

Low dose of arsenic trioxide inhibits multidrug resistant-related P-glycoprotein expression in human neuroblastoma cell line

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Abstract. This study investigated arsenic trioxide (As₂O₃), cisplatin (DDP) and etoposide (Vp16) on the anticancer effects and P-glycoprotein (P-gp) expression in neuroblastoma (NB) SK-N-SH cells. The potential influence of As₂O₃, DDP and Vp16 currently included in NB routine treatment protocols on cytotoxicity in SK-N-SH cells was measured by flow cytometry and drug half-maximal inhibitory concentration (IC₅₀) was established. Moreover, chemotherapeutic agent-mediated changes of cellular expression levels of resistant-related P-gp, was monitored using western blotting. The data showed that As₂O₃, DDP and Vp16 significantly inhibited the growth and survival of the SK-N-SH cells at different concentration. Notably, the levels of apoptosis were upregulated in SK-N-SH cells with an acceleration of the exposure time and the concentration of As₂O₃, DDP and Vp16. As₂O₃, DDP and Vp16 were observed with their IC₅₀ values on SK-N-SH cells being 3 μM, 8 and 100 μg/ml, respectively. Flow cytometry analysis showed that As₂O₃ at low concentrations in SK-N-SH cells led to enhanced accumulation of cell populations in G2/M phase with increasing the exposure time, and increased levels of apoptosis. In contrast, we observed that SK-N-SH cell populations arrested in S phase by DDP and Vp16. *In vitro* examination revealed that following pretreatment of SK-N-SH cells with As₂O₃, the expression of P-gp was not increased. The expression of P-gp downregulation were noted following the group treated by As₂O₃ at 2 and 3 μM. Exposed to As₂O₃ at 3 μM for 72 h, SK-N-SH cells exhibited lower expression of P-gp than 2 μM As₂O₃ for 72 h. In contrast, the expression of P-gp was upregulated by DDP and VP16. In summary, SK-N-SH cells were responsive to chemotherapeutic

agent-induced apoptosis in a dose-dependent and time-dependent manner. In particular, our findings showed that low dose of As₂O₃ markedly reduced the P-gp expression and increased apoptotic cell death in human NB cell line.

Introduction

Neuroblastoma (NB) is the most commonly occurring extra-cranial solid neoplasm diagnosed in young children that shows aggressive behaviour. NB is a childhood malignancy, which may originate from multipotent crest cells of the sympathetic nervous system and accounts for 8 to 10% of all childhood cancers, yet disproportionately responsible for 15% of all childhood cancer deaths (1,2). Approximately 40% of the children with NB have an aggressive form with a 5-year event-free survival rate <50% (3). Despite intensive treatment protocols including myeloablative cytotoxic therapy and 13-*cis*-retinoic acid (4) or anti-GD2 immunotherapy (5) that were applied, the majority of the children diagnosed with high-risk NB experience a recurrence and the long-term survival <40% (6,7). Although the initial tumor response is very good in children with high-risk NB, >60% of the treated patients experience relapse and still die of this disease (8,9). Currently, in the clinic relapsed/refractory patients still lack effective treatment strategies which has been recognized as a critical problem, and the poor outcome remains.

Initially, responsiveness to chemotherapy in NB tumors is high (10), but eventually, these patients often relapse and develop a multiple drug resistant (MDR) phenotype. Acquired MDR is a major contributing factor to chemotherapeutic treatment failure (11,12). MDR is a phenomenon in which tumor cells become resistant to diverse chemical structures and functionally unrelated chemotherapeutic agents (13). Once NB develops MDR, as a consequence, the long-term survival rate of NB patients is poor (11). Strategies to improve the outcomes in high-risk NB include 13-*cis*-retinoic acid, anti-GD2 immunotherapy and bone marrow transplantation. Still, the high-risk NB patients eventually develop progressive disease.

The mechanism of NB developing MDR is complicated and several factors are involved, including loss of function of p53 (14), overexpression of the membrane bound ATP-binding

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cassette transporters (ABC proteins) (13), inactivation of the detoxification system, changing drug targets and significant downregulation of apoptotic cell death-related genes (12). Especially, the most often observed the main mechanism for MDR includes loss of function of p53, high levels of P-glycoprotein (P-gp) and MRP1 (multidrug resistance-related protein). When overexpressed in neoplastic tissue, P-gp and MRP1 effectively extrude various cytotoxic agents and eliminate chemotherapeutics, may contribute to their refractoriness to large group of cytotoxic drugs, such as anthracycline, epipodophyllotoxin, vinca alkaloids, taxanes and methotrexate (15,16).

A number of conventional anticancer agents show cytotoxic effects by interfering with DNA or RNA replication and protein synthesis in cancer cells, as well as activation of the expression of p53 gene, but unfortunately, most of chemotherapy drugs can also increase gene mutations and change tumor suppressor gene expression. In some highly malignant tumor cells, apoptotic mechanisms of secondary damage due to chemotherapy, such as inactivation of p53 gene containing cysteine aspartic protease (caspase) fail, limiting their application. These include increased DNA repair, altered target sensitivity, decreased apoptotic response, and numerous aberrant signal transduction pathways. Commonly used chemotherapy drugs such as Dox and DDP induce tumor cells formed MDR in that they can increase the leukemia cells or NB the expression of P-gp. While increasing the dose of chemotherapeutic agents may successfully eliminate MDR to some extent, but high-dose chemotherapy can cause inevitable severe side-effects, such as heart, liver, kidney and hearing damage, thus patients can not tolerate and abandon treatment (17). This prompted us to investigate new treatment strategies against MDR NB caused by diverse mechanisms.

It is necessary to develop potential chemotherapeutic strategies to enhance chemosensitivity and to overcome the MDR. While in the last few years, arsenic was also seen as a carcinogen, but recent reports have indicated that arsenic trioxide (As_2O_3) has been found to inhibit cell growth and/or induce apoptosis in acute promyelocytic leukemia (APL) (18,19) and successfully employed as a highly effective agent for other malignancies. As_2O_3 has been associated with complicated anticancer mechanisms, including induction of tumor cell differentiation, inhibition of tumor cell growth and induce apoptosis (20), although the efficacy of As_2O_3 on different tumors remains unsure (21). In the APL cells, As_2O_3 exert antitumor effect by acting on the PML/RARA as target (22); in other tumor cells, the antitumor effect was associated with activation of caspase cascade leading to apoptotic cell death and reduced expression of Bcl-2 gene (23).

Many studies have confirmed that As_2O_3 has the capacity to kill NB cells *in vitro* and *in vivo* (24-27). Despite that invasive tumor in the body often is in a low oxygen environment, Karlsson *et al* (26) show that the cytotoxic effect of As_2O_3 is retained under hypoxic conditions, even in MDR NB cells. In contrast, the cell death induced by the conventionally used drug etoposide (Vp16) was significantly impaired in Vp16-sensitive NB cell lines cultivated at hypoxia. These results support the idea of using As_2O_3 as an efficient treatment strategy also in solid tumors with hypoxic areas like NB. Memorial Sloan-Kettering Cancer Center has completed phase II clinical trials of treatment of NB and other malignancies, preliminarily

showing that As_2O_3 treatment of MDR NB has better safety, the primary efficacy outcome are not yet available.

Some investigators have shown that As_2O_3 -induced NB apoptosis is caused by downregulation of Bcl-2 protein, activate caspase, the generation of reactive oxygen species, loss of the mitochondrial transmembrane potential, and cell cycle arrest in G1 or G2/M phase (23). Little is known about As_2O_3 impact on the expression of multidrug resistant-related P-gp in NB, however, further studies are required. We examined the effect of As_2O_3 and conventionally used drugs on P-glycoprotein by western blotting; flow cytometric assay was used to examine the impact of various antitumor drugs on cell cycle and cell apoptosis in SK-N-SH cells.

