# Synergistic antitumor activity of oridonin and arsenic trioxide on hepatocellular carcinoma cells

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**Abstract.** Although arsenic trioxide ($As_2O_3$) has been successfully employed in treatment of patients with APL (acute promyelocytic leukemia), the sensitivity of solid tumor cells to this treatment was much lower than APL cells. The single agent of $As_2O_3$ was inefficient for treatment of hepatocellular carcinoma (HCC) in phase II trial demonstrating that new modalities of treatment with enhanced therapeutic effect are needed. In this study, we showed that oridonin, a diterpenoid isolated from traditional Chinese medicine *Rabdosia rubescences*, greatly potentiated apoptosis induced by $As_2O_3$ in hepatocellular carcinoma cells. The synergistic pro-apoptosis effect of combination of these two drugs led to increase in intracellular reactive oxygen species (ROS) level and N-acetyl-L-cysteine (NAC), a thiol-containing anti-oxidant, was able to completely block the effect. The combination treatment induced ROS-dependent decrease in mitochondrial membrane potential (MMP) decrease, and relocation of Bax and cytochrome C. Besides, oridonin dramatically increased the intracellular $Ca^{2+}$ overload triggered by $As_2O_3$. Furthermore, the co-treatment of oridonin and $As_2O_3$ induced ROS-mediated down-regulation of Akt and XIAP, and inhibition of NF-κB activation. The two drug combination enhanced tumor suppression activity in murine HCC model compared with single agent treatment *in vivo*. These findings demonstrate that oridonin can sensitize hepatocellular carcinoma cells to $As_2O_3$ treatment and will facilitate the optimization of $As_2O_3$ therapy for HCC patients.

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Surgery is curative in only a minority of patients due to coexisting advanced cirrhosis, multifocal disease, invasion and extrahepatic metastases (1). Although locoregional treatments have been shown to be somewhat effective, their therapeutic efficiency based on highly selected patients, possibly limiting its generalizability to other patients with different clinical profiles. Thus, systemic chemotherapy remains the main options for the majority of HCC patients with unresectable tumors (2). Various clinical trials have reported that the response rates of single-agent chemotherapy for patients with unresectable HCC vary from 0 to 20%. Combination chemotherapy including doxorubicin, cisplatin, fluorouracil and interferon, has been studied and the results have yielded more effectiveness than those found for single agents (3).

Arsenic trioxide ($As_2O_3$), a traditional Chinese medicine, has been widely employed to treat APL (acute promyelocytic leukemia). It is well established that $As_2O_3$ induces complete remissions in approximately 80-90% of patients diagnosed with APL, as well as 60-90% of all-trans-retinoic acid (ATRA)-refractory patients (4-6). Besides APL cells, $As_2O_3$ also induced apoptosis of a variety of solid tumor cell lines including hepatocellular (7) gastric (8) esophageal (9) prostate (10) colorectal cancers (11). However, the anti-tumor efficacy of $As_2O_3$ against solid tumors has not been as good as APL. It requires much higher dosages to inhibit solid cancer growth than those required to inhibit hematologic malignancies. The high dosage of $As_2O_3$ could cause adverse effects such as leukopenia, anemia, fever, vomiting and the dose-limiting toxicity is the main hindrance for its application in solid cancers (12). Although, extensive studies have been demonstrated that $As_2O_3$ could induce hepatocellular tumor growth inhibition and cells apoptosis (7,13), single-agent arsenic trioxide was not active against advanced HCC at the dosages of 0.16-0.24 mg/kg per day in a phage II trial (12). Thus, new strategies of treatment with enhanced therapeutic effect and alleviated toxicity are needed for application of $As_2O_3$ on patients with HCC.

Oridonin is a diterpene compound extracted from the plant *isodon rubescens* which has been used as anti-inflammation and anti-tumor agent in traditional Chinese medicine for thousands of years. Oridonin has been shown to possess cell cycle arrest, apoptosis and autophagy-inducing activities towards a variety of cancer cells (14). Besides, oridonin is able to trigger ROS generation and perturb redox balance in hepatocellular carcinoma cells (15). An important event during treatment of cancer cells with $As_2O_3$ involves the generation of reactive

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oxygen species (ROS), and elevation of the cellular ROS could facilitate $As_2O_3$-induced apoptosis (16,17). In this study, we aimed to verify our hypothesis that oridonin could improve the anti-tumor efficacy and reduce the adverse effects of $As_2O_3$ for treatment of HCC.

## Materials and methods

*Cell lines and reagents.* Human hepatocellular cancer cell lines Bel7402, SMMC7721, HepaG2 and murine hepatoma cell line Hepa1-6 were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and incubated at 37˚C in 5% $CO_2$. $As_2O_3$ was purchased from Sigma (St. Louis, MO, USA) and oridonin was obtained from Biopurify (Cheng Du, China).

