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The synergistic antitumor activity of arsenic trioxide and vitamin K_2 in HL-60 cells involves increased ROS generation and regulation of the ROS-dependent MAPK signaling pathway

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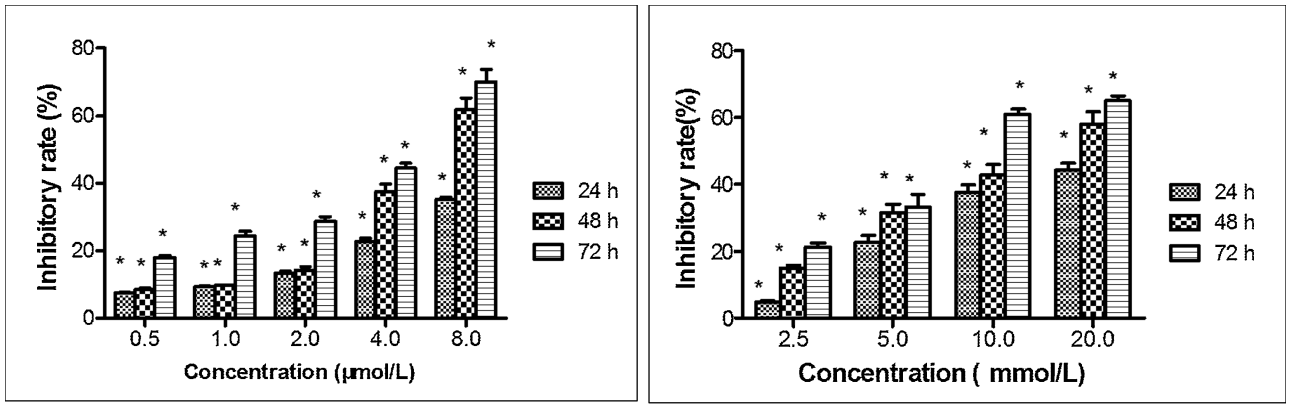
Objective: The aim of this study was to investigate the synergistic anticancer effects of arsenic trioxide (ATO) and vitamin K_2 (VK_2) in HL-60 cells, and elucidate the potential mechanisms. *Methods:* HL-60 cells were exposed to ATO and VK_2, either alone or in combination. Cell proliferation and apoptosis were assessed. The combination index (CI) method was used to evaluate whether the action of the drug combination was synergistic, additive or antagonistic. Reactive oxygen species (ROS) and the mitogen-activated protein kinase (MAPK) signaling pathway were also studied, to provide insight into potential mechanisms. *Results:* The results showed that combining ATO with VK_2 significantly inhibited HL-60 cell growth more than either agent alone, indicating a synergistic effect with $CI < 1$. Annexin V staining demonstrated that the inhibition of cell growth by the drug combination was mediated through an increase in apoptosis; this was supported by examination of caspase-3 and caspase-9 with Western blot assays. Furthermore, induction of ROS, and phosphorylation and activation of the JNK and p38 (but not ERK1/2) pathways, was observed in cells administered the drug combination. Prior treatment with the antioxidant, *N*-acetylcysteine, partly blocked the apoptosis and expression of caspase-3 induced by the drug combination; apoptosis and expression of caspase-3 were also reversed by inhibitors of JNK or p38. *Conclusion:* These results suggest that ATO and VK_2 act synergistically to increase HL-60 cell apoptosis, through ROS generation and regulation of the MAPK signaling pathway.

1. Introduction

Arsenic trioxide (As_2O_3, ATO) is an effective anticancer therapeutic agent that has been used successfully for many years in the treatment of acute promyelocytic leukemia (APL, a subset of acute myeloid leukemia, AML); this is due to the high rate of complete remission achieved with ATO, and a remarkable biological activity even in patients who have relapsed and are resistant to all-trans retinoic acid (ATRA) or conventional chemotherapy (Shen et al. 1997). Moreover, myelosuppression and other major clinical side effects do not seem to be as severe in ATO-treated patients as in those administered other forms of conventional cytotoxic chemotherapy. ATO has been approved for the treatment of newly-diagnosed and relapsed APL by the US Food and Drug Administration (FDA) (Soignet et al. 2001). Although ATO shows potent antitumor activity for the treatment of APL, resistance develops in some cases (Takahashi 2010). Non-APL hematologic or lymphoid malignancies and solid tumors may also be treated with ATO, but these are less sensitive to the drug and require higher concentrations (> 5 μmol/L) that are generally not clinically tolerable due to toxicity (Jing et al. 1999). Since ATO is an environmental toxin that is recognized as a human class 1 carcinogen, severe adverse effects and chronic toxicities remain serious obstacles that hamper the wider clinical use of ATO. These adverse effects include gastrointestinal disturbances, cardiac toxicity, carcinogenicity and potential genetic abnormalities, especially in children. However, attempts are being made to overcome these issues. Studies have shown that the adverse effects of, and sensitivity to, ATO being are closely related to the dosage used, with ATO more toxic at relatively high exposures (Ratnaike 2003). One possible approach to circumvent the problems of toxicity is to co-administer another compound that acts synergistically with ATO, thus allowing an increase in the efficacy of ATO and a reduction in the dose used. One of the mechanisms underlying the antitumor effect of ATO involves the triggering of tumor cell apoptosis through generation of intracellular reactive oxygen species (ROS) (Han et al. 2010; Miller et al. 2002). Studies have shown that the sensitivity of tumor cells to ATO, including those deemed relatively resistant to ATO, is closely related to the intracellular ROS concentration (Dai et al. 1999). ROS-inducing agents have been demonstrated to sensitize tumor cells and increase the efficacy of ATO, both *in vitro* and *in vivo* (Sturlan et al. 2003). Vitamin K (VK) has the potential to generate ROS that can exert a cytotoxic action in tumor cells (Chiou and Tzeng 2000). VK_2 (menaquinone) is one form of VK that has essential functions in blood coagulation and bone metabolism; in recent years, it has also attracted attention due to its anticancer properties. There is evidence that VK_2 can inhibit proliferative growth and angiogen-