Overall, we compared and contrasted the effects of As_2O_3 and conventional chemotherapeutic drugs at varying concentrations on the expression of P-gp and the impact of cell cycle in NB SK-N-SH cells. In this study, our results indicated that compared to conventional chemotherapeutic drugs, As_2O_3 mediates this effect by inducing apoptosis and provides a suggestion that As_2O_3 is a potential chemotherapeutic reagent for the treatment of NB including MDR NB.

Materials and methods

Cell line. Human NB cell line, SK-N-SH, was purchased from Cell Bank of Sun-Yet Sen Medical School. SK-N-SH cells were grown in DMEM supplemented with 12% fetal calf serum (FCS), 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were maintained at 37°C in a humidified incubator of 5% $CO_2/95\%$.

Reagents. As_2O_3 , VP16 and DDP was purchased from Sun Yat-Sen Memorial Hospital. As_2O_3 was dissolved in phosphate-buffered saline as 10 mM stock and kept at 4°C. MDR1/ABCB1 (D3H1Q) rabbit mAb and anti-GAPDH antibody rabbit antibody was obtained from Cell Signaling Technology. HRP affini-pure goat anti-rabbit IgG for western blotting was purchased from Sigma Chemical Co. Immobilon western chemiluminescent HRP substrate was purchased from Millipore, and DMEM medium and trypsin were purchased from Hyclone. PVDF membrane was purchased from Millipore, and FBS was purchased from NBQQ.

Flow cytometry with Annexin V-PI staining for apoptosis. Logarithmically growing cells were exposed to various concentrations and exposure intervals of As_2O_3 , VP16 and DDP. For the experiments, cells were seeded in 6-well plates at a density of $\sim 3 \times 10^5$ cells per milliliter at 37°C in a humidified incubator of 5% for 24 h. For determination of cell growth inhibition, cells were exposed to varying concentrations of As_2O_3 , VP16 and DDP at 37°C for the indicated time. After treatment, the cells at $1-5 \times 10^5$ /ml harvested from culture and washed by PBS, and then resuspended in 200 μ l staining solution that contained 5 μ l of Annexin V-FITC and 10 μ l propidium iodide, according to the protocol of the Annexin V Staining kit. The proportion of cell apoptosis was analyzed by flow cytometry within one hour. The IC_{50} value resulting from 50% inhibition of cell growth was calculated graphically as a comparison with the control. All treatments were done in triplicates and the experiments were repeated three times.

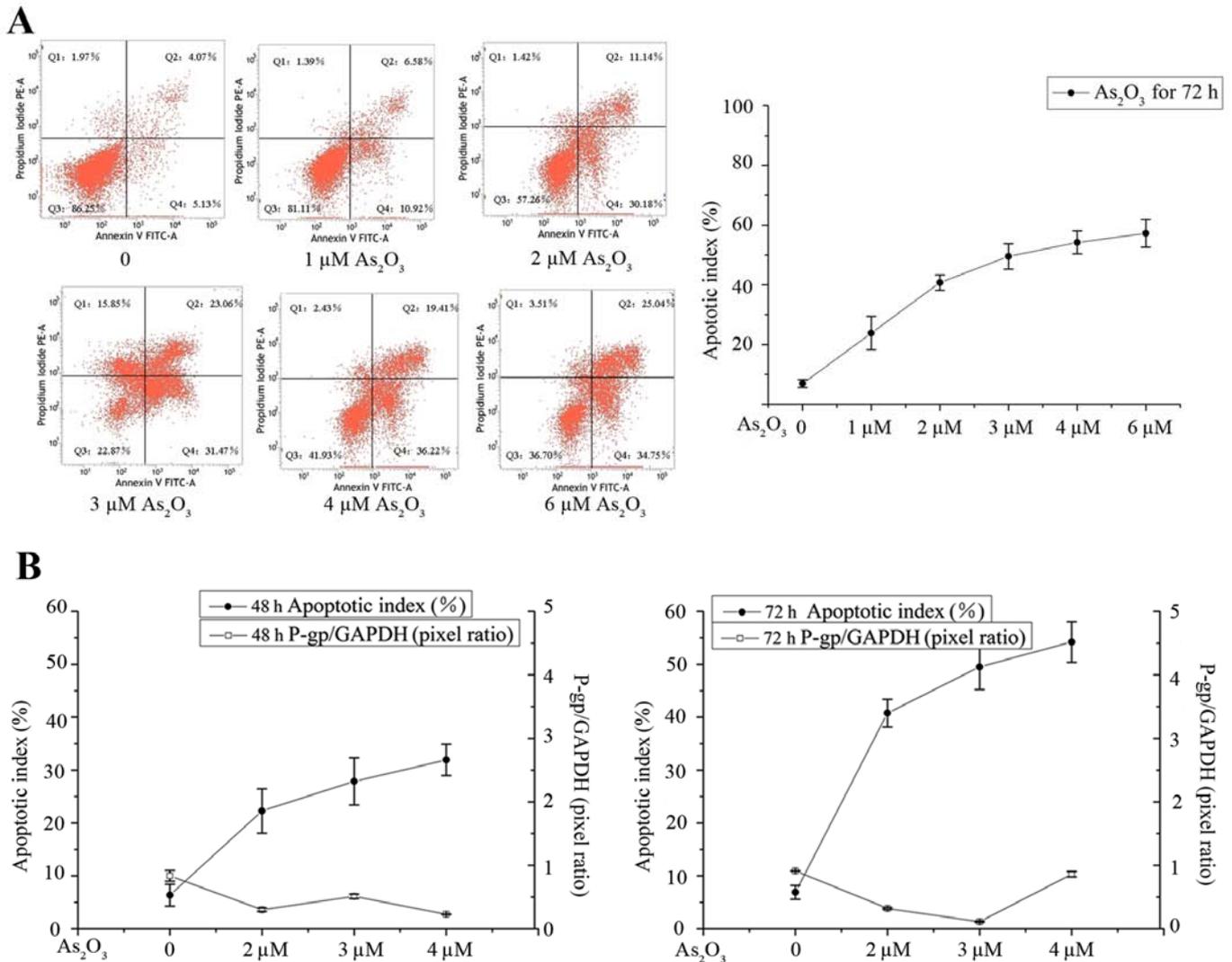


Figure 1. Different cytotoxic agents affect specific apoptosis of SK-N-SH cells and expression levels of P-gp. (A) Cytotoxicity of As₂O₃ after various time induction at given concentrations in SK-N-SH cells. After 72-h exposure to various concentrations of As₂O₃, cell viability was assayed by flow cytometry. Dose-response curves of SK-N-SH cells upon As₂O₃ treatment. The As₂O₃ IC₅₀ of SK-N-SH cells was 3 μM. (B) Cells with decreased expression levels of P-gp show increased sensitivity to As₂O₃ induced apoptosis. Cells were exposed to the indicated concentrations of As₂O₃ for 48 and 72 h and exhibit decreased expression levels of P-gp. All data are presented as the mean (± SEM) from at least 3 independent experiments.

