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Tetramethylpyrazine potentiates arsenic trioxide activity against HL-60 cell lines

Yuni Wu^{1,2,3}, Youhua Xu^{1,2,3}, Yali Shen^{1,2,3}, Cuicui Wang^{1,2,3}, Gaili Guo^{1,2,3}
and Tiantian Hu^{1,2,3}

¹Key Laboratory of Developmental Diseases in Childhood, Chongqing, China

²Key Laboratory of Pediatrics in Chongqing, Chongqing, China

³Chongqing International Science and Technology Cooperation Center for Child Development and Disorders, Chongqing, China

Abstract

The objective of this study was to evaluate the effects of tetramethylpyrazine (TMP) in combination with arsenic trioxide (As_2O_3) on the proliferation and differentiation of HL-60 cells. The HL-60 cells were treated with 300 $\mu\text{g/mL}$ TMP, 0.5 μM As_2O_3 , and 300 $\mu\text{g/mL}$ TMP combined with 0.5 μM As_2O_3 , respectively. The proliferative inhibition rates were determined with MTT. Differentiation was detected by the nitroblue tetrazolium (NBT) reduction test, Wright's staining and the distribution of CD11b and CD14. Flow cytometry was used to analyze cell cycle distribution. RT-PCR and Western blot assays were employed to detect the expressions of c-myc, p27, CDK2, and cyclin E1. Combination treatment had synergistic effects on the proliferative inhibition rates. The rates were increased gradually after the combination treatment, much higher than those treated with the corresponding concentration of As_2O_3 alone. The cells exhibited characteristics of mature granulocytes and a higher NBT-reducing ability, being a 2.6-fold increase in the rate of NBT-positive ratio of HL-60 cells within the As_2O_3 treatment versus almost a 13-fold increase in the TMP + As_2O_3 group. Cells treated with both TMP and As_2O_3 expressed far more CD11b antigens, almost 2-fold compared with the control group. Small doses of TMP potentiate As_2O_3 -induced differentiation of HL-60 cells, possibly by regulating the expression and activity of G0/G1 phase-arresting molecules. Combination treatment of TMP with As_2O_3 has significant synergistic effects on the proliferative inhibition of HL-60 cells.

Key words: Tetramethylpyrazine; Acute promyelocytic leukemia; Arsenic trioxide; HL-60 cell; Proliferation; Differentiation

Introduction

A recent study revealed that differentiation may be associated with the induction of apoptosis, and thus, differentiation-inducing therapy may be useful in combination with intensive chemotherapy to increase the susceptibility of leukemia blast cells to drug-induced apoptosis (1). It has also been reported that hematopoietic tumors often arise as a consequence of uncontrolled proliferation of immature blasts that fail to terminally differentiate into mature blood cells; therefore, initiating a maturation program requires cell cycle arrest (2).

Arsenic trioxide (As_2O_3) has been successfully used to treat acute promyelocytic leukemia (PML). *In vitro* studies have shown that micromolar concentrations of As_2O_3 can induce apoptosis of leukemia cells, while at lower concentrations (0.1-0.5 μM) As_2O_3 induces cell differen-

tiation (3,4). These preclinical studies were confirmed by controlled clinical trials showing that treatment with As_2O_3 led to complete remission in acute PML patients (5). Because As_2O_3 at higher concentrations induces many side effects (ventricular arrhythmia, skin reaction, peripheral neuropathy, electrolyte changes, hepatic dysfunction, gastrointestinal reactions, etc.), low-dose combination therapy is required. In particular, combination treatment using two chemotherapeutic agents at low concentrations has been reported to have improved cytotoxic effects on cancer cells with minimal side effects.

Tetramethylpyrazine (TMP) is a compound extracted from the Chinese medicinal plant *Ligusticum chuanxiong*. The structure of the TMP is 2,3,5,6-tetramethylpyrazine and TMP is an amide alkaloid (6). The tubers of *L. chuanxiong*

Correspondence: Youhua Xu, Department of Hematology, Affiliated Children's Hospital, Chongqing Medical University, No. 136, Zhongshan 2nd Road, Yuzhong District, Chongqing 86 400014, China. E-mail: xuyouhua0504@163.com or 1983711c@163.com (Yuni Wu)

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have been used in traditional Chinese medicine for the treatment of cancer. TMP has been synthesized and widely used in oriental medicine to effectively treat several cardiovascular complications. Several studies have shown that TMP has various biological activities, such as antioxidant activity (7), the ability to modulate nitric oxide production (8), and cytotoxicity against various tumor cells (9). The hypotheses are clear. A previous study has found that TMP could promote apoptosis of the human promyelocytic leukemia cell line HL-60 *in vitro* (10); however, the effects of TMP in combination with As₂O₃ on the proliferation and differentiation of HL-60 cells are unknown, and the mechanisms by which this drug acts in the treatment of acute PML have not been established. Here, we report the effects of TMP alone and in combination with As₂O₃ on proliferation, cell cycle regulation, and differentiation of HL-60 cells.