(A) (B)

Fig. 1: Effects of ATO or VK_2, applied for 24, 48 or 72 h, on HL-60 cell growth. (A) Inhibition of HL-60 cell growth after treatment with the indicated concentrations of ATO. (B) Inhibition of HL-60 cell growth after treatment with the indicated concentrations of VK_2. Symbols and bars represent the mean and SE values, respectively. *$P < 0.01$ compared with the control.

esis, and induce apoptosis and differentiation in various cancer cells, including leukemia as well as solid tumors (Kawakita et al. 2009; Miyazawa et al. 2001). VK_2 is produced by naturally occurring bacteria in the intestines. Compared to other forms of VK (such as natural VK_1, and chemically-synthesized VK_3 [menadione] and VK_5), natural VK_2 is thought to be safe for long-term administration to humans without toxic effects (Sasaki et al. 2005). In view of its safety and efficacy, VK_2 has been used as an active agent for the treatment of osteoporosis (Shiraki et al. 2000) and myelodysplastic syndromes (MDS) (Miyazawa et al. 2000). More recently, (Vos et al. 2012) have suggested that VK_2 could be a possible treatment for Parkinson's disease and amyotrophic lateral sclerosis.

Recent studies have indicated that increases in ROS production are associated with the activation of mitogen-activated protein kinases (MAPK) (Boutros et al. 2008). The MAPK signaling pathway, stimulated by ROS, has multiple roles in the regulation of cell proliferation, differentiation and apoptosis (Blenis 1993). The MAPK superfamily includes three major subgroups: extracellular signal-regulated kinase (ERK), c-JUN NH_2-terminal kinase (JNK) and p38 MAPK; these play a key role in the cellular responses to extracellular stimuli (Genestra 2007).

Although the anticancer effects of ATO and VK_2 have been studied separately, and each agent has been used clinically for the treatment of different diseases, no studies have been done so far to investigate the combined action of these two agents. Based on previous reports, we were interested in determining the effects of a combination of ATO and VK_2 on HL-60 cells, an AML-derived cell line. Our aim was to characterize some of the potential mechanisms contributing to the cytotoxic effects of this drug combination, namely ROS production and the ROS-mediated MAPK signaling pathway. This is the first study to report the combined effects of ATO and VK_2 in a non-APL AML cell line, and evaluate the potential mechanism of action. Our investigation may prove to be of clinical benefit for the treatment of APL and other tumors that are resistant or less sensitive to ATO.

2. Investigations and results

2.1. Inhibition of cell growth by ATO or VK_2

We evaluated the inhibition of proliferative activity in HL-60 cells, after these were treated with ATO or VK_2 for 24, 48 or 72 h. The results showed that ATO inhibited the proliferation of HL-60 cells in a time- and concentration-dependent manner (Fig. 1A).