Flow cytometry for cell cycle. Logarithmically growing cells were exposed to various concentrations and exposure intervals of As₂O₃, VP16 and DDP. For the experiments, cells were seeded in 6-well plates at a density of approximately 3x10⁵ cells per milliliter at 37°C in a humidified incubator of 5% for 24 h. IC₅₀ of As₂O₃ was added to the experimental group. According to the literature (28) and our result of IC₅₀ of As₂O₃ on SK-N-SH cells, we used 3 μM As₂O₃-treated SK-N-SH cells for 48 and 72 h. IC₅₀ of DDP and Vp16 were added to the drug control groups. According to our prior research, we chose 8 μg/ml DDP-treated cells for 12 and 24 h, 100 μg/ml Vp16-treated cells for 24 and 48 h. Each group was set in three parallel wells. Untreated or drug-treated cells were centrifuged, washed with PBS and fixed with ice-cold 75% ethanol overnight. Then, the cells were centrifuged and the cell pellet was resuspended in 50 μl of PBS. The fixed cells were incubated with 50 μl of RNase for 30 min in 37°C water and 450 μl of propidium iodide incubated for 30 min in the dark. The percentage of cells at a particular phase was calculated by flow cytometry.

Western blot analysis was performed to investigate the expression of P-gp. Logarithmically growing cells were exposed to various concentrations and exposure intervals of As₂O₃, VP16 and DDP. For the experiments, cells were seeded in 6-well plates at a density of ~3x10⁵ cells per milliliter at 37°C in a humidified incubator of 5% for 24 h. The concentrations were based on our results of IC₅₀ of As₂O₃, DDP and Vp16 (the concentration gradients based on the results of preliminary experiments). Each group was set in three parallel wells. As₂O₃ 2, 3 and 4 μM (incubation 48, 72 and 96 h); DDP 5, 8 and 10 μg/ml (incubation 12 and 24 h); Vp16 50, 100 and 150 μg/ml (incubation 24 and 48 h). After treatment with appropriate drug concentration, protein lysate was prepared in lysis buffer and the protein concentration was determined by bicinchonic acid (BCA) protein assay. Equal amount of proteins were loaded on 8% SDS-polyacrylamide gel. Voltage 80 V was prepared for the stacking gel; 100 V was prepared for separating gel in the end. The proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane by semidry

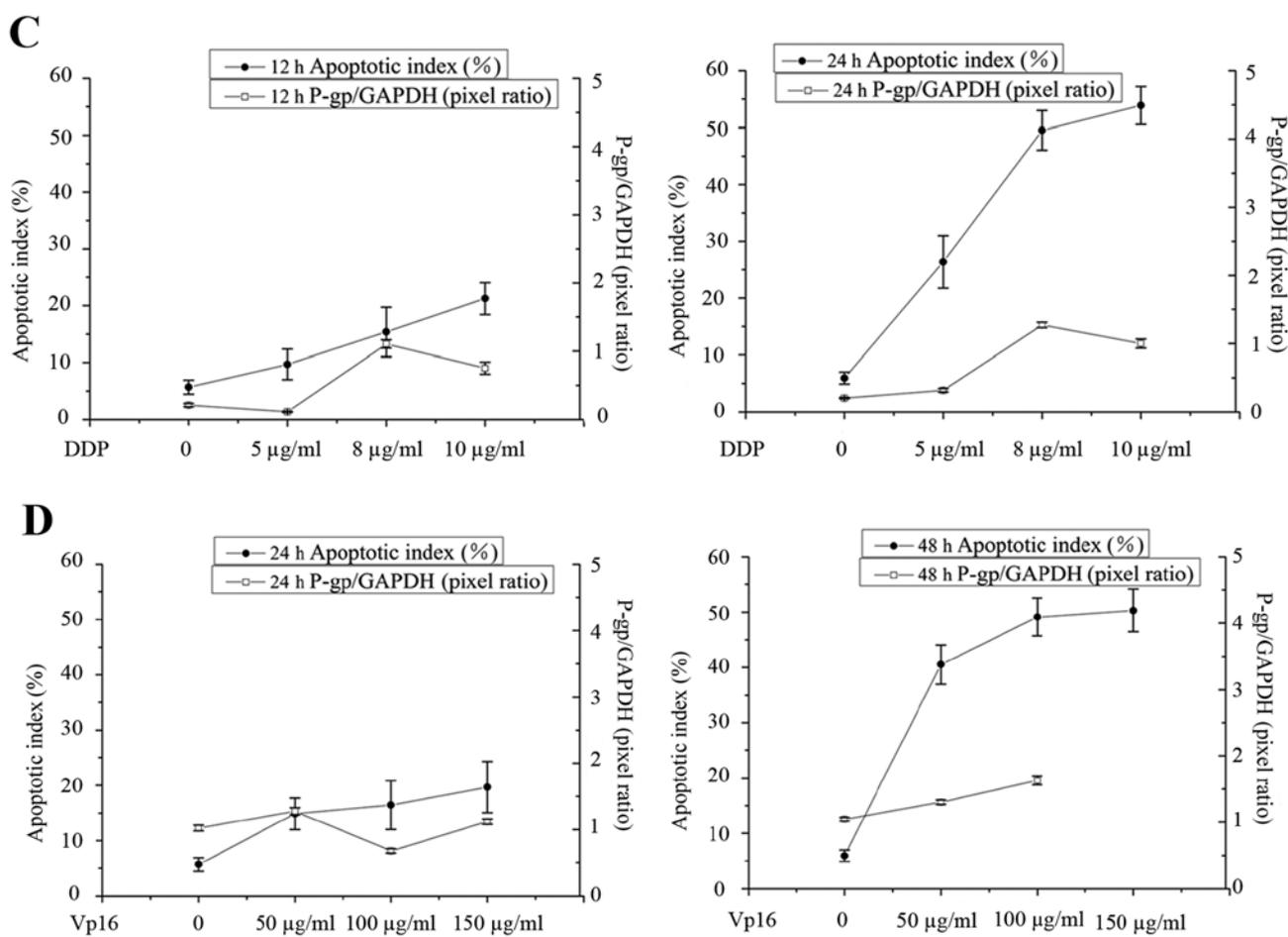


Figure 1. Continued. (C) SK-N-SH cells were cultured with varying amounts of DDP as shown. Cells were exposed to the indicated concentrations of DDP for 12 and 24 h and exhibit increased expression levels of P-gp. (D) Cells were exposed to the indicated concentrations of Vp16 for 24 and 48 h and exhibit increased expression levels of P-gp. The experiment described is one representative experiment of three.

transfer system. After blocking the non-specific space of the membrane with 5% non-fat milk in TBST (TBS with 0.1% Tween-20) for one hour, membranes were first incubated with monoclonal antibody against P-glycoprotein (1:1,000) (Cell Signaling Technology) overnight at 4°C with constant shaking. Then the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:1,000) (Sigma Chemical Co.) for 1 h with constant shaking. After electrophoresis, proteins were transferred to a nitrocellulose membrane and probed by corresponding antibodies. The quantification of protein levels in each sample was performed by ImageJ software. All experiments were performed in triplicate unless otherwise noted.

Statistical analysis. Each apoptosis value represents the mean \pm SD. Subsequently, each experiment was performed at least three times. Statistical analysis of data was carried out using a one-way ANOVA and a probability value of <0.05 ($P < 0.05$) was accepted as a significant difference. Computations were performed using SPSS17 software.

Results

Effects of As_2O_3 , DDP and Vp16 on SK-N-SH cells. To characterize the effects of As_2O_3 , DDP and Vp16 on SK-N-SH

cells, we performed flow cytometry analyses of the viability of SK-N-SH cells. It has previously been shown that As_2O_3 efficiently induces apoptosis of human NB cells *in vitro*. The initial experiments were conducted for evaluation of As_2O_3 , DDP and Vp16 induced anti-proliferation in SK-N-SH cell lines. SK-N-SH cells were treated with either PBS or drugs in each well. At first, according to the literature review and the results of our previous study, we determined the treatment conditions (drug concentration and treatment duration). The growth inhibitory effects of various concentrations of As_2O_3 , DDP and Vp16 were determined as described in Materials and methods. The viability of the cells was measured at different therapy times. Each point represents the mean \pm SD of three independent experiments. The IC_{50} values were determined by FACS.