Material and Methods

Material

HL-60 cells were bought from KeyGen Biotech Co. Ltd., China, and preserved in our laboratory. RPMI-1640, DMSO, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), nitroblue tetrazolium (NBT), and 12-O-tetradecanoylphorbol 13-acetate (TPA) were obtained from Sigma, USA. PCR primers were synthesized by Shanghai Sangon Biotechnology Co. Ltd., China. PE-conjugated CD14 and FITC-conjugated CD11b antibodies were purchased from Beijing 4A Biotech Co. Ltd., China. Nuclear-cytosol extraction kit was purchased from KENGEN Biotechnology, China. Antibodies against p27^{KIP1}, cyclin E1, cyclin-dependent kinase 2 (CDK2), and c-myc were obtained from Epitomics Co., USA. β -actin and goat anti-rabbit IgG-horseradish peroxidase (HRP) were obtained from Santa Cruz Biotechnology, USA. TMP was supplied by Shanghai Modern Hasen Pharmaceutical Co., Ltd., China. As₂O₃ was purchased from Beijing SL Pharmaceutical Co., Ltd., China.

Cell culture

HL-60 cells were maintained in RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, 1 mM L-glutamine, and 10% heat-inactivated fetal bovine serum (FBS). The cells were grown in humidified atmosphere at 37°C in 5% CO₂. This study was approved by the Animal Ethics Committee of the Institute of Zoology at Chongqing Medical University.

Cell counting and MTT assay

To quantify the number of cells after each treatment, cells were counted at different incubation times using a hemocytometer under a light microscope. Cell viability was determined by the MTT assay (11). The blank wells were full of RPMI-1640 medium only, control wells contained

untreated cells, and test cells were treated with the substance to be assayed.

Cell growth inhibition rate (%) = (the absorbance (A) at 590 nm of treated wells - the A590 value of blank wells) / (the A590 value of control wells - the A590 value of blank wells) x 100.

Wright's staining

Cell morphology was examined using microscopy with Wright's staining. Briefly, cells were centrifuged onto glass slides, dried, stained, and examined at 1000X magnification using an Olympus light microscope (Olympus, Japan).

Nitroblue tetrazolium reduction test

The extent of differentiation was assessed by measurement of superoxide production, monitored by reduction of NBT (12). A total of 200 cells were counted under a light microscope, and the percent of cells containing membrane deposits of reduced blue-black formazan was recorded.

Cell surface differentiation antigen expression assay

Approximately 1 x 10⁶ cells were collected from each experimental group and washed twice with ice-cold phosphate-buffered saline (PBS), pH 7.4. Expression of the cell surface antigens CD11b and CD14 was determined by cytofluorometric analysis. Briefly, 20 μ L of either FITC-conjugated CD11b or PE-conjugated CD14 antibody was added to 100 μ L PBS and incubated at 4°C for 2 h. The cells were then resuspended in PBS solution containing paraformaldehyde, after which cytofluorometric analysis was performed (BD FACS Calibur Co., USA) with a sample size of at least 10,000 cells. The expression of CD14 and CD11b is reported as cell percent. The gate excludes debris, outliers, and events that lie far away from the mean. Data were stored and processed using the FACSscan software.

Cell cycle analysis

Approximately 0.5 x 10⁶ cells were collected from cultures, washed twice with PBS, fixed in 70% ice-cold ethanol, and stored at -20°C overnight. The fixed cells were then collected and resuspended in PBS containing 50 mg/mL PI and 100 μ g/mL DNase-free RNase A. Cells were incubated for 1 h at room temperature and then analyzed by flow cytometry.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of CDK2, cyclin E1, c-myc, and p27^{KIP1}

At the indicated times, 1 x 10⁷ cells of each experimental group were collected, and total cell RNA was isolated using Trizol reagent. RNA concentration was determined by UV absorption at 260 nm. The RNA was reverse transcribed into cDNA, which then underwent PCR in a final volume of 25 μ L as follows: 95°C for 5 min, 30 cycles at 95°C for 1 min, specific annealing temperature for 1 min, 72°C for 1

Table 1. Primers and annealing temperatures.

Gene	Sense	Antisense	Size (bp)	Annealing temperature
c-myc	5'-GATTCTCTGCTCTCCTCGAC-3'	5'-TCCAGACTCTGACCTTTTGC-3'	180	55°C
p27 ^{KIP1}	5'-ATGTCACGTGCGAGTGTC-3'	5'-TCTGTAGTAGAACTCGGGCAA-3'	270	55°C
CDK2	5'-CCTTGTGGTCCCTTCTAC-3'	5'-CAAATCCACCCACTATGA-3'	395	55°C
Cyclin E1	5'-CTGGATGTTGACTGCCTTGA-3'	5'-CCGCTGCTCTGCTTCTTAC-3'	359	52°C
β-actin	5'-AAGATGACCCAGATCATGTTTGAGACC-3'	5'-GCCAGGTCCAGACGCAGGAT-3'	191	52°C

PCR primers were synthesized by Shanghai Sangon Biotechnology Co. Ltd., China.

min, and an additional extension at 72°C for 10 min. The primer sequences and annealing temperatures are shown in Table 1. The PCR products were detected by gel electrophoresis and ultraviolet transillumination.