*Cell viability assay.* Cells were seeded in 96-well plates at $5x10^3$ cells per well and exposed to agents for 48 h. After treatment, the MTT assay was performed. Absorbance at 570 nm was directly proportional to the number of living cells in culture.

*Apoptosis assessment.* Apoptosis was detected by annexin V/propidium iodide (PI) double stain and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. The annexin V/PI stain was performed as previously described (18). Briefly, Bel7402 cells were stained with EGFP-tagged annexin V for 20 min post-treatment, then PI was added. The stained cells were analyzed by flow cytometry. TUNEL analysis was performed using DNA fragmentation detection kit following the manufacturer's instructions (Merck Chemicals, Germany). The number of TUNEL positive cells in the field at magnifications x200 was quantified.

*Western blotting.* After treatment, Bel7402 cells were harvested and lysed. Extracted proteins were separated on SDS-PAGE and then transferred onto PVDF membranes. After blocking in 5% milk, the membranes were firstly hybridized with primary antibody, and then with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz, USA). The Western blots were detected using enhanced chemiluminescence sytem (Amersham Pharmacia Biotech, USA). The mitochondria and cytoplasm fractions were extracted using the mitochondria extraction kit (Beyotime, China).

*ROS detection and intracellular glutathione (GSH) measurement.* 2,7-Dichlorodihydrofluorescein diacetate (DCF-DA) (Invitrogen, USA) was used to detect intracellular ROS level. DCF-DA is intracellularly cleaved by non-specific esterases and oxidized by ROS to the fluorescent 2,7-dichlorofluorescein (DCF). After washing once with PBS, treated cells were incubated with 20 $\mu$M DCF-DA in serum-free DMEM at 37˚C for 30 min, then detected by flow cytometry. Cellular GSH levels were analyzed using 5-chloromethylfluorescein diacetate (CMFDA) (Invitrogen). CMFDA is converted to fluorescent 5-chloromethylfluorescein (CMF) by cytoplasmic esterases and then reacted with glutathione. Cells were incubated with 5 $\mu$M CMFDA at 37˚C for 30 min after washed once with PBS, then 1 $\mu$g/ml PI was added to stain the dead cells before analyzing by flow cytometry.

*Measurement of mitochondrial membrane potential and cytosolic $Ca^{2+}$.* Mitochondrial membrane potential was measured using 3,3'-dihexyloxacarbocyanine iodide [DiOC6(3)] (Invitrogen). Briefly, cells were washed once with ice-cold PBS after treatment and then incubated with 50 nM DiOC6(3) in serum-free DMEM for 30 min in 5% $CO_2$ at 37˚C, and then analyzed by flow cytometry. For detection of cytosolic $Ca^{2+}$, cells after treatment were washed once with PBS and then incubated in serum-free DMEM containing 2 $\mu$M fluo-4 acetoxymethylester (AM) (Invitrogen) for 30 min in 5% $CO_2$ at 37˚C. The cells were left at room temperature for 15 min and then analyzed.

*Luciferase assay.* Bel7402 cells were transiently transfected with NF-κB luciferase reporter plasmid (Stratagene, USA) using Lipofectamine 2000™ (Invitrogen) according to the manufacturer's instruction. Twelve hours post transfection, cells were treated with 8 $\mu$M $As_2O_3$, 5 $\mu$g/ml oridonin, their combination, the combination plus 2.5 mM NAC or 0.5 $\mu$M PMA (as positive control) for 24 h. Luciferase activity was then measured by the Dual Luciferase Reporter Assay system (Promega, USA).

*Tumor implantation and evaluation of antitumor effects in vivo.* 6 to 8-week old female C57BL/6 mice were implanted subcutaneously (s.c) on the mid-right side with $1x10^7$ Hepa 1-6 mouse hepatoma cells in 0.1 ml PBS. After the tumors reached the size of ~0.1 $cm^3$, mice were randomized into four groups with 7 mice per group. Group I received 3 mg/kg $As_2O_3$ in 0.2 ml of 0.9% NaCl solution, group II received equal volume of oridonin at dose of 10 mg/kg, Group III received 3 mg/kg $As_2O_3$ plus 10 mg/kg oridonin respectively, Group IV received 0.2 ml 0.9% NaCl solution as control. Mice were administered drugs intraperitoneally (i.p.) daily with an interval once every 6 times. Tumor volumes were measured at the 0 and 3 weeks after administration and determined using the formula: tumor volume = length x width$^2$ x 0.52 (19). The animal study protocol was in according with the guideline of administration of lab animals issued by the Ministry of Science and Technology (Benjing, China) and approved by the Jiangsu Institutional Animal Care and Use Committee (IACUC).

*Statistical analysis.* Quantitative data were expressed as mean ± standard deviation (SD) and analyzed using SPSS software. Paired Student's t-test was performed to assess statistical significance. Differences between experimental groups were considered significant at $p<0.05$.