As_2O_3 exhibited dose- and time-dependent inhibitory effects on SK-N-SH cells with an IC_{50} of 3 μM (Fig. 1A and B). Furthermore, these results also indicated that As_2O_3 exerted its cytotoxic effect in SK-N-SH cells via induction of apoptosis. For example, without the As_2O_3 treatment, there were only $6.9 \pm 1.3\%$ apoptotic cells in the SK-N-SH cell line, but the apoptotic cells were gradually increased and reached $49.51 \pm 4.27\%$ with a 3 μM concentration of As_2O_3 at 72 h of exposure. As shown in Fig. 1A and B, only 40.77% of cells were apoptotic at 2 μM As_2O_3 of exposure, 49.51% of cells

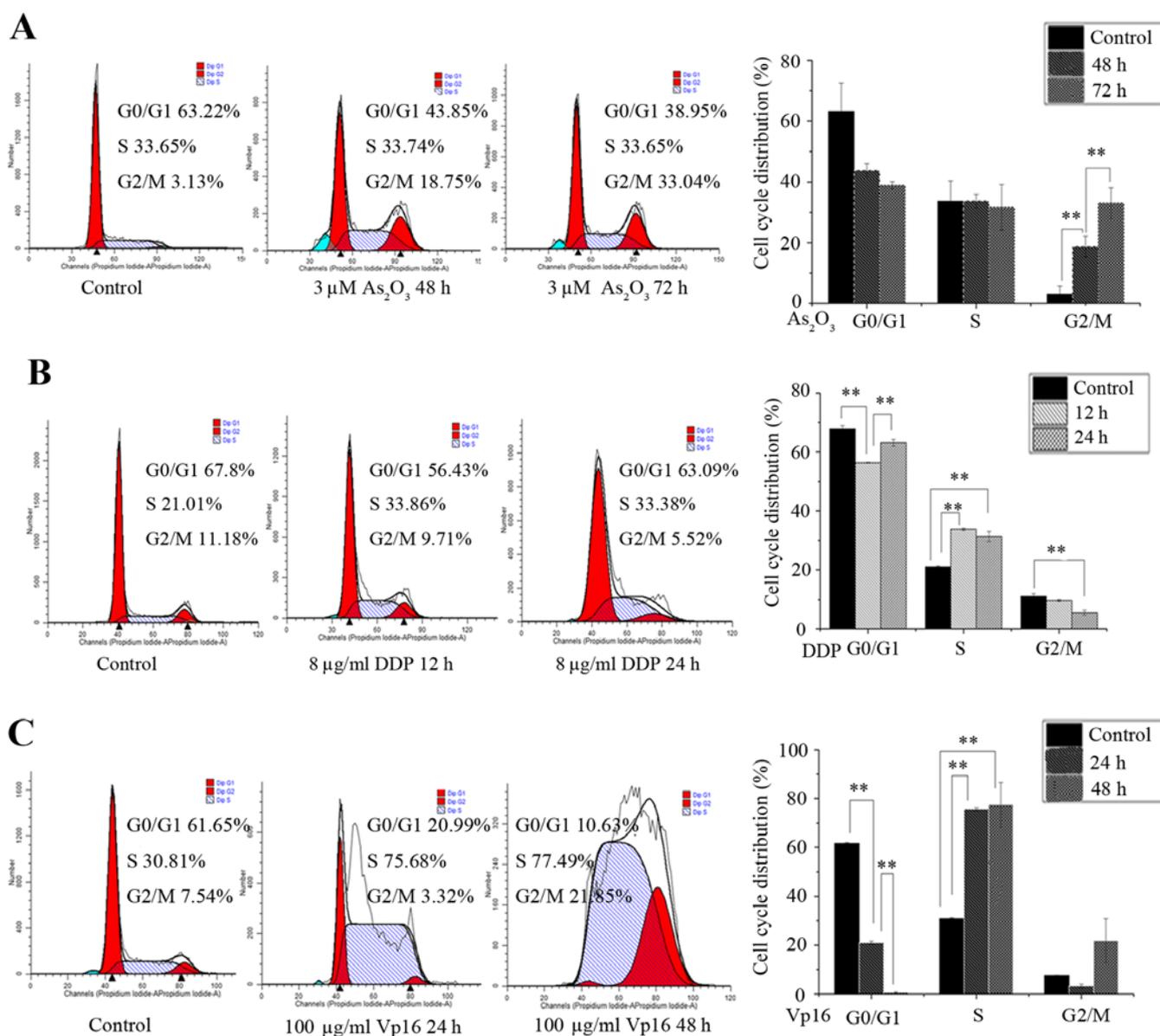


Figure 2. Cell cycle distribution analysis. One representative image of three is shown. (A) After 48- and 72-h exposure to 3 μM As_2O_3 , estimation of cell cycle distribution was determined by flow cytometry. SK-N-SH cells accumulate in G2/M phase in a dose-dependent manner. All data are expressed as mean \pm SEM from three independent experiments. (B and C) SK-N-SH cells arrest in S phase after exposure to DDP and VP16. ** $P < 0.05$ a significant difference between the two groups.

were apoptotic at 3 μM As_2O_3 , and 54.21% were apoptotic at 4 μM As_2O_3 after 72 h of exposure. Thus, the concentrations of As_2O_3 (2-4 μM) were the doses chosen for subsequent experiments. Moreover, flow cytometry analysis of As_2O_3 -treated cells revealed that following treatment with As_2O_3 (3 μM) for 48 and 72 h, the percentage of apoptotic cells increased from 27.87 \pm 4.47 to 49.51 \pm 4.27%; As_2O_3 (4 μM) for 48 and 72 h, the percentage of apoptotic cells increased from 31.92 \pm 2.97 to 54.21 \pm 3.82%. These data suggest that As_2O_3 may inhibit NB growth *in vitro*.

The growth and survival of SK-N-SH cells were markedly inhibited by the DDP and Vp16 treatment in a dose- and time-dependent manner (Fig. 1C and D). Fig. 1C and D demonstrate the results of flow cytometry with Annex in V-PI staining on SK-N-SH cells for IC₅₀ of DDP and Vp16 found to be 8 and 100 $\mu\text{g/ml}$; consistent with our previous results.

DDP significantly reduced SK-N-SH cell viability at concentrations of 8 $\mu\text{g/ml}$ or more (Fig. 1C). By 24-h treatment high levels of apoptosis were observed in SK-N-SH cells.

At 8 $\mu\text{g/ml}$, DDP strongly reduced the viability of SK-N-SH cells compared with the effects at 5 $\mu\text{g/ml}$ (Fig. 1C) for 12 and 24 h. Essentially the same results were obtained in previous research results. After 5, 8 and 10 $\mu\text{g/ml}$ DDP treatment of SK-N-SH cells for 12 h, the apoptosis rates were (9.68 \pm 2.69)%, (15.38 \pm 4.36)% and (21.25 \pm 2.82)%; after 5, 8 and 10 $\mu\text{g/ml}$ DDP treatment of SK-N-SH cells for 24 h, the apoptosis rates were (26.37 \pm 4.6)%, (49.55 \pm 2.48)% and (53.89 \pm 3.31)%, DDP cytotoxicity to SK-N-SH cells in a concentration-dependent manner. The apoptosis rates significantly increased after the same concentration of DDP treatment of SK-N-SH cells for 24 h relative to 12 h, DDP cytotoxicity to SK-N-SH cells in a time-dependent manner.