Western blotting

HL-60 cells were collected, washed twice with ice-cold PBS, and harvested in sodium dodecyl sulfate (SDS) lysis buffer (2% SDS, 50 mM dithiothreitol, 62.5 mM Tris-HCl, pH 6.8). The protein sample concentration was quantified using the BCA method, and an equal amount of protein (30 µg) was loaded onto each well of a 10% SDS polyacrylamide gel. Cell extracts were separated by polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane (PVDF). The primary antibodies used were anti-β-actin (1:500), anti-p27^{KIP1} (1:1000), anti-cyclin E1 (1:800), anti-CDK2 (1:1000), and anti-c-myc (1:800). HRP-conjugated anti-IgG was used as the secondary antibody.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 Student Edition for Windows. Differences between groups were examined for statistical significance by analysis of variance (ANOVA). P < 0.05 was considered to be statistically significant.

Results

TMP, alone or in combination with As₂O₃ has effects on proliferative inhibition

TMP has an anticancer effect on HL-60 cells with HL-60 cell viability being reduced in a concentration- and time-dependent manner (Figure 1A). However, this inhibitory effect became apparent as no more than 50% at a concentration of 300 µg/mL TMP; therefore, this concentration was used throughout the study. Combined treatment with 300

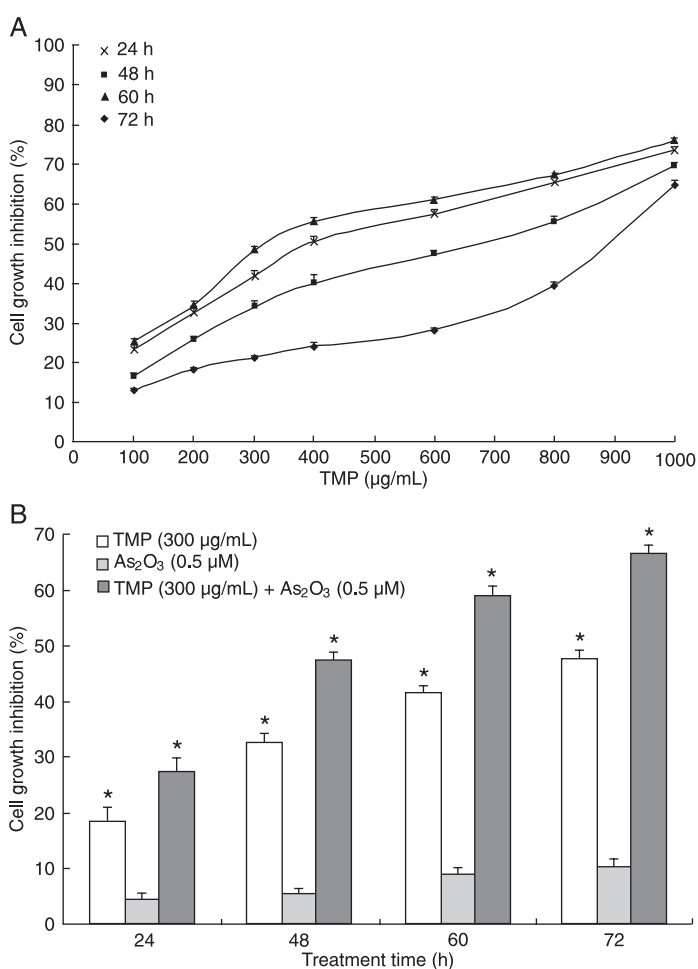


Figure 1. Effect of tetramethylpyrazine (TMP) and arsenic trioxide (As₂O₃) on the rate of HL-60 cell growth inhibition. Growth inhibition was determined using the MTT assay. *A*, Cells were treated with various concentrations of TMP for the indicated times. The growth of HL-60 cells was significantly inhibited by TMP in a dose- and time-dependent manner. *B*, Cells were treated with 300 µg/mL TMP plus 0.5 µM As₂O₃ for the indicated periods. The results are reported as means ± SD for three independent experiments. *P < 0.05 compared to cells treated with As₂O₃ alone in every group (groups of 24, 48, and 72 h; ANOVA).

$\mu\text{g/mL}$ TMP and $0.5 \mu\text{M}$ As_2O_3 had a synergistic effect on inhibiting the proliferation of HL-60 cells. This synergistic effect occurred in a time-dependent manner (Figure 1B). Importantly, the proliferation was inhibited much more effectively with the combined treatment than with treatment with As_2O_3 alone ($P < 0.01$).

Effects of TMP and As_2O_3 on the morphology of HL-60 cells

After incubation for 3 days, treated cells were selected and stained with Wright's solution (Figure 2A-D). HL-60 cells treated with the combination of TMP and As_2O_3 were characterized by a decreased nuclear/cytoplasmic ratio and irregular nuclear shape (kidney-shaped nuclei, nuclear lobulation, and broad bean nuclei). The morphologic changes in the combination group were more obvious than those observed in cells treated with As_2O_3 alone.

