_3$ analog with potent ability to induce HL-60 cell differentiation without modulating calcium metabolism. Blood 1991;78:75–82.
9. Jung SJ, Lee YY, Pakkala S, et al. 1,25(OH)2-16-ene-vitamin $D_3$ is a potent antileukemic agent with low potential to cause hypercalcemia. Leuk Res 1994; 18:453–63.
10. Anzano MA, Smith JM, Uskokovic MR, et al. 1α,25-Dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol (Ro24-5531), a new deltanoid (vitamin D analogue) for prevention of breast cancer in the rat. Cancer Res 1994;54:1653–6.
11. Pakkala S, de Vos S, Elstner E, et al. Vitamin $D_3$ analogs: effect on leukemic clonal growth and differentiation, and on serum calcium levels. Leuk Res 1995;19:65–72.
12. Koike M, Elstner E, Campbell MJ, et al. 19-Nor-hexafluoride analogue of vitamin $D_3$: a novel class of potent inhibitors of proliferation of human breast cell lines. Cancer Res 1997;57:4545–50.
13. Kubota T, Koshizuka K, Koike M, Uskokovic M, Miyoshi I, Koeffler HP. 19-Nor-26,27-bishomo-vitamin $D_3$ analogs: a unique class of potent inhibitors of proliferation of prostate, breast, and hematopoietic cancer cells. Cancer Res 1998;58:3370–5.
14. Hisatake J, Kubota T, Hisatake Y, Uskokovic M, Tomoyasu S, Koeffler HP. 5,6-*Trans*-16-ene-vitamin $D_3$: a new class of potent inhibitors of proliferation of prostate, breast, and myeloid leukemic cells. Cancer Res 1999;59:4023–9.
15. Hisatake J, O'Kelly J, Uskokovic MR, Tomoyasu S, Koeffler HP. Novel vitamin D(3) analog, 21-(3-methyl-3-hydroxy-butyl)-19-nor D(3), that modulates cell growth, differentiation, apoptosis, cell cycle, and induction of PTEN in leukemic cells. Blood 2001;97:2427–33.
16. Llach F, Keshav G, Goldblat MV, et al. Suppression of parathyroid hormone secretion in hemodialysis patients by a novel vitamin D analogue: 19-nor-1,25-dihydroxyvitamin $D_2$. Am J Kidney Dis 1998;32:S48–54.
17. Martin KJ, Gonzalez EA, Gellens M, Hamm LL, Abboud H, Lindberg J. 19-Nor-1-α-25-dihydroxyvitamin $D_2$ (paricalcitol) safely and effectively reduces the levels of intact parathyroid hormone in patients on hemodialysis. J Am Soc Nephrol 1998;9:1427–32.
18. Chen TC, Schwartz GG, Burnstein KL, Lokeshwar BL, Holick MF. The *in vitro* evaluation of 25-hydroxyvitamin $D_3$ and 19-nor-1α,25-dihydroxyvitamin $D_2$ as therapeutic agents for prostate cancer. Clin Cancer Res 2000;6:901–8.
19. Kumagai T, O'Kelly J, Said JW, Koeffler HP. Vitamin $D_2$ analog 19-nor-1,25-dihydroxyvitamin $D_2$: antitumor activity against leukemia, myeloma, and colon cancer cells. J Natl Cancer Inst 2003;95:896–905.
20. Molnar I, Kute T, Willingham MC, Powell BL, Dodge WH, Schwartz GG. 19-Nor-1α,25-dihydroxyvitamin D(2) (paricalcitol): effects on clonal proliferation, differentiation, and apoptosis in human leukemic cell lines. J Cancer Res Clin Oncol 2003;129:35–42.
21. Grignani F, Ferrucci PF, Testa U, et al. The acute promyelocytic leukemia-specific PML-RARα fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. Cell 1993;74:423–31.
22. Cossarizza A, Baccarani-Contri M, Kalashnikova G, Franceschi C. A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Biochem Biophys Res Commun 1993;197:40–5.
23. Jones G, Strugnell SA, DeLuca HF. Current understanding of the molecular actions of vitamin D. Physiol Rev 1998;78:1193–231.
24. Zierold C, Darwish HM, DeLuca HF. Identification of a vitamin D-response element in the rat calcidiol (25-hydroxyvitamin $D_3$) 24-hydroxylase gene. Proc Natl Acad Sci U S A 1994;91:900–2.
25. Ohyama Y, Ozono K, Uchida M, et al. Identification of a vitamin D-responsive element in the 5′-flanking region of the rat 25-hydroxyvitamin $D_3$ 24-hydroxylase gene. J Biol Chem 1994;269:10545–50.
26. Christakos S, Barletta F, Huening M, et al. Vitamin D target proteins: function and regulation. J Cell Biochem 2003;88:238–44.
27. Ji Y, Studzinski GP. Retinoblastoma protein and CCAAT/enhancer-binding protein β are required for 1,25-dihydroxyvitamin $D_3$-induced monocytic differentiation of HL60 cells. Cancer Res 2004;64:370–7.
28. Norman AW, Nemere I, Zhou LX, et al. 1,25(OH)2-vitamin $D_3$, a steroid hormone that produces biologic effects via both genomic and nongenomic pathways. J Steroid Biochem Mol Biol 1992;41:231–40.
29. Wang X, Studzinski GP. Activation of extracellular signal-regulated kinases (ERKs) defines the first phase of 1,25-dihydroxyvitamin $D_3$-induced differentiation of HL60 cells. J Cell Biochem 2001;80:471–82.
30. Chen GQ, Zhu J, Shi XG, et al. *In vitro* studies on cellular and molecular mechanisms of arsenic trioxide ($As_2O_3$) in the treatment of acute promyelocytic leukemia: $As_2O_3$ induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RARα/PML proteins. Blood 1996;88:1052–61.