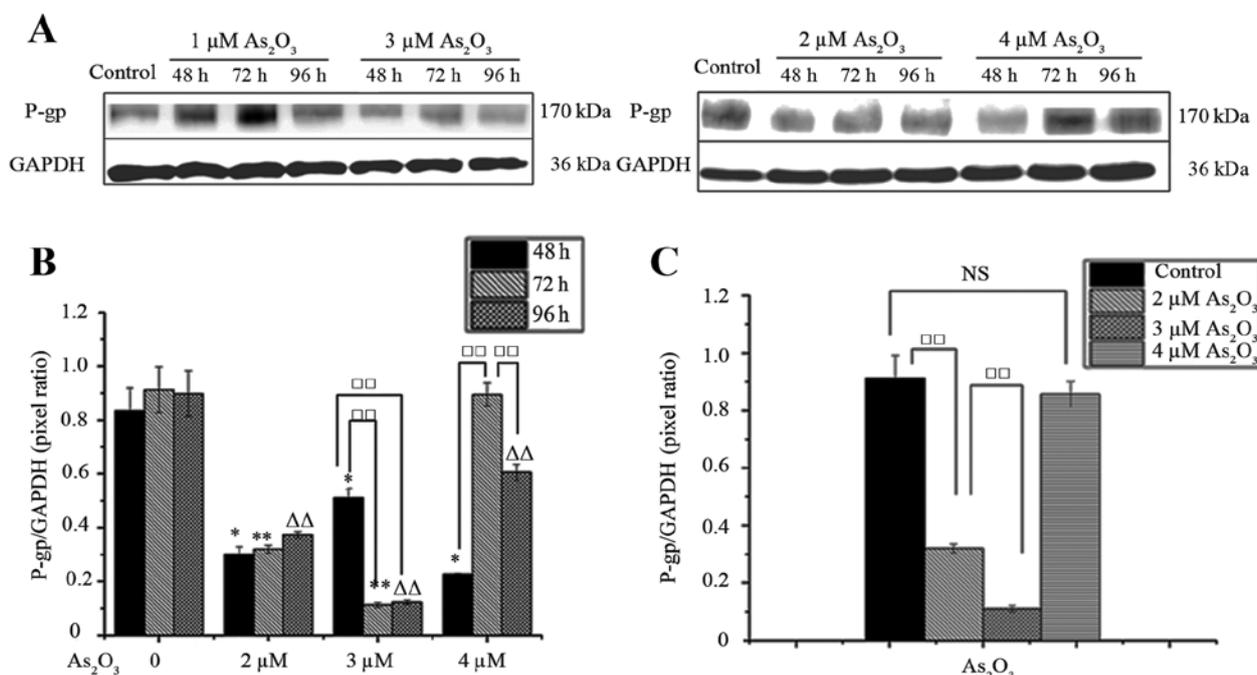


Figure 3. As₂O₃ inhibits multidrug resistant-related P-gp expression in SK-N-SH cell lines. (A) Expression levels of P-gp were detected in SK-N-SH cell lines by western blotting. As₂O₃ has decreased P-gp expression relative to the controls. Detection of GAPDH was used as a loading control. (B) The histogram shows quantifications of western blot analyses obtained with ImageQuant 5.2 software. Bars are expressed as mean \pm SEM. (C) Cells expressing P-gp show no alterations in control and 4 μM As₂O₃ for 72 h. *P<0.05, compared with 48 h control. **P<0.05, compared with 72 h control. $\Delta\Delta$ P<0.05, compared with 96 h control. $\square\square$ P<0.05, compared with the two groups. ^{NS}P>0.05, compared with the two groups.

After 50, 100 and 150 $\mu\text{g/ml}$ Vp16 (Fig. 1D) treatment of SK-N-SH cells for 48 h, the apoptosis rates were (14.86 \pm 2.86)%, (16.44 \pm 4.41)% and (19.67 \pm 4.62)%; after 50, 100 and 150 $\mu\text{g/ml}$ Vp16 treatment of SK-N-SH cells for 72 h, the apoptosis rates were (40.54 \pm 3.53)%, (49.12 \pm 3.96)% and (53.78 \pm 3.85)%, Vp16 cytotoxicity to SK-N-SH cells in a concentration-dependent manner. The apoptosis rates significantly increased after the same concentration of Vp16 treatment of SK-N-SH cells for 48 h relative to 24 h, Vp16 cytotoxicity to SK-N-SH cells in a time-dependent manner.

Effect of As₂O₃, DDP and Vp16 on cell cycle arrest of SK-N-SH cells. Analysis of cell cycle phase distribution was carried out to study the anti-proliferative mechanism of As₂O₃ on SK-N-SH cells. IC₅₀ of As₂O₃ was added to the experimental group. According to the literature (28) and our result of IC₅₀ of As₂O₃ on SK-N-SH cells, we chose 3 μM As₂O₃-treated SK-N-SH cells for 48 and 72 h. IC₅₀ of DDP and Vp16 were added to the drug control groups. According to our prior research, we chose 8 $\mu\text{g/ml}$ DDP-treated cells for 12 and 24 h, 100 $\mu\text{g/ml}$ Vp16-treated cells for 24 and 48 h. Each group was set in three parallel wells. The result indicated that As₂O₃ inhibited the cell proliferation of SK-N-SH cells was partly due to the cell cycle arrest, not only cytotoxic effect.

As₂O₃ treatment resulted in a time-dependent accumulation of SK-N-SH cells in the G2/M phase (Fig. 2A). At time 0, only 3.13 \pm 2.6% of cells were in the G2/M phase. After 48 h of treatment, 18.75 \pm 3.44% of cells were in the G2/M phase. The value of G2/M phase cells peaked at 72 h (33.04%).

As shown in Fig. 2B, 21.01 \pm 0.35% of cells were at S-phase at time 0 and 33.86 \pm 0.39% of cells were at S-phase at 12 h.

Treatment with DDP for 24 h caused a small decrease in the S peak in SK-N-SH cells, with no statistical difference. Treatment with Vp16 showed similar results in SK-N-SH cells (Fig. 2C). S-phase cells reached peak value (77.49 \pm 9.08) at 48 h.

Effects of As₂O₃, DDP and Vp16 on the expression changes of P-gp in SK-N-SH cells. Overexpression of P-gp as a membrane-bound energy-dependent efflux pump, in neoplastic tissue is responsible for MDR formation. Correspondingly, it has been reported that As₂O₃ inhibits the P-gp expression of leukemia cells (29). To examine whether As₂O₃, DDP and Vp16 affect P-gp expression in SK-N-SH cells, we initially monitor the expression of P-gp at various time-points and various concentrations using western blotting. The concentrations were based on our results of IC₅₀ of As₂O₃, DDP and Vp16 (the concentration gradients based on the results of preliminary experiments). Each group was set in three parallel wells.