Effects of TMP and As_2O_3 on NBT reduction ability in HL-60 cells

Consistent with the morphologic change, the ability of HL-60 cells to reduce NBT significantly increased following a 3-day combined treatment with TMP and As_2O_3 when compared to either a control group or a group treated with

As_2O_3 alone ($P < 0.01$; Figure 3). Compared to the control group, there was a 2.6-fold increase in NBT-positive cells in the As_2O_3 group versus a nearly 13-fold increase in the TMP plus As_2O_3 group. Also, there was a significant difference in percent NBT-positive cells between samples treated with TMP and samples treated with As_2O_3 plus TMP. These data provide further evidence that TMP could have a stimulatory effect on As_2O_3 -induced differentiation.

TMP and As_2O_3 induce G1 cell cycle arrest

Differentiation resulting from treatment has been shown to accompany decreased proliferation (13). Based on the observed increase in differentiation following TMP and As_2O_3 treatment, we examined cell cycle arrest following treatment in HL-60 cells. Cells cultured with TMP exhibited a significant decrease in percent arrest in the S phase. There was also an increase in percent cells in G0/G1 following TMP treatment, from 39.1% in the control group to 53.7% after TMP treatment ($P < 0.001$; Figure 4). There was no cell cycle arrest following As_2O_3 treatment alone (Figure 4). However, the combined treatment decreased the percentage of cells in the S phase and increased the percentage of cells in the G0/G1 phase when compared to both the control group and the As_2O_3 -treated group ($P < 0.001$).

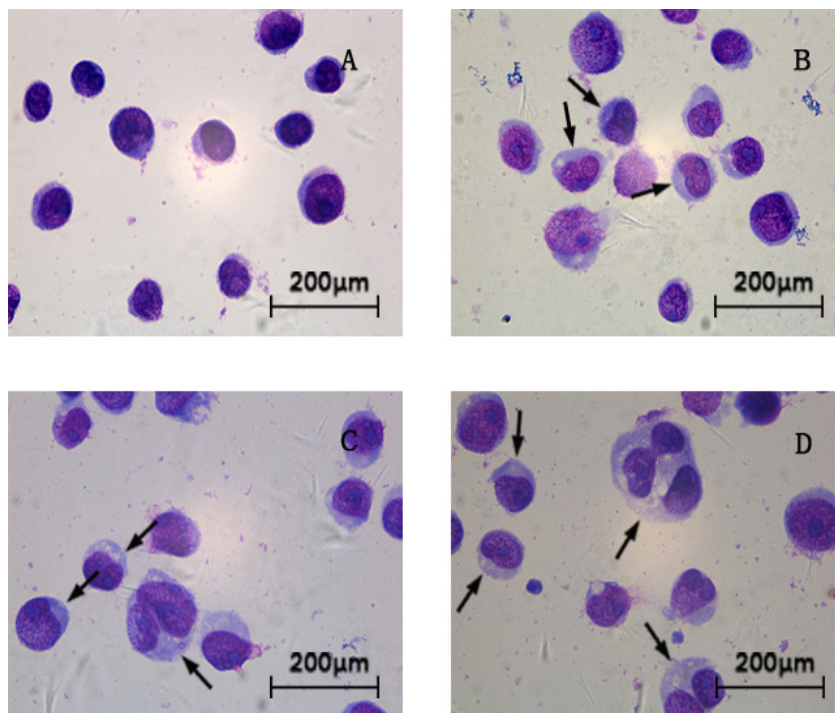


Figure 2. Effects of tetramethylpyrazine (TMP) and arsenic trioxide (As_2O_3) on the morphology of HL-60 cells. Cells were treated with A, control (untreated); B, $300 \mu\text{g/mL}$ TMP; C, $0.5 \mu\text{M}$ As_2O_3 , and D, $300 \mu\text{g/mL}$ TMP + $0.5 \mu\text{M}$ As_2O_3 , and then stained with Wright's solution and examined at a 1000X magnification using a light microscope. The experiments were repeated three times. Cells (arrows) were characterized by decreased nuclear/cytoplasmic ratio and irregular nuclear shape.

Flow cytometry analysis of cell-surface differentiation and antigen expression

HL-60 differentiation yields both monocytic and granulocytic cells; thus, cell differentiation was analyzed after incubating the cells with TMP alone or in combination with As_2O_3 for 72 h. CD14 is expressed exclusively when cells differentiate into monocytes, while CD11b is a monocyte- and granulocyte-specific marker. When compared to the control group, combined treatment with TMP plus As_2O_3 increased the expression of CD11b nearly 2-fold ($P < 0.05$; Figure 5). Both CD11b and CD14 expression differed significantly between samples treated with As_2O_3 plus TMP and control samples (Table 2), indicating that HL-60 cells differentiated through both the granulocytic and monocytic pathway following combined treatment with TMP plus As_2O_3 .

Effects of TMP and As_2O_3 on the expressions of cell cycle regulatory proteins

To determine the possible mecha-

nism by which TMP causes cell cycle arrest, we analyzed cell cycle-related protein expression using Western blotting. The results revealed that combined treatment with As₂O₃ and TMP resulted in a reduction in the levels of cyclin E1 and CDK2 (Figure 6B), while the levels of the related CDK

inhibitor p27^{KIP1} increased after the combined treatment. Based on the observed protein levels, mRNA levels of p27^{KIP1} were also found to be increased; however, the expression of CDK2 and cyclin E1 mRNA did not change (Figure 6A). The c-myc protein is a member of the region/helix-loop-helix/leucine zipper (bHLHZip) transcription factor family, which is implicated in regulation of proliferation and differentiation in multiple cell types. In agreement with the RT-PCR results, there was also decreased c-myc protein expression in response to combined treatment with TMP plus As₂O₃ (Figure 6A,B). These results provide new insight into c-myc function in the regulation of the fate of HL-60 cells.