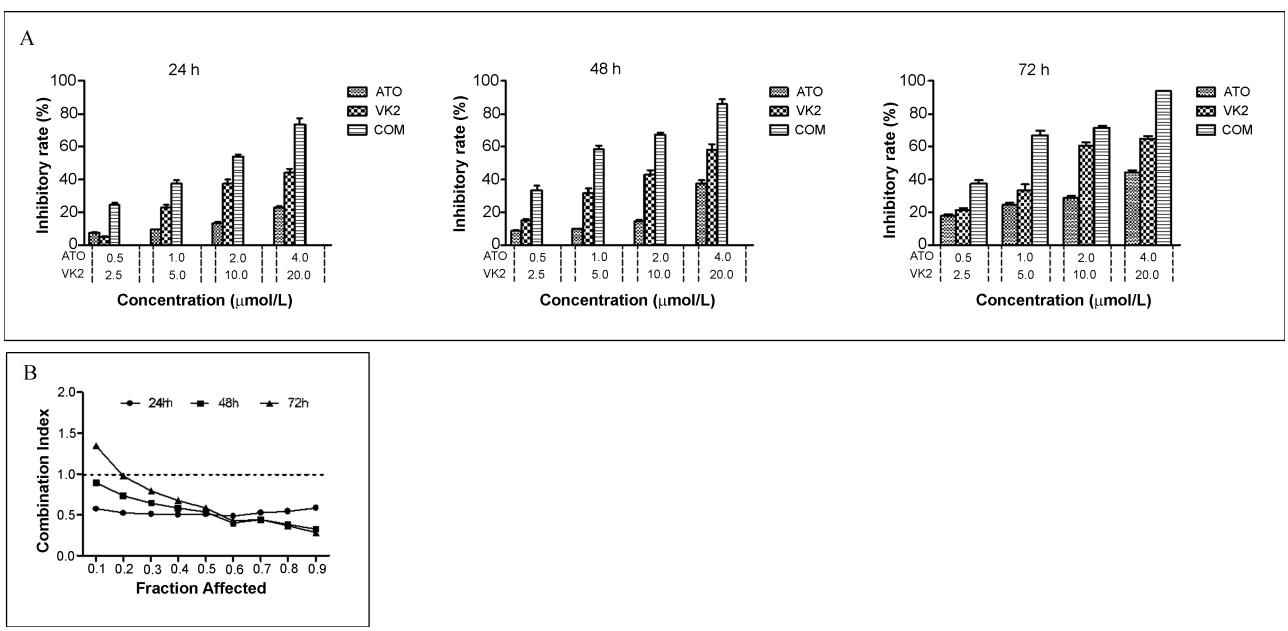


Fig. 2: The synergistic effects of ATO and VK_2, administered in combination. A. The anti-proliferative effects of ATO and VK_2, given alone or together. Symbols and bars represent the mean and SE values, respectively. B. The effects of a combination of ATO and VK_2. $CI > 1$, $CI = 1$ and $CI < 1$ indicate antagonism, an additive effect or synergism, respectively.

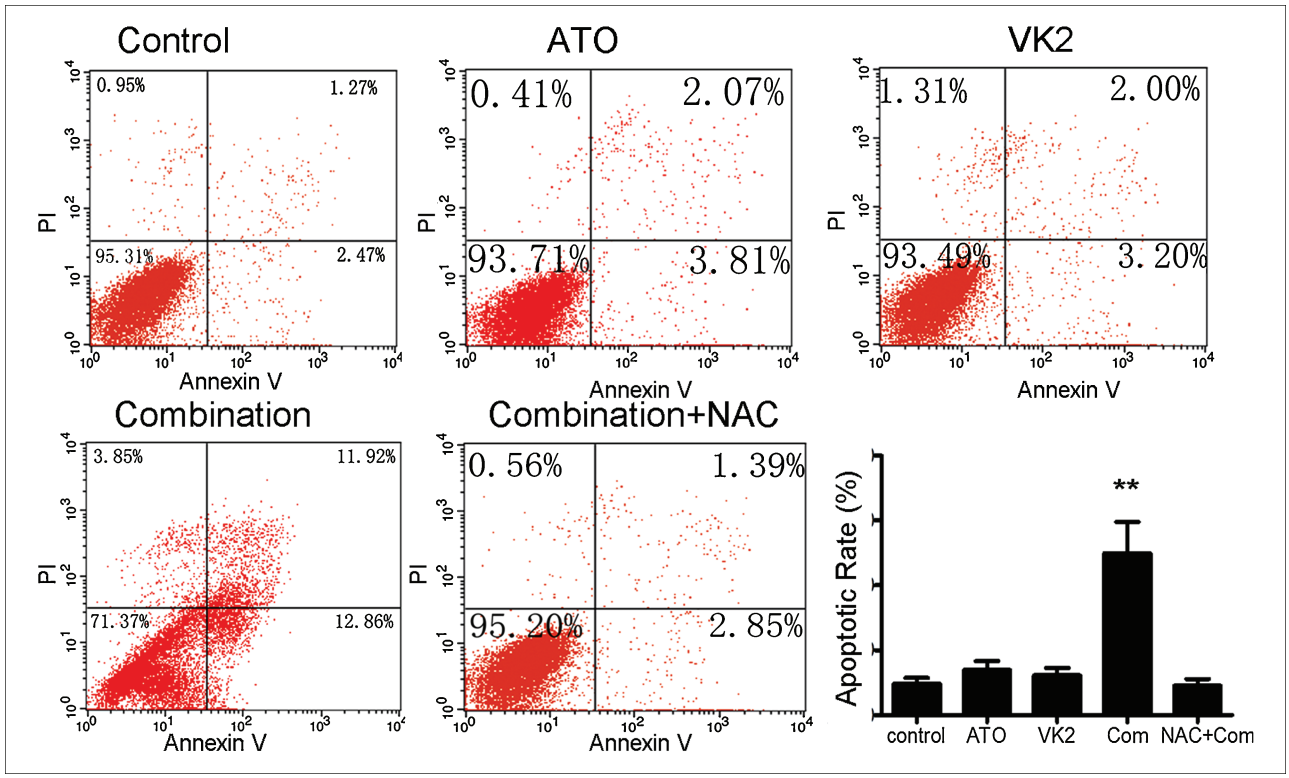


Fig. 3: Effects of ATO and VK_2, alone or in combination, on apoptosis of HL-60 cells. Cells were treated for 48 h with 1 μmol/L ATO and 2.5 μmol/L VK_2, either alone or in combination. Values are expressed as the mean ± SD of three independent experiments. *P < 0.01 compared with the control.