As shown in Fig. 3A and B, western blotting revealed that As₂O₃ treatment did not result in a time- and dose-dependent upregulation of P-gp (P<0.05). To determine the extent of the observed striking reduction in P-gp expression, we performed western blot analysis on P-gp expression using the column chart (Fig. 3B). We found that 2 and 3 μM As₂O₃ treatment for 48, 72 and 96 h leads to reduced expression of P-gp (Fig. 3B), and the expression levels of P-gp were downregulated in 3 μM As₂O₃ treatment for 72 h more notably than in 2 μM As₂O₃ for 72 h (Fig. 3C). As₂O₃ did not lead to decreasing levels of P-gp expression in a time- and dose-dependent manner. In particular, P-gp expression was profoundly decreased under 3 μM (IC₅₀) As₂O₃ treatment for

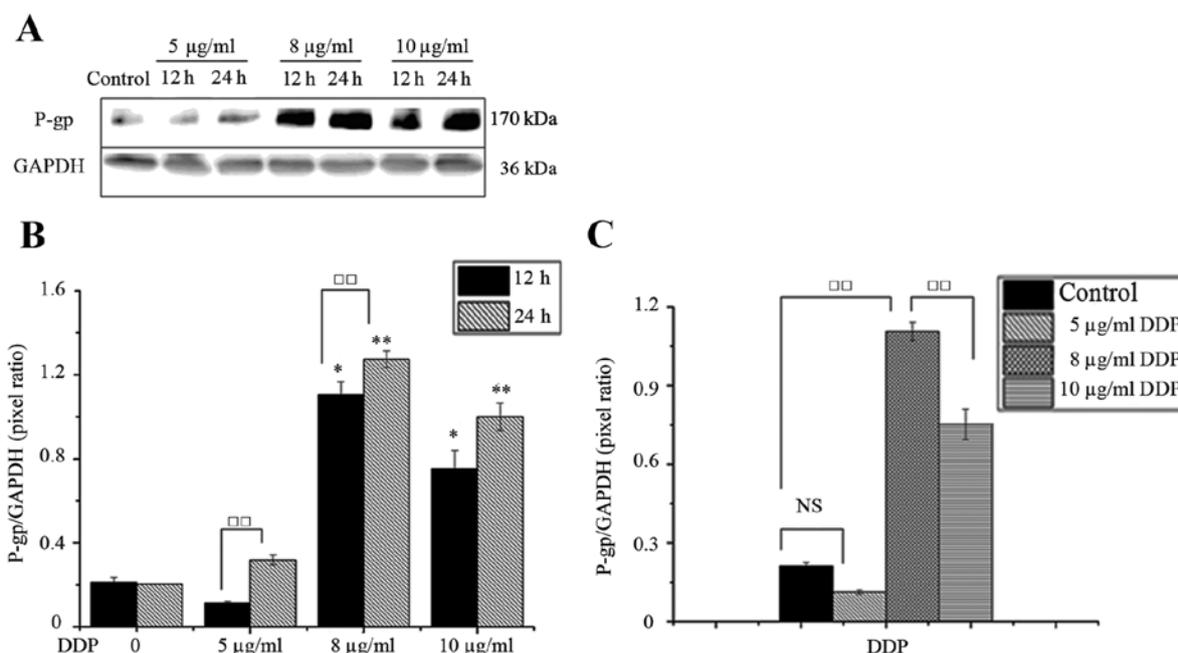


Figure 4. P-gp expression is increased by incubating the cells in the presence of DDP. (A) Western blot analysis of P-gp was performed with total cellular extracts prepared at 12 and 24 h after various concentrations of DDP. GAPDH was used as a control for protein loading. (B) The histogram shows quantifications of western blot analyses obtained with the ImageQuant 5.2 software. Results (mean \pm SD) are reported as relative expression normalized to GAPDH the used as loading control. (C) SK-N-SH cells were treated with various concentrations of DDP for 12 h. P-gp appeared overexpressed in SK-N-SH cells in comparison to controls. * $P < 0.05$, compared with 12 h control. ** $P < 0.05$, compared with 24 h control. $^{\Delta\Delta}P < 0.05$, compared with the two groups. $^{NS}P > 0.05$, compared with the two groups.

72 h. At 4 μM As_2O_3 for 48 and 96 h, the expression of P-gp was noted to gradually decrease, but no differences were found at 72 h after As_2O_3 exposure. Taken together, our data reveal that after As_2O_3 treatment, we observed a reproducible reduction in the induced P-gp expression (Fig. 3B). Upon treatment with different concentrations of As_2O_3 , expression of P-gp was substantially decreased (Fig. 3B). These results may imply that As_2O_3 may not be a substrate of P-gp and so it cannot stimulate and sustain the expression of P-gp in SK-N-SH cells during incubation.

On the contrary, the expression of P-gp in SK-N-SH cells was significantly retained and upregulated when treating with DDP and VP16. Western blots further identified that substantial amounts of P-gp were expressed in treatment with 8 and 10 $\mu\text{g/ml}$ DDP for 12 and 24 h in comparison to the control (Fig. 4). As shown in Fig. 4B, expression levels of P-gp, were significantly lower in the same concentration of DDP for 24 h compared to DDP for 12 h. Furthermore, the highest levels of P-gp were observed in SK-N-SH cells with IC_{50} of DDP for 24 h. This difference was statistically significant in 5 $\mu\text{g/ml}$ DDP for 12 and 24 h.

Similarly, the increase in the P-gp expression level was detected in SK-N-SH cells that were treated with Vp16 in comparison with the basal level identified in control cells (Fig. 5). Furthermore, 50 and 100 $\mu\text{g/ml}$ of Vp16 for 24 and 48 h elicited increase over the P-gp expression in SK-N-SH cells. These histograms illustrate the pattern of the dose-dependent response to various concentrations of Vp16 (Fig. 5B and C). As shown in Fig. 5B, the P-gp expression was higher in 100 $\mu\text{g/ml}$ Vp16 of 48 h than in 100 $\mu\text{g/ml}$ Vp16 of 24 h. On the other hand, upon IC_{50} of Vp16 administration for 48 h, the P-gp

expression was the highest. The P-gp was consistently induced by 150 $\mu\text{g/ml}$ Vp16 (Fig. 5B).

To examine whether P-gp levels correlated with apoptosis, western blot analyses and flow cytometry were performed. Our results showed that DDP and Vp16 induced apoptosis in SK-N-SH cells, and they increased the level of P-gp expression (Fig. 1C and D). In contrast, As_2O_3 in the micromolar range had a concentration-dependent toxic effect on SK-N-SH cells with decreased P-gp expression (Fig. 1B). In conclusion, this study demonstrates that As_2O_3 is efficacious in reversing P-gp-mediated MDR by inhibiting transport function and expression of P-gp.

Discussion

NB is characterised as the most common extracranial solid tumor with great malignancy that ranges from spontaneous regression in 10% of all cases to rapid and largely treatment resistant progression with fatal outcome (30). Despite treatment advances including chemotherapy, radiotherapy and stem cell transplantation, advanced NB still remains one of the most challenging problems that physicians must deal with in pediatric oncology and long-term survival rate is <40% (6). However, much of the literature points to the fact that the prolonged treatment with chemotherapy is often ineffectual due to the appearance of MDR.

The availability of reducing MDR drugs is a prerequisite to develop novel effective therapies. Decades ago, As_2O_3 was known as a carcinogen, however, according to research at present it now becomes a hotspot in the treatment of tumors.