Discussion

TMP is widely used in the treatment of ischemic stroke and has been reported to possess a diverse array of pharmacological functions, such as modulation of arterial resistance, cerebral blood flow, platelet function, microcirculation, and capillary permeability (14-16). TMP has been in clinical use for decades at doses ranging from 40 to 2000 mg in adults. Few adverse reactions to TMP have been reported, supporting the safety of treatment with this agent.

The present study demonstrates that TMP inhibits HL-60 cell proliferation in a dose- and time-dependent manner. Moreover, we found that TMP exerts a stimulatory effect on As₂O₃-induced HL-60 cell cycle arrest and that this arrest leads to the inhibition of cell proliferation and the induction of differentiation. This observation prompted us to investigate the effects of TMP and As₂O₃ on cell cycle regulation and the characteristics of HL-60 differentiation.

The induction of differentiation and cell cycle arrest are often used to assess the efficacy of novel anti-tumor compounds. Blockade of the cell cycle is regarded as an effective strategy for eliminating cancer cells (17). Our study indicates that the inhibitory effect of combined TMP and As₂O₃ treatment on cell proliferation is a result of induction of G1 phase arrest. The induction of differentiation in malignant cells can have positive clinical implications, such as loss of proliferative potential and induction

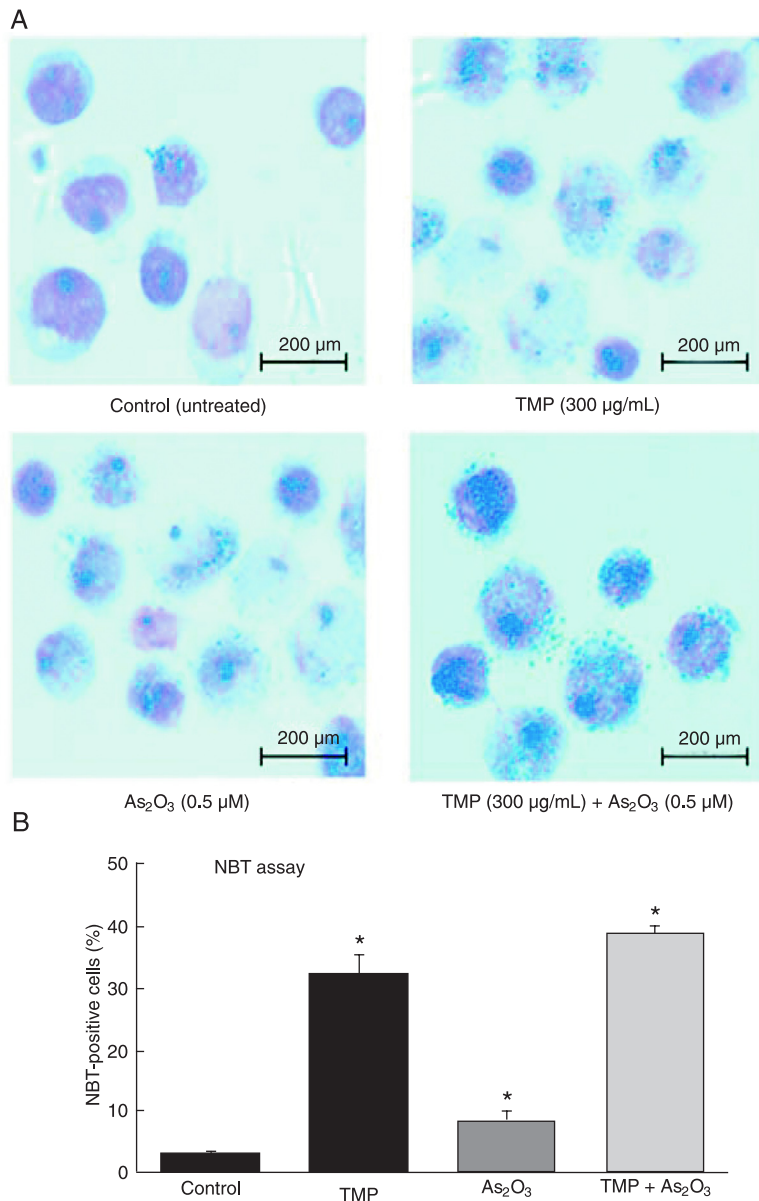


Figure 3. Effects of tetramethylpyrazine (TMP) and arsenic trioxide (As₂O₃) on the nitroblue tetrazolium (NBT) reduction ability of HL-60 cells. HL-60 cells were untreated (control) or treated with TMP, As₂O₃, or TMP combined with As₂O₃. A, Cells containing membrane deposits of reduced blue-black formazan were counted and examined at 1000X magnification. B, We found that the NBT reduction ability of HL-60 cells incubated with TMP combined with As₂O₃ for 3 days was significantly increased when compared with either the control group or the As₂O₃-alone group. All assays were performed in triplicate. *P < 0.05 compared to control (ANOVA).