The IC_{50} values calculated for ATO were 22.86 ± 2.44 μmol/L, 6.66 ± 0.34 μmol/L and 4.14 ± 0.41 μmol/L, after 24, 48 and 72 h of exposure, respectively. Moreover, VK_2 also exhibited a time- and concentration-dependent effect on HL-60 cell growth (Fig. 1B). The IC_{50} values for VK_2 were 18.40 ± 1.12 μmol/L, 13.48 ± 0.73 μmol/L and 8.95 ± 0.40 μmol/L, after 24, 48 and 72 h of exposure, respectively.

2.2. *Synergistic cytotoxicity of ATO and VK_2*

To analyze the interaction between ATO and VK_2, HL-60 cells were treated with various concentrations of each agent alone or in combination, and cell viability assessed using the cell counting kit-8 (CCK-8) test (Fig. 2A). We found that the combination of the two agents exhibited superior cytotoxicity to each agent alone, indicating that VK_2 enhanced ATO-mediated cytotoxicity in HL-60 cells. The combination index (CI) was then calculated by the method of Chou and Talalay (1984). In Fig. 2B, the CI values have been plotted against the values of the affected fraction. As shown by the CI/Fraction affected (F_a) curve in Fig. 2B, the CI values at 24, 48 and 72 h, in HL-60 cells exposed simultaneously to the two agents at different concentrations, were slightly lower than 1, indicating that the inhibitory actions of the two drugs were synergistic. These data suggest that HL-60

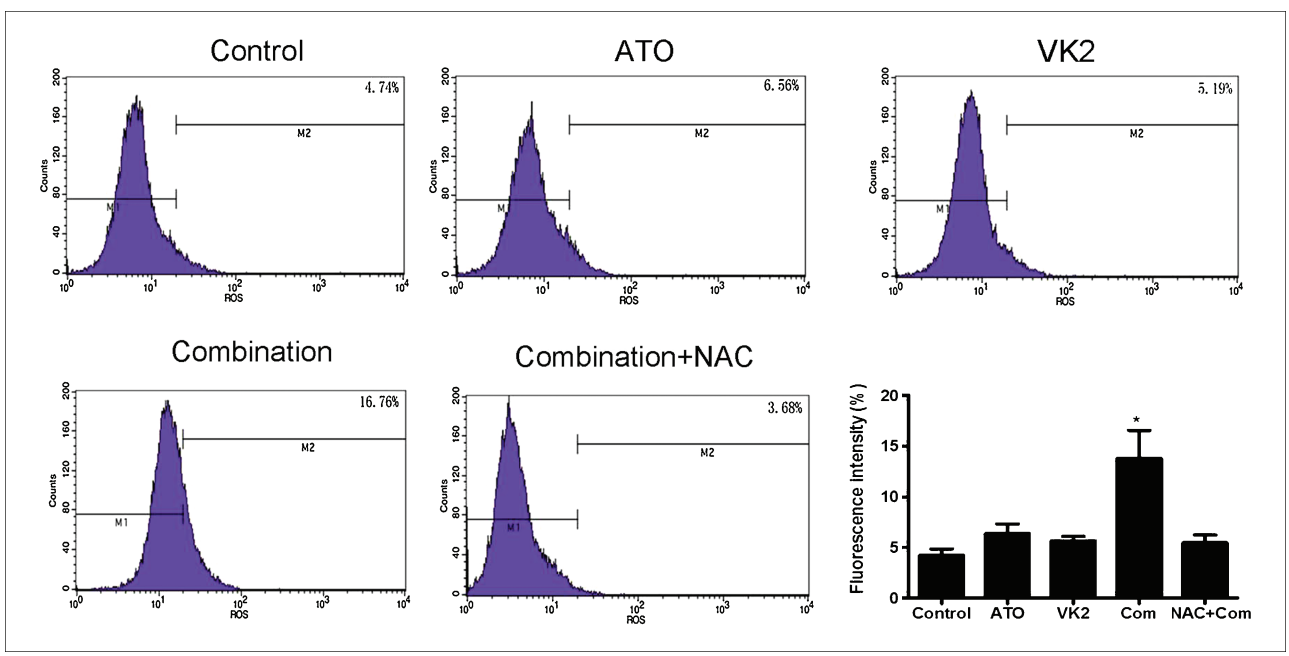


Fig. 4: Effects of ATO and VK_2, either alone or in combination, on the intracellular levels of ROS in HL-60 cells. The intracellular levels of ROS after treatment with 1 μmol/L ATO and 2.5 μmol/L VK_2, either alone or in combination. Values are expressed as the mean ± SD of three independent experiments. *P < 0.05 compared with the control.

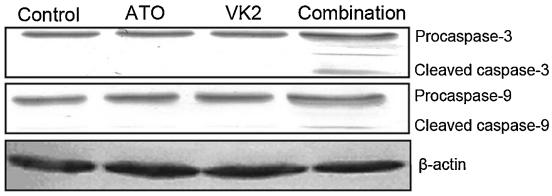


Fig. 5: Western blot analysis of caspase-3 and caspase-9 proteins. HL-60 cells were treated for 48 h with 1 μmol/L ATO, 2.5 μmol/L VK_2, or the combination of both, and then analyzed by Western blot. Expression of β-actin was used as an internal control.

cells were more sensitive to the combination of these two agents, and that this combination induced greater cytotoxicity.