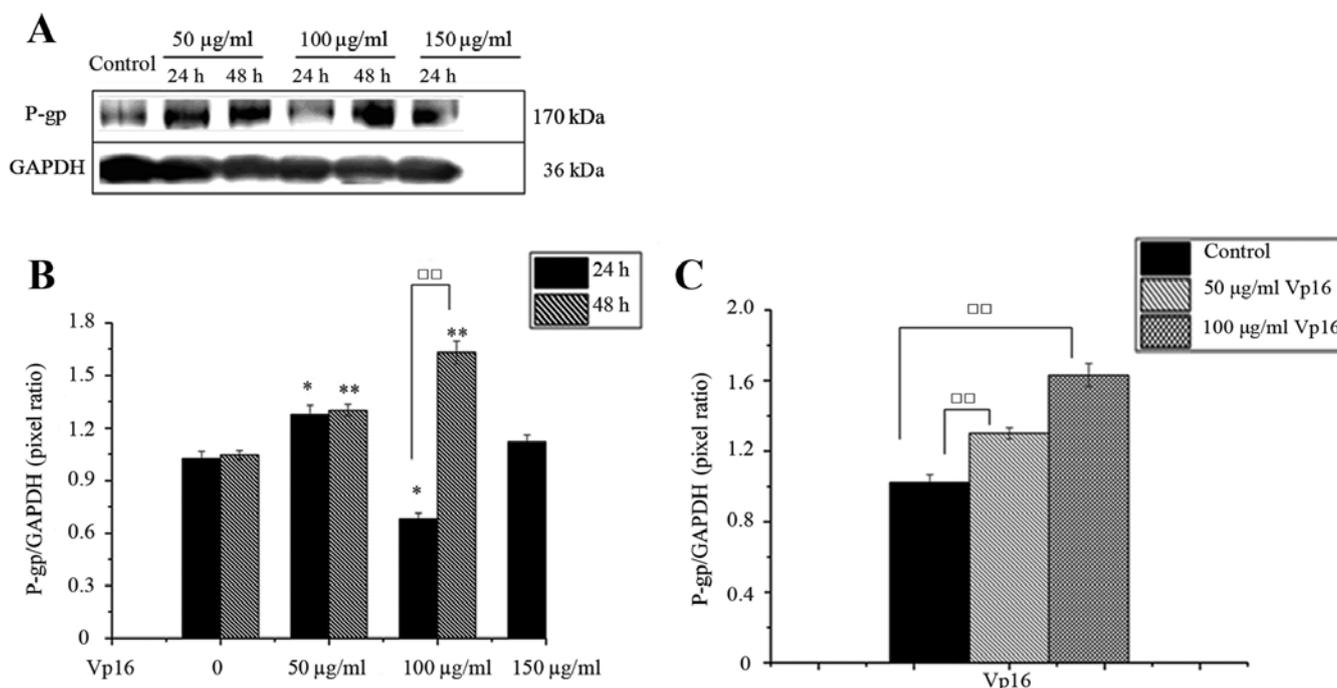


Figure 5. The VP16-inducible expression of P-gp determined by western blotting. (A) SK-N-SH cells were treated with various doses of VP16 for the indicated times. Expression levels of P-gp were detected in SK-N-SH cell lines by western blotting. GAPDH is employed as the internal control. Result is a representative image of three. P-gp expression was determined by western blotting. (B) P-gp appeared overexpressed in SK-N-SH cells. (C) SK-N-SH cells were treated with various concentrations of VP16 for 48 h. * $P < 0.05$, a significant difference from 24 h controls and ** $P < 0.05$, a significant difference from 48 h controls. ^{AA} $P < 0.05$, compared with the two groups.

The clinical use of As_2O_3 as an anticancer agent is based on the drug's ability to induce APL cell differentiation and promotes apoptosis. As_2O_3 is a poor substrate for transport by P-glycoprotein and recommended for cancer therapy especially in reversing the MDR *in vitro*, such as leukemia (31), osteosarcoma (32), myeloma (33), gastric (34) and hepatic carcinoma (35). In addition, we found that As_2O_3 lacked cross-resistance in both P-glycoprotein- and MRP-overexpressing cell lines (36).

As_2O_3 partially reduce MDR to DOX in K562/A02 cells via reducing function of efflux pumps such as GST-p and modulate the expression of MDR-related proteins such as Topo-II and bcl-2 (31). Although our understanding of As_2O_3 clinical treatment of MDR NB has many advantages, less is known about the effect of As_2O_3 on the P-gp expression in NB.

To address the possibility that As_2O_3 has a role in reducing drug resistance, the expression levels of P-gp were assayed by western blotting. In this study we investigated the mechanism involved in the suppression of MDR NB. We analyzed the effect of As_2O_3 on P-gp expression, in order to investigate the mechanism involved in As_2O_3 treatment of MDR NB and provide new evidence for As_2O_3 clinical treatment NB and thereby reinforcing that As_2O_3 has potential in clinical treatment of NB, contributing to reducing drug resistance. To our knowledge, this is the first demonstration of As_2O_3 directly affecting expression levels of P-gp in SK-N-SH cell lines. Therefore, our approach, which utilizes As_2O_3 and conventional chemotherapy drug treatment of NB cell lines and detects the P-gp levels, provides a more comprehensive system in which to investigate the roles of As_2O_3 in apoptosis

events, ultimately leading to establishment of the possibility of As_2O_3 clinical treatment MDR NB. This is the first report characterizing the P-gp alterations in SK-N-SH cells treated with As_2O_3 , DDP and VP16.

Herein, we report the effect of As_2O_3 on cytotoxicity of SK-N-SH human neuroblastoma cells *in vitro*. Following treatment with As_2O_3 at different low doses for 48 h, apoptosis was observed in SK-N-SH cells, and the apoptosis rate has a positive correlation with acting time; coincident with the results of Karlsson *et al* (28). Our data also demonstrate that As_2O_3 is efficacious in inhibiting the proliferation of SK-N-SH cells in a dose-dependent manner, with an IC_{50} of $\sim 3 \mu M$ following treatment with As_2O_3 for 72 h. In this study, As_2O_3 inhibited cell viability and/or induced apoptosis in SK-N-SH cells depending on the drug concentration, suggesting that by prolonging the time of effective drug and increased the drug concentration, can significantly improve drug efficacy.

In a previous report, we first demonstrated that TrkA, TrkB and TrkC gene expression were upregulated with increasing concentration of As_2O_3 administration, more apparent at 48 h than 24 h. Advanced NB tumors carrying amplified MYCN oncogene which is the most unfavorable prognostic factor and prominent genetic marker of high-stage disease often exhibit an aggressive phenotype (37). Yin *et al* (38) found that MYCN mRNA in LA-N-5 cell lines was strongly inhibited by treatment with different concentrations of As_2O_3 , and decline the most obvious at $3 \mu M$ As_2O_3 . This, together with our study, indicates that As_2O_3 may exert a greater anti-carcinogenesis effect on MYCN positive high-risk NB than other drugs.

To overcome the limitation, one strategy is to increase doses of chemotherapy drugs; however, it causes serious toxic effects on heart, liver, kidney and hearing damage, which patients are not able to tolerate, abandoning the treatment. When As₂O₃ was combined with DDP or DOX in ALL, it showed strong synergy with chemotherapeutic agent *in vitro* and reversed the effect of DDP or DOX on multidrug resistance of tumor cell lines (15,31,39). In fact, a number of recent publications indicate that As₂O₃ combined with DDP or DOX effect on leukemia cells lead to synergy and reverse tumor resistance. We speculate that the cytotoxic efficiency of As₂O₃ combined with conventional chemotherapy drugs, is important from a clinical point of view.

In conclusion, our data shows that the IC₅₀ value for As₂O₃ in SK-N-SH cells was ~3 μM, a concentration very close to clinically achievable concentration for successfully treating patients with acute promyelocytic leukemia (40). Thus it is suggested that As₂O₃ might be suitable in the treatment of some instances of NB.

Suppression of cell growth by As₂O₃ can be explained in part by its capacity to affect cell cycle distribution. Cell cycle analysis has revealed that As₂O₃ significantly induced a time-dependent accumulation of cells in the G2/M phase more significantly increased 72 h later. Therefore, to explain the mechanism that As₂O₃ induced rapid and extensive apoptosis concomitant with arrest of cells in G2/M phase of the cell cycle, due to G2/M block, unlike G1 block, is more toxic to the cells, and not reversible. Thus As₂O₃-induced G2/M arrest may be a requirement for the activation of apoptotic pathways.

Park *et al* (41) showed that As₂O₃ arrest cell cycle in G2/M phase in leukemia U937 cells and trigger apoptosis. Gao *et al* (42) showed that As₂O₃ could arrest NB4 cell lines in G2/M phase, and G2/M cells which possessed higher ROS levels were more susceptible to As₂O₃-induced apoptosis (42). More strikingly, Hassani *et al* (43) showed that the G2/M arrest plays a critical role in the induction of apoptosis by As₂O₃ in NB cells.