of apoptosis (18). To further investigate the mechanism by which TMP exerts its stimulatory effect on As₂O₃-induced differentiation, we investigated the effect of the expression of several cell cycle regulatory proteins. The results provided convincing evidence that TMP in combination with As₂O₃ exerts its effects on cell cycle progression mainly via up-

regulation of p27^{KIP1} protein and mRNA expression. The cell cycle is regulated by a complex machinery, in which CDKs, CKIs, and cyclins play essential roles (19). CKIs, including p27^{KIP1}, are tumor suppressor proteins that down-regulate cell cycle progression by binding to active CDK-cyclin complexes, thereby inhibiting their kinase activities

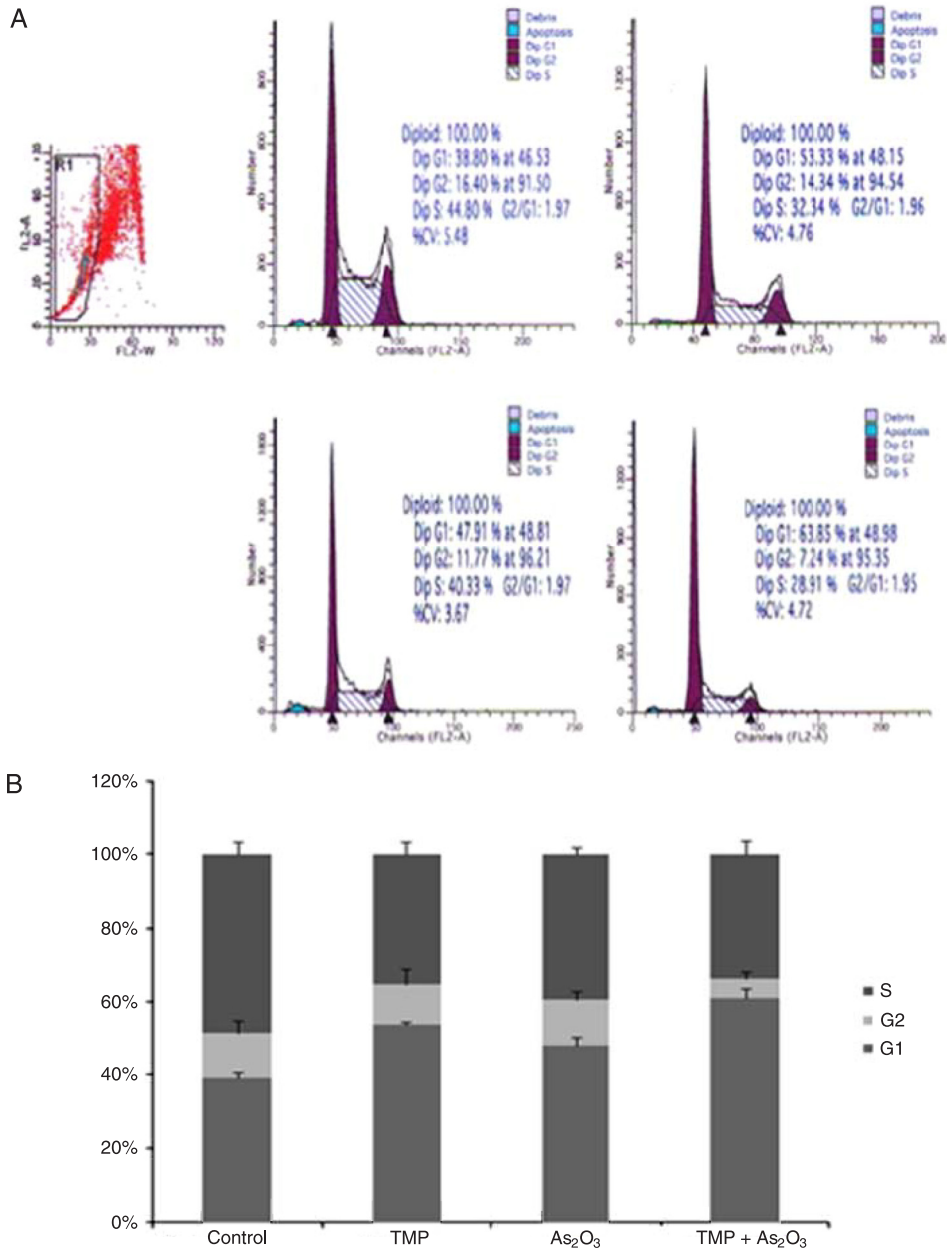


Figure 4. Tetramethylpyrazine (TMP) and arsenic trioxide (As₂O₃) induce G1 cell cycle arrest in HL-60 cells. *A*, Cells were treated with TMP, As₂O₃, or TMP combined with As₂O₃ for the indicated duration before analysis by flow cytometry. *B*, We found that combined treatment decreased the percent of cells in the S phase and increased the percent of cells in the G0/G1 phase. The results are reported as means ± SD for three independent experiments.