2.3. Enhancement of apoptosis by combining ATO with VK_2

The annexin V-FITC apoptosis detection kit and flow cytometry were employed to evaluate whether the reduction in HL-60 cell proliferation caused by the combination of ATO and VK_2 was due to the induction of apoptosis. As shown in Fig. 3, neither 1 μmol/L ATO nor 2.5 μmol/L VK_2 caused significant apoptosis when these were given as single agents, but a combination of these drugs induced an increase in apoptosis (with both early- and late-stage apoptotic cells evident) to 24.95 ± 4.82 %, markedly higher than that seen with either agent alone ($P < 0.01$). Since ROS plays an important role in ATO-mediated apoptosis, the effects of the ATO and VK_2 combination were also assessed in cells pre-treated for 2 h with 5 mmol/L N-acetyl-L-cysteine (NAC), a well-known antioxidant. We observed that the apoptotic rate was significantly decreased when NAC was added before the drug combination, equaling that observed in the control or with use of either agent alone. This indicates that apoptosis induced by the ATO/VK_2 combination was inhibited by a ROS antagonist, implying that the combination of ATO plus VK_2 may act by increasing the intracellular ROS level.

2.4. Assessment of ROS levels

To understand the mechanism underlying the synergistic cytotoxicity of the two agents, the generation of ROS was measured using an oxidation-sensitive fluorescent probe, DCFH-DA, which is oxidized in the presence of ROS. After incubation with DCFH-DA for 30 min, cells were treated for 1 h with 1 μmol/L ATO, 2.5 μmol/L VK_2, or a combination of both. As shown in Fig. 4, when HL-60 cells were treated with ATO or VK_2 alone, the ROS level did not increase significantly when compared with the control. However, after co-application of these agents simultaneously to HL-60 cells, a significant elevation in the ROS level was observed, to above that seen for each agent alone ($P < 0.05$). When HL-60 cells were pre-treated with 5 mmol/L NAC for 2 h, ROS generation in response to a combination of ATO and VK_2 was significantly decreased. These data demonstrate that the combination of ATO and VK_2 significantly increased ROS production in HL-60 cells; this, in turn, may promote mitochondrial dysfunction and trigger mitochondrial-mediated apoptosis.

2.5. Caspase-3 and caspase-9 are activated by the combination of ATO with VK_2

The caspase cascade is a critical step in the ROS-mediated mitochondrial apoptosis signaling pathway. To evaluate whether combined treatment of HL-60 cells with ATO and VK_2 was associated with caspase activation, we examined caspase-3 and caspase-9. As shown in Fig. 5, caspase-3 and caspase-9 were cleaved into their active fragments after HL-60 cells were treated for 48 h with a combination of 1 μmol/L ATO and 2.5 μmol/L VK_2. In contrast, no activation of caspase-3 and caspase-9 was observed when the cells were treated with ATO or VK_2 alone. These findings suggest that the intrinsic pathway was involved in mediating the apoptosis induced by the co-administration of ATO and VK_2.

2.6. The combination of ATO and VK_2 affects the MAPK signaling pathway

To further explore the actions of ATO and VK_2, we examined the MAPK signaling pathway. As shown in Fig. 6A, when given alone, neither 1 μmol/L ATO nor 2.5 μmol/L VK_2 were able to influence JNK or p38 phosphorylation. In contrast, a significant increase in the phosphorylation of both JNK and p38 were observed in response to a combination of ATO and VK_2 at low concentrations. However, ERK1/2 was not affected after treatment of cells with the drug combination. To further analyze the involvement of ROS and the MAPK signaling pathway in the pro-apoptotic action of the drug combination, we utilized NAC and inhibitors of JNK (SP600125) and p38 (SB203580). As shown in Fig. 6B, when cells were pretreated for 2 h with NAC (5 mmol/L), SP600125 (1 μmol/L) or SB203580 (5 μmol/L), the expressions of p-JNK and p-p38 were reduced, and the cleavage of caspase-3 was decreased. These findings indicate that ROS, and activation of JNK and p38, are involved in mediating the synergistic antitumor effects of an ATO and VK_2 combination.