31. Krishnan AV, Peehl DM, Feldman D. Inhibition of prostate cancer growth by vitamin D: regulation of target gene expression. J Cell Biochem 2003;88:363–71.
32. Krishnan AV, Peehl DM, Feldman D. The role of vitamin D in prostate cancer. Recent Results Cancer Res 2003;164:205–21.
33. Peehl DM, Krishnan AV, Feldman D. Pathways mediating the growth-inhibitory actions of vitamin D in prostate cancer. J Nutr 2003;133:2461–9S.
34. Albertson DG, Ylstra B, Segraves R, et al. Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. Nat Genet 2000;25:144–6.
35. Cai X, Shen YL, Zhu Q, et al. Arsenic trioxide-induced apoptosis and differentiation are associated respectively with mitochondrial transmembrane potential collapse and retinoic acid signaling pathways in acute promyelocytic leukemia. Leukemia 2000;14:262–70.
36. Park WH, Seol JG, Kim ES, et al. Arsenic trioxide-mediated growth inhibition in MC/CAR myeloma cells via cell cycle arrest in association with induction of cyclin-dependent kinase inhibitor, p21, and apoptosis. Cancer Res 2000;60:3065–71.
37. Seol JG, Park WH, Kim ES, et al. Potential role of caspase-3 and -9 in arsenic trioxide-mediated apoptosis in PCI-1 head and neck cancer cells. Int J Oncol 2001;18:249–55.
38. Chen Z, Chen GQ, Shen ZX, Chen SJ, Wang ZY. Treatment of acute promyelocytic leukemia with arsenic compounds: *in vitro* and *in vivo* studies. Semin Hematol 2001;38:26–36.
39. Drexler HG, Quentmeier H, MacLeod RA, Uphoff CC, Hu ZB. Leukemia cell lines: *in vitro* models for the study of acute promyelocytic leukemia. Leuk Res 1995;33:681–91.
40. Farhan H, Wahala K, Adlercreutz H, Cross HS. Isoflavonoids inhibit catabolism of vitamin D in prostate cancer cells. J Chromatogr B Analyt Technol Biomed Life Sci 2002;777:261–8.
41. Farhan H, Cross HS. Transcriptional inhibition of CYP24 by genistein. Ann N Y Acad Sci 2002;973:459–62.
42. Zhang P, Wang S, Hu XH. Arsenic trioxide treated 72 cases of acute promyelocytic leukemia. Chin J Hematol 1996;17:58–62.
43. Ruthardt M, Testa U, Nervi C, et al. Opposite effects of the acute promyelocytic leukemia PML-retinoic acid receptor α (RARα) and PLZF-RARα fusion proteins on retinoic acid signalling. Mol Cell Biol 1997;17:4859–69.
44. Rego EM, Wang ZG, Peruzzi D, He LZ, Cordon-Cardo C, Pandolfi PP. Role of promyelocytic leukemia (PML) protein in tumor suppression. J Exp Med 2001;193:521–9.
45. Puccetti E, Obradovic D, Beissert T, et al. AML-associated translocation products block vitamin D(3)-induced differentiation by sequestering the vitamin D(3) receptor. Cancer Res 2002;62:7050–8.
46. Grignani F, De Matteis S, Nervi C, et al. Fusion proteins of the retinoic acid receptor-α recruit histone deacetylase in promyelocytic leukaemia. Nature 1998; 391:815–8.
47. Hong SH, Yang Z, Privalsky ML. Arsenic trioxide is a potent inhibitor of the interaction of SMRT corepressor with its transcription factor partners, including the PML-retinoic acid receptor α oncoprotein found in human acute promyelocytic leukemia. Mol Cell Biol 2001;21:7172–82.
48. Zhu J, Lallemand-Breitenbach V, de The H. Pathways of retinoic acid- or arsenic trioxide-induced PML/RARα catabolism, role of oncogene degradation in disease remission. Oncogene 2001;20:7257–65.
49. Seeler JS, Dejean A. SUMO: of branched proteins and nuclear bodies. Oncogene 2001;20:7243–9.
50. Koken MH, Daniel MT, Gianni M, et al. Retinoic acid, but not arsenic trioxide, degrades the PLZF/RARα fusion protein, without inducing terminal differentiation or apoptosis, in a RA-therapy resistant t(11;17)(q23;q21) APL patient. Oncogene 1999;18:1113–8.
51. Kitamura K, Hoshi S, Koike M, Kiyoi H, Saito H, Naoe T. Histone deacetylase inhibitor but not arsenic trioxide differentiates acute promyelocytic leukaemia cells with t(11;17) in combination with all-*trans* retinoic acid. Br J Haematol 2000;108:696–702.
52. Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 1995;80:179–85.
53. Marcinkowska E, Wiedlocha A, Radzikowski C. 1,25-Dihydroxyvitamin $D_3$ induced activation and subsequent nuclear translocation of MAPK is upstream regulated by PKC in HL-60 cells. Biochem Biophys Res Commun 1997;241:419–26.
54. Iwama K, Nakajo S, Aiuchi T, Nakaya K. Apoptosis induced by arsenic trioxide in leukemia U937 cells is dependent on activation of p38, inactivation of ERK and the $Ca^{2+}$-dependent production of superoxide. Int J Cancer 2001;92:518–26.
55. Wang ZG, Rivi R, Delva L, et al. Arsenic trioxide and melarsoprol induce programmed cell death in myeloid leukemia cell lines and function in a PML and PML-RARα independent manner. Blood 1998;92: 1497–504.
56. Davison K, Mann KK, Miller WH Jr. Arsenic trioxide: mechanisms of action. Semin Hematol 2002;39:3–7.