(20,21). When we compared cell cycle phase distribution with alternations in cell cycle regulatory molecules, it was apparent that CKI up-regulation is one of the major causes of combination chemotherapy-induced G₁ arrest and cell

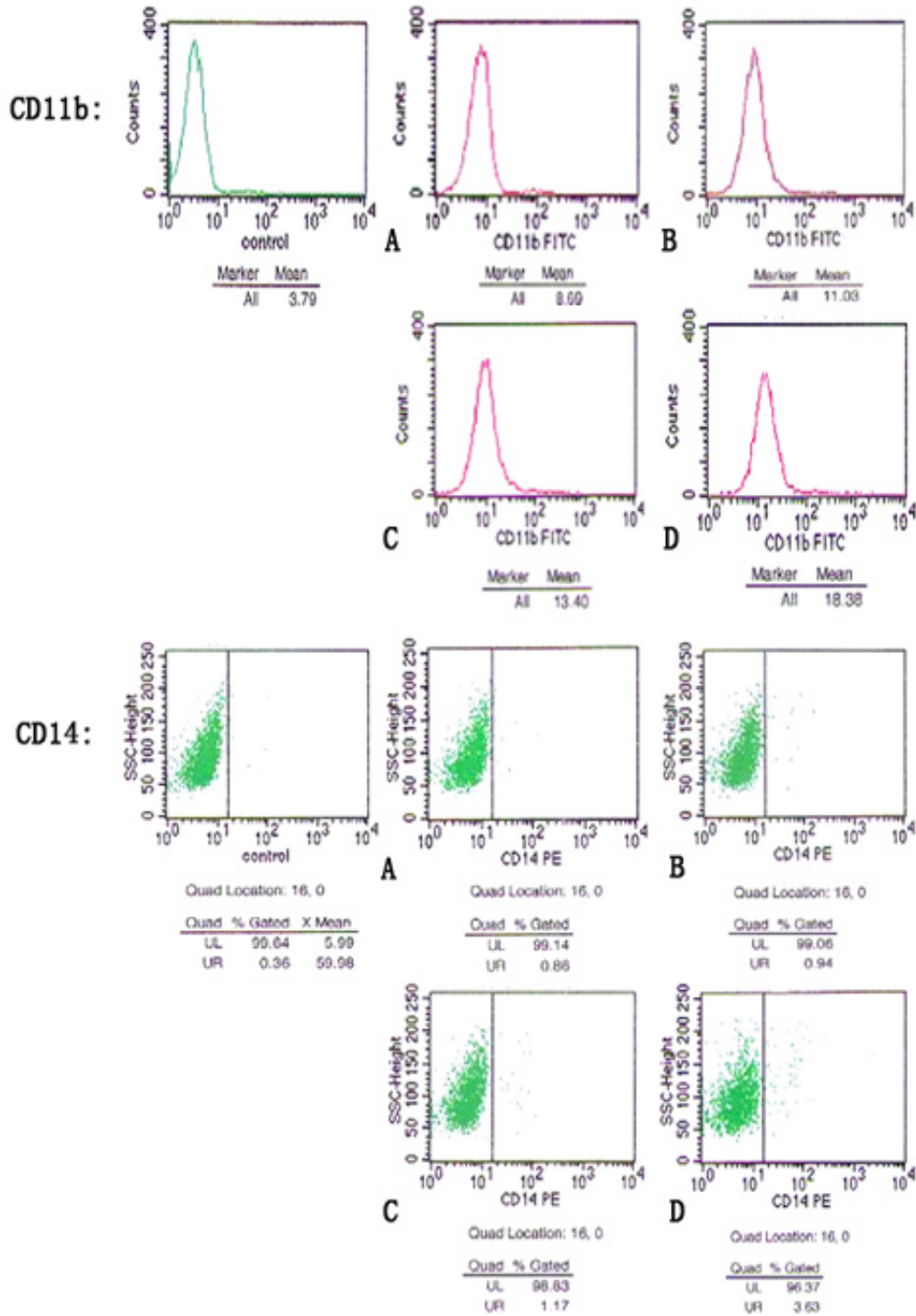


Figure 5. Expression of the CD11b granulocytic mature cell surface marker and CD14 monocyte surface marker determined with fluorescent-labeled antibodies. Cells were cultured without treatment (A, control); B, 300 µg/mL TMP; C, 0.5 µM As₂O₃, and D, 300 µg/mL TMP + 0.5 µM As₂O₃ for 72 h.

growth inhibition. Among the CDKs that regulate the cell cycle, CDK2 is known to be activated in association with cyclin E1 during G1 progression and G1-S transition. Our study reveals that, although the mRNA expression of CDK2 and cyclin E1 did not change, there was a marked reduction in the protein levels of cyclin E1 and CDK2 after the combined treatment. This suggests that the p27^{KIP1} protein is binding to active cyclin E1 and CDK2, inhibiting their kinase activities.