3. Discussion

The antitumor effects of ATO have been studied for many years, with regard to the treatment of not only APL, but also other hematopoietic and lymphoid malignancies, and solid tumors. The main aims of these previous studies have been to diminish the dosage, reduce the toxicity and improve the anticancer activity of ATO, as well as enhance the sensitivity of tumor cells and delay drug resistance to ATO. To achieve these aims, many agents have been studied for use in combination with ATO; appropriate drug combinations are known to have greater efficacy, and have been widely used in the treatment of cancer.
In our study, we investigated whether use of a combination of ATO with VK_2, at low concentrations, resulted in a synergistic antitumor effect on HL-60 cells. This AML-M_2 cell line has been shown previously to be resistant or less sensitive to clinically achievable concentrations of ATO (< 2 μmol/L), as a consequence of its well-established intracellular antioxidant defense mechanisms (Dai et al. 1999). We specifically did not select NB4 cells for this study as this APL-derived cell line is known to be sensitive to lower concentrations of ATO, which causes generation of sufficient amounts of ROS to induce apoptosis. Our study is therefore the first to evaluate the antitumor effects of a combination of ATO and VK_2 in a non-APL cell line.
In this study, we found that treatment of HL-60 cells with low concentrations of ATO or VK_2 resulted in cytotoxic actions, but that a more significant cytotoxic effect was evoked by simultaneous administration of both agents. The induction of apoptosis is a major mechanism by which anticancer drugs exert their actions. The apoptotic signaling pathways are generally divided into two types: the extrinsic pathway and the intrinsic pathway (Elmore 2007). The extrinsic, or death receptor, pathway involves cell surface death receptors, such as tumor necrosis factor or Fas: upon binding of their ligands, these receptors initiate a signaling cascade that activates caspase-8, which in turn

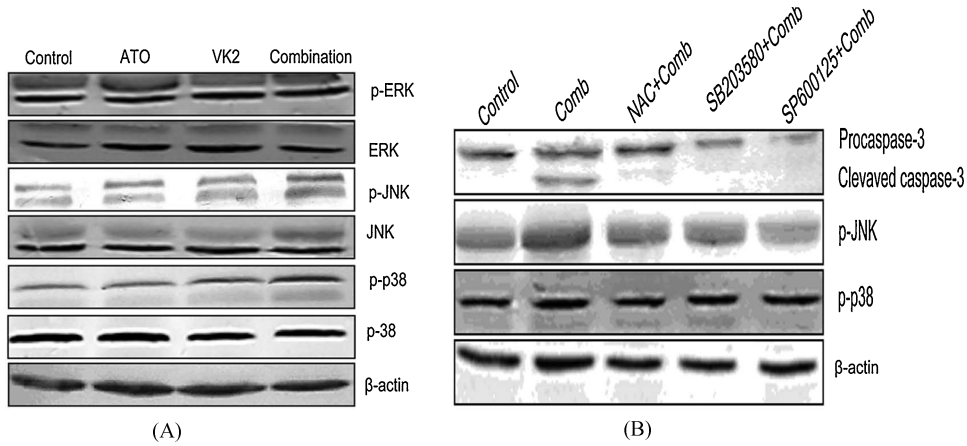


Fig. 6: Western blot analysis of the MAPK pathway in HL-60 cells. A. Involvement of MAPK members in HL-60 cells, in response to 1 μmol/L ATO, 2.5 μmol/L VK_2, or the combination. B. Effects of the combination of 1 μmol/L ATO and 2.5 μmol/L VK_2 on p-JNK, p-p38 and caspase-3, after pretreatment with NAC, SP600125 or SB203580. Expression of β-actin was used as an internal control.

directly cleaves caspase-3 to induce apoptosis. The intrinsic, or mitochondrial, pathway involves mitochondrial changes that trigger the release of cytochrome c, leading to the activation of caspase-9 and then caspase-3 (Kaufmann and Hengartner 2001). Irrespective of whether the extrinsic or intrinsic pathway is involved, the caspase cascade and caspase-3 play a key role in triggering apoptotic cell death, and may be activated by many different stimuli. In this study, the co-administration of ATO and VK_2 was found to result in cleavage and activation of caspase-3. Furthermore, by evaluating the expression of the intrinsic apoptotic protein, caspase-9, we found that caspase-9 was also activated by combined treatment with ATO and VK_2. Interestingly, use of either agent alone did not significantly alter the expression of caspase-3 and caspase-9. Taken together, these data suggest that the combination of ATO and VK_2 induced HL-60 cell apoptosis by activation of the intrinsic apoptotic pathway. Such a synergism between ATO and VK_2 would be predicted to result in an enhanced therapeutic benefit.

Previous studies have indicated that ROS production and ROS-dependent MAPK mediate the cytotoxic effects of ATO (Sanchez et al. 2009), including when ATO is given in combination with other interventions (Ho et al. 2011). In order to investigate the potential mechanisms underlying the synergistic anticancer effects of the two agents, we measured the ROS concentration. Generation of ROS may mediate the pro-apoptotic actions of both agents, such that increasing the intracellular ROS level would sensitize the tumor cells to ATO-induced apoptosis (Yi et al. 2002). In our experiment, treatment of HL-60 cells with a combination of ATO and VK_2 was associated with an increase in intracellular ROS concentration, whereas addition of each agent alone (at low concentrations) only resulted in low levels of ROS that were indistinguishable from that measured in untreated cells. To obtain further insight into the potential involvement of oxidative stress in the cytotoxic actions of the drug combination, we determined the effects of NAC: we found that NAC reversed the effects of the drug combination on both the ROS level and induction of apoptosis, indicating that ROS plays a crucial role in the synergistic effect of ATO and VK_2. ROS may lead to disruption of the mitochondrial membrane potential, release of cytochrome c with consecutive activation of the caspase cascade, and ultimately to programmed cell death through apoptosis (Jing et al. 1999). ATO-resistance at lower concentrations of the drug may be due to the generation of ROS in insufficient amounts to induce apoptosis; the addition of VK_2 may increase ROS levels and thereby impair the mitochondrial membrane potential, resulting in a higher sensitivity of cells to ATO.