Here, we confirmed that that As₂O₃ treatment resulted in accumulation of cells in the G2/M phase in a time-dependent manner. Furthermore, activation of caspases occurred only in As₂O₃-induced mitotic cells, not in interphase cells (36), suggesting As₂O₃ has a higher cytotoxicity against NB cells blocked in G2/M phase. This finding provides a more comprehensive understanding of restraining cell cycle at G2/M closely related to the initiation of apoptosis.

The exact mechanisms underlying As₂O₃ that could markedly arrest cell cycle in G2/M phase are not fully understood. It is possible that As₂O₃ affects the expression of the cell cycle regulatory elements and Akt signal transduction pathway. The results of Han *et al* (44) suggest that, the levels of cdc2 protein which is related to the progression of G2/M phase were downregulated in cells treated with As₂O₃. There are a variety of reports suggesting that Akt is one of the important survival factors and plays an essential role stimulating of cellular growth and chemotherapeutic resistance to apoptosis (45,46). PTEN as the tumor suppressor gene inactivated in a wide variety of cancers downregulates active Akt (47) regulates many biological processes including metabolism, growth, survival, and proliferation. Data from Zhang *et al* (48) and Li *et al* (49) showed that As₂O₃ is associated with upregulated

Wee1 and PTEN levels and downregulated cdc2 protein and Akt signal pathway resulting in induction of G2/M cell cycle arrest.

In addition, As₂O₃ treatment blocked the cell cycle in G1 or at G2/M depending on the cell line. We have also observed that As₂O₃ was able to induce an M phase arrest of the cell cycle in MCF-7, H460, HeLa and K562 cell lines (36), related with tubulin aggregation, like paclitaxel (50). Liu *et al* (51) reported that As₂O₃-induced G1 arrest in myeloma cells with wild-type (wt) p53 but G2/M arrest in myeloma cells with mutated p53. Thus As₂O₃ induces cell cycle arrest in two distinct pathways including G1 or G2/M cell cycle arrest, depending on varying p53 status.

Here, we confirmed that As₂O₃ could markedly arrest cell cycle in G2/M phase with a time-dependent manner. Furthermore, activation of caspases occurred only in As₂O₃-induced mitotic cells, not in interphase cells (36), suggesting As₂O₃ has a higher cytotoxicity against NB cells blocked in G2/M phase. This finding provides a more comprehensive understanding of As₂O₃-induced mitotic arrest closely related to the initiation of apoptotic pathways. Taken together, our data demonstrate that As₂O₃ markedly arrests cell cycle at G2/M phase, and induces apoptosis. Our study on cell cycle revealed that As₂O₃ causes accumulation of SK-N-SH cells in the G2/M phase, in rational combinatorial strategies involving As₂O₃ in combination with vinorelbine (NVB) that G2/M phase is sensitive to and might clinically intensify As₂O₃-induced apoptosis.

Currently, the overexpression of membrane proteins in cancer cells functioning as drug pumps results in MDR to chemotherapeutic agents is a major impediment to the successful treatment of cancer. The adenosine triphosphate (ATP)-binding cassette (ABC) membrane transporter family is well known including ABCB1 (P-glycoprotein, P-gp), ABCC1 (multidrug-resistant protein 1, MRP1), ABCC2 (MRP2) and ABCG2 (breast cancer resistance protein, BCRP). As far as is known, multidrug resistance P-glycoprotein, an ABCB1 member of the ABC transporter family, acts as a membrane-bound energy-dependent efflux pump lowering intracellular drug levels to sublethal concentrations and helps cells to escape from death. P-gp which pumps a broad range of structurally unrelated cells encoded by the MDR1 gene, overexpression of it in neoplastic tissue is responsible for the emergence of the MDR phenotype in cancer cells. P-gp has a basal ATPase activity and can be activated in the presence of its substrates such as commonly used chemotherapy drugs of VCR, DOX and Vp16, then expelling antitumor agents from the cytoplasm by ATP hydrolysis (52). Overexpression of P-gp confers reduced sensitivity to large group of cytotoxic drugs.

It is generally believed that the overexpression of P-gp pumping out intracellular commonly used chemotherapy drugs in NB chemotherapy regimens such as anthracycline, epipodophyllotoxin and vinca alkaloids, leads to NB developing MDR. Gibalová *et al* (53) indicated that P-gp, independent of its drug efflux activity as DDP is not transportable by P-gp, altering DDP-induced apoptosis pathway of a decrease in DDP-induced caspase-3 activation that confers a partial loss of DDP sensitivity. Moreover, Topo-II (54) and glutathione S-transferase π (GSTπ) (55) overexpression

contributes to MDR formation. In contrast, accumulating evidence indicates that P-gp has an antiapoptotic function by pumping some caspase-dependent apoptotic substance and toxic chemicals (56,57).

Up to now, the regulatory mechanisms underlying the expression of P-gp have not yet been well clarified. Duraj *et al* (58) verified that that mitogen-activated protein kinase (MAPK) pathway was associated with regulatory P-gp expression in P-gp-mediated MDR. In addition, Wei *et al* (52) demonstrated that tetrandrine, an inhibitor of MAPK pathway, effectively reverses the MDR of KBv200 and MCF-7/adr cells presumably via downregulating P-gp expression of SK-N-SH. In addition, the hepatocarcinogen 2-acetylaminofluorene 2-AAF could also upregulate the human MDR1 expression in human hepatoma (59). Zhou *et al* (60) provided the first direct evidence that the c-Jun NH 2-terminal kinase (JNK) pathway could indeed downregulate P-glycoprotein in a dose- and time-dependent manner, and reverses P-glycoprotein mediated MDR in cancer cells.

Although As₂O₃ had potent reversal effect on leukemia cell P-gp-mediated MDR *in vitro* (29), little information is available regarding this compound's effect on the P-gp expression in NB cells. Our study aims to verify that. We actually did observe that As₂O₃ inhibited P-gp expression in SK-N-SH cells. In particular, P-gp protein levels were strongly decreased with 2 and 3 μM As₂O₃ for 48, 72 and 96 h. The data in this study showed upregulation of P-gp on SK-N-SH cells following treatment with DDP or VP16, which is consistent with Gu *et al* (61) and Yang and Page (62). Collectively, our result showed high levels of P-gp observed when SK-N-SH cells were treated with DDP and Vp16, however, P-gp expression is strongly reduced upon treatment with different concentrations of As₂O₃. It is therefore very likely that appropriately increased As₂O₃ accumulation and acting time, P-gp expression would not be upregulated. In general, the results presented in this study indicate As₂O₃ is a poor substrate for transport by P-glycoprotein, suggesting that As₂O₃ may be useful for treatment of human solid tumors, particularly in patients with MDR. There is evidence that decreasing protein expression level of P-gp is one of the effective ways to reverse P-gp-mediated MDR (52).

Therefore, these findings support the notion that inhibition of P-gp expression may reverse the MDR phenotype through enhancing intracellular accumulation of anticancer drugs. To circumvent MDR, much effort has focused on finding P-gp inhibitors and identification of molecules targeting multiple drug-resistant mechanisms to effectively reverse P-gp-mediated MDR. However, the failure of this type of drugs with poor clinical effectiveness has substantially dampened the initial enthusiasm. One reasonable explanation is the side-effects of these drugs which now has affected its wide application in the clinic.

In conclusion, As₂O₃ inhibited the growth of SK-N-SH cells by inducing a G2/M arrest of the cell cycle and by triggering apoptosis. These findings demonstrated that As₂O₃ is effective and a potential agent in reversing P-gp-mediated MDR. Our results support As₂O₃ as an effective complement to conventional chemotherapy for high-risk NB patients and possibly also for potential use for reversing P-gp-mediated MDR when synergistic with chemotherapy.

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