C-myc promotes both apoptosis and differentiation in a transcriptional activity-dependent manner. The repression of c-myc has been demonstrated to be required for terminal differentiation of many cell types, including myeloid cells, and deregulated c-myc has been found to induce

growth arrest (22). Inhibition of c-myc/Max dimerization affects multiple cellular activities in acute myeloid leukemia cells and represents a potential antileukemic approach (23). Here, we establish that c-myc expression affects the differentiation pattern in PML-RAR α -negative HL-60 cells when induced by TMP, alone or in combination with As₂O₃. The results show that c-myc down-regulation is concomitant with down-regulation of cyclin E1 and up-regulation of p27^{KIP1}, as expected from previous reports (24). To further investigate the mechanism by which 0.5 μ M As₂O₃ can induce HL-60 cells to undergo partial differentiation without a statistically significant cell cycle arrest, it is important to observe the c-myc expression changes. Since c-myc protein expression decreased persistently, while cyclin E1 and

Table 2. Expression of CD11b and CD14 in HL-60 cells.

Drug concentration	Percent of CD11b expression	Percent of CD14 expression
Control group	2.246 \pm 0.092	0.503 \pm 0.065
TMP (300 μ g/mL)	3.123 \pm 0.110*	0.587 \pm 0.070
As ₂ O ₃ (0.5 μ M)	3.493 \pm 0.139*	0.857 \pm 0.042
TMP (300 μ g/mL + As ₂ O ₃ 0.5 μ M)	4.833 \pm 0.166*	1.986 \pm 0.387

The percentages of CD11b and CD14 expression are reported as means \pm SD for three independent experiments. TMP = tetramethylpyrazine; As₂O₃ = arsenic trioxide. *P < 0.01 compared to control cells (ANOVA).

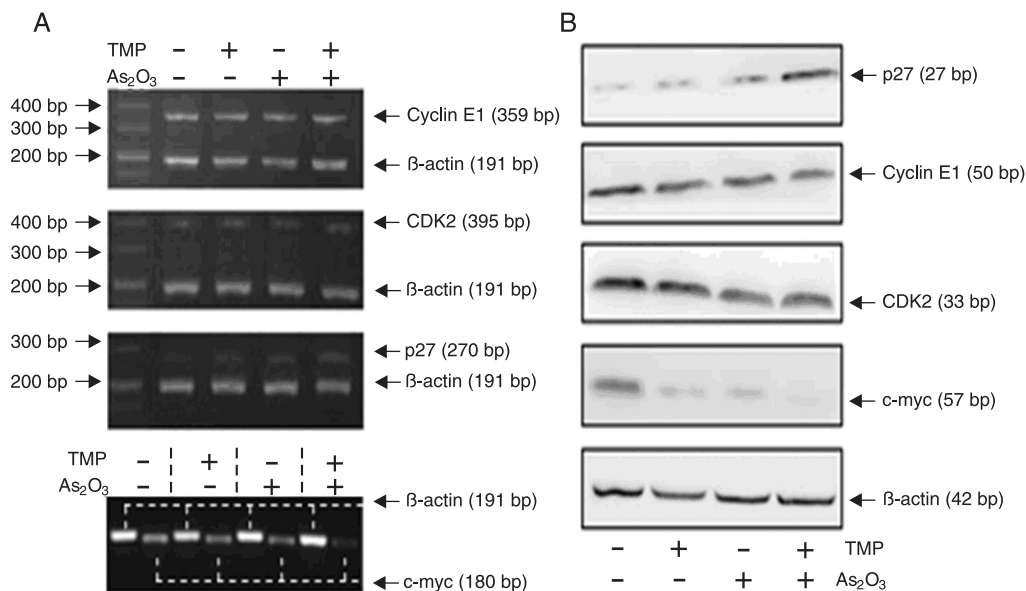


Figure 6. A, Expression of p27, c-myc, CDK2, and cyclin E1 mRNA determined by RT-PCR. Cells were cultured without treatment or treated with tetramethylpyrazine (TMP), arsenic trioxide (As₂O₃), or TMP combined with As₂O₃ for 72 h. B, Expression of p27, c-myc, CDK2, and cyclin E1 protein was determined using Western blotting.

CDK protein levels were slightly affected, we speculate that c-myc may be responsible for the partially blocked differentiation observed. The presence of decreased c-myc expression throughout the analysis confirms previous findings obtained with PML cells (25). The increase in p27^{KIP1} expression at this As₂O₃ concentration suggests that cells are preparing to induce cell cycle arrest. From these results, we suggest a model whereby TMP plus As₂O₃-induced G0/G1 arrest during terminal HL-60 cell differentiation is associated with up-regulation of p27, down-regulation of c-myc, and a slight down-regulation of CDK2 and cyclin E1.

Both efficacy and side effects need to be considered when evaluating a drug. As₂O₃ is a well-known chemical compound that, despite its high toxicity, has been used since the fifteenth century (26). Because As₂O₃ at higher concentrations induces many side effects and chronic arsenic exposure is linked to carcinogenesis, low-dose combination therapy is required (27,28). Together with increasing doses, As₂O₃ caused an increase in the number of cells undergoing apoptosis (29). In our study, a low-dose combination of two chemotherapeutic agents (300 µg/mL TMP plus 0.5 µM As₂O₃) markedly decreased cell viability and induced differentiation throughout the cell cycle, as detected by a slower rate of accumulation in G0/G1. In summary, our results indicate that As₂O₃ in combination with TMP is of potential chemotherapeutic use in PML-RAR α -negative leukemia.

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