Although the cell signaling pathways of ATO and other anticancer drugs have been extensively studied, there remains debate about the precise mechanisms. With regard to pro-apoptotic mechanisms that are 'upstream' of the caspase cascade, the ROS-dependent MAPK signaling pathway has been shown to play a key role in the response to stress or extracellular stimuli (Herskowitz 1995). The MAPK superfamily includes three major subgroups, ERK1/2, JNK and p38 MAPK, which are involved in cell proliferation, differentiation and apoptosis. Some studies have demonstrated that activation of JNK and p38 is involved in the apoptotic effect of ATO (Davison et al. 2004; Kang and Lee 2008). In contrast, others have reported that inhibition of the JNK or/and p38 pathway can increase ATO-induced apoptosis in some cancer cells (Huang et al. 2010; Wen et al. 2008). Two studies (Jin et al. 2006; Park et al. 2008) have reported that a combination of ATO and sulindac induces generation of ROS and activation of JNK that contribute significantly to cell killing in lung cancer. However, others (Stepnik et al. 2012) have found that ROS and the MAPK signaling pathway are not the major mechanisms responsible for the cytotoxicity of this drug combination in leukemia cells. It has also been reported that the co-administration of ATO and ATRA can activate JNK and increase apoptosis in APL cells (Mathieu and Besancon 2006). Our current study demonstrated that low concentrations of ATO (1 μmol/L) or VK_2 (2.5 μmol/L),when used individually, did not alter the level of MAPK family proteins. However, the combination (at low concentrations) resulted in activation of p-JNK and p-p38, but not p-ERK1/2; furthermore, inhibitors of JNK and p38 reversed the activation of p-JNK, p-p38 and caspase-3. These data suggest that the activation of JNK and p38 is necessary for the apoptosis induced by the drug combination. ATO has occasionally been observed to down-regulate ERK1/2 in some leukemia cell models (Redondo-Munoz et al. 2010; Shim et al. 2002). In addition, inactivation of ERK1/2 and activation p38 have been reported to be associated with apoptotic effects of ATO on U937 leukemia cells (Iwama et al. 2001). Activated ERK seems to have an important role in the monocytic differentiation of HL-60 cells induced by vitamin D3 (Wang and Studzinski 2001). ATO, either alone or together with vitamin D_3, has been shown to activate ERK1/2 in HL-60 cells within one day, and this activation of ERK1/2 was considered to be related to cell proliferation (Kumagai et al. 2005). Furthermore, the combination of vitamin D_3 and ATO has been demonstrated to potently induce differentiation and reduce apoptosis of myeloid leukemia cells, with these effects associated with ERK1/2 activation and decreased ROS levels (Kumagai et al. 2005; Mozdarani and Asghari 2010). We therefore pre-

sume that the combination of ATO and VK_2 resulted mainly in an induction of apoptosis in HL-60 cells, rather than differentiation. The activation or inhibition of cell signaling pathways is dependent on many factors, including the stimulus (the compounds/anticancer drugs used and their concentrations), the time of exposure and the origin of the tumor cell lines. In addition, other signaling pathways may contribute to the synergistic effects.

In conclusion, the combination of ATO and VK_2 can produce a synergistic effect to cause apoptosis of HL-60 cells through generation of ROS and activation of the JNK and p38 MAPK pathway. VK_2 might be an effective agent to enhance ATO-mediated apoptosis in AML, other than APL, that is less sensitive to the drug. Further studies are required to explore other tumor cell lines and elucidate the additional mechanisms responsible for the synergism. Our study may provide a promising therapeutic strategy to reduce the dosage and increase the efficacy and safety of ATO for treating AML and other tumors, thereby extending the clinical spectrum of its therapeutic use.

4. Experimental

4.1. Reagents and antibodies

ATO was obtained from the Pharmaceuticals Limited Company of Harbin Medical University (Heilongjiang, China). VK_2 was purchased from Sigma-Aldrich (St. Louis, MO, USA). The cell counting kit-8 (CCK-8 kit), N-acetylcysteine (NAC), reactive oxygen species (ROS) assay kit, SDS lysis buffer, phenylmethanesulfonyl fluoride (PMSF), HRP-labeled secondary antibody, JNK inhibitor (SP600125), p38 inhibitor (SB203580), bicinchoninic acid (BCA) protein assay kit and BeyoECL Plus were all purchased from Beyotime (Jiangsu, China). The annexin V-FITC apoptosis detection kit was sourced from BD Biosciences (San Jose, CA, USA). Rabbit caspase-3, rabbit casepase-9, antibodies against MAPK proteins (anti-ERK1/2, anti-phospho-ERK1/2, anti-JNK, anti-phospho-JNK, anti-p38 MAPK and anti-phospho-p38 MAPK) and β-actin were all obtained from Cell Signaling Technology (Beverly, MA, USA).

4.2. Cell culture and treatment

The human AML cell line, HL-60, was obtained from the Cell Bank, Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). HL-60 cells were cultured and maintained in RPMI-1640 medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich). Cells were kept in a humidified, 5% CO_2-air incubator at 37 °C. The cells were passaged two or three times per week to maintain them in an exponential growth state. Before use, working concentrations of ATO were made by serial dilution of the stock solution in RPMI-1640. VK_2 was dissolved in 99.9% ethanol at a stock concentration of 100 mmol/L, and then diluted with medium to the appropriate working concentration before use.

4.3. Cytotoxicity assays

Cell viability was assessed using the CCK-8 proliferation assay kit. HL-60 cells were plated at a concentration of 1×10^5 cells/mL in 96-well plates, and then treated for 24, 48 or 72 h with ATO (0.5, 1, 2, 4 or 8 μmol/L) or/and VK_2 (2.5, 5, 10 or 20 μmol/L), *i.e.*, alone or in combination. The control group was treated with RPMI-1640 medium alone. 10 μL CCK-8 reagent was added to each well, and the cells were incubated at 37 °C for the last 4 h of culture. The optical density (OD) was then measured at a wavelength of 450 nm, using a microplate reader (Thermo Molecular Device Co., Union City, NJ, USA). In each sample, three parallel experimental groups were used to assess cell viability. All experiments were performed in triplicate, independently. The cell inhibitory ratio was calculated by the following formula: Inhi bitory ratio (%) = $[(OD_{Control}\text{-}OD_{Treated})/(OD_{Control})] \times 100\%$.

4.4. Evaluation of the effect of the drug combination

Before testing the combined effect of the two agents, the individual IC_{50} values of ATO or VK_2, against the viability of HL-60 cells, were determined by constructing a concentration-response curve using the CCK-8 assay, with the viability of the control group described as 100%. IC_{50} values were calculated by determining the concentration of drug needed to inhibit half of the viability of cells, using linear regression analysis. To analyze the effect of the drug combination, the combination index (CI) was calculated according to the median-effect method of Chou and Talalay (1984). Values of CI > 1, CI = 1 and CI < 1 were taken to indicate antagonism, an additive effect and synergism, respectively.

4.5. Assessment of apoptosis

The % apoptosis was measured using an annexin V-FITC apoptosis detection kit. HL-60 cells (1×10^6 cells/mL) were plated in 6-well flat-bottomed plates. Cells were treated for 48 h with 1 μmol/L ATO (IC_{15}), 2.5 μmol/L VK_2 (IC_{20}), or the combination. Untreated cells were used as controls. Cells were then harvested, washed twice with cold phosphate buffered saline (PBS; Gibco, USA), gently re-suspended in 100 μL annexin V binding buffer, and incubated with 5 μL fluorescein isothiocyanate (FITC)-conjugated annexin-V and 5 μL propidium iodide (PI), at room temperature for 15 min in the dark. Binding buffer 400 μL was then added to each tube, and the cells were analyzed by flow cytometry (FCM, FACScan; Becton Dickinson, San Diego, CA, USA). Cells staining negative for FITC-annexin V and PI were considered alive. Cells staining positive for FITC-annexin V and negative for PI were deemed to be in early apoptosis, while cells staining positively for both FITC-annexin V and PI were considered to be post-apoptotic or necrotic cells.

4.6. Analysis of intracellular ROS production

The intracellular ROS level was measured by means of a ROS assay kit with an oxidation-sensitive fluorescent probe, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), as described by Zegura et al. (2004). HL-60 cells were incubated with 20 μL DCFH-DA for 30 min at 37 °C, before being treated for 60 min with 1 μmol/L ATO and 2.5 μmol/L VK_2, either alone or in combination. The cells were collected, washed twice with PBS and then analyzed using flow cytometry.

4.7. Western blot analysis

After 48 h of treatment with 1 μmol/L ATO (IC_{15}), 2.5 μmol/L VK_2 (IC_{20}) or the combination, all the HL-60 cells were harvested, washed twice with cold PBS and then lysed in lysis buffer containing: 20 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl; 1% Triton X-100; 2.5 mmol/L sodium pyrophosphate; 1 mmol/L EDTA; 1% Na_2CO_3; 0.5 μg/mL leupeptin; 1 mmol/L PMSF. The lysates were collected and centrifuged at 12,000 rpm for 15 min at 4 °C. Before immunoblotting, the protein concentrations were determined with a BCA detection kit. Equal amounts of protein (30 μg/lane) from each extract were resolved using 10% SDS-PAGE, and the proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) transfer membrane (Millipore; Billerical, MA, USA). The membranes were blocked with 5% nonfat dry milk in TBST (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% Tween-20) for 2 h at room temperature, and then incubated with primary antibodies overnight at 4 °C. The membranes were washed three times with TBST for 15 min each, and subsequently incubated with secondary antibody diluted to 1:1000. The expression of β-actin was used as an internal control for equal protein loading. Bands were recorded with an ECL kit.

4.8. Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Differences between groups were assessed by ANOVA using Student's t-test analyses. A value of $P < 0.05$ was considered statistically significance.

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