## Results

*Synergistic inhibition of oridonin and $As_2O_3$ on hepatic carcinoma cell growth.* Various doses of oridonin were used in combination with $As_2O_3$ in human hepatic carcinoma cell lines including Bel7402, HepaG2, SMMC7721, and murine Hepa 1-6 cells. Compared with treatment of $As_2O_3$ alone, combined treatment with oridonin induced significant decreases of cells viability (Fig. 1). For Bel7402, treated with low dose (6 $\mu$g/ml) of oridonin alone reduced cell growth by ~5%. When this dose of oridonin was combined with 6 $\mu$M $As_2O_3$ the reduction in growth was 70% (Fig. 1A). Besides, similar treatment causes no synergic effect on non-malignant 293 T (human embryonic kidney cell line) cells (data not shown).

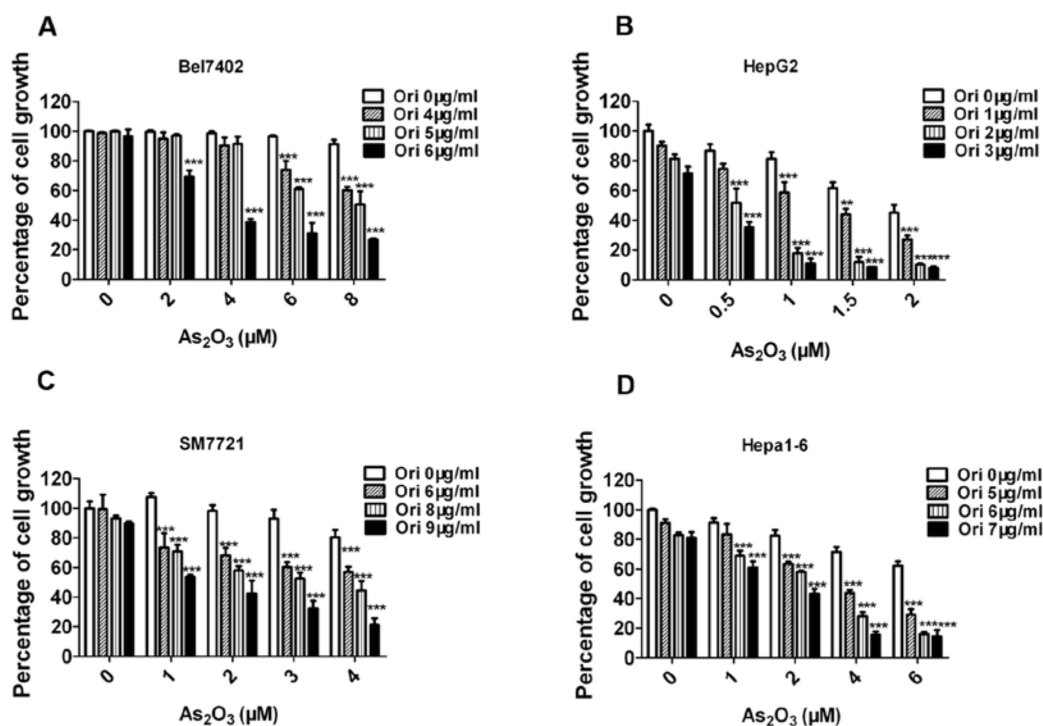


Figure 1. Cell viability assay. Human hepatocarcinoma cells Bel7402 (A), HepG2 (B), SM7721 (C) and mouse hepatocarcinoma cells Hepa1-6 (D) were co-treated with the indicated concentrations of $As_2O_3$ and oridonin (ori) for 48 h. Cell number was determined by the absorbance at 570 nm and expressed as a percent of control cells. Representative experiments, n=3, mean ± SD. $^{**}p<0.01$; $^{***}p<0.001$ when compared with 0 $\mu$g/ml oridonin (ori 0 $\mu$g/ml, white bar).

*Oridonin-enhanced apoptosis induced by $As_2O_3$.* To examine the apoptosis of Bel7402 cells treated with low dose of oridonin, $As_2O_3$ and their combination, annexin V/PI stain and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay were performed. As shown in Fig. 2A-D, though 5 $\mu$g/ml oridonin caused no apoptosis, it significantly potentiated $As_2O_3$-induced apoptosis. $As_2O_3$ at dose of 8 $\mu$M alone induced slight apoptosis (~10%) at 48 h, when it combined with oridonin, apoptosis was greatly enhanced, up to ~35% (Fig. 2B). Anti-oxidization agent NAC could completely blocked this synergistic pro-apoptosis effect of the two-drug combination (Fig. 2A-D), which indicates that intracellular ROS play a critical role in the induction of apoptosis.

Caspases are essential components of mammalian apoptosis machinery, their activation level directly reflects the extent of apoptosis. During intrinsic apoptosis, procaspase 9 is cleaved into an active caspase, which in turn cleaves downstream pro-caspase 3 into an executor. Active caspase 3 cleaves many substrates such as poly (ADP)-ribose polymerase (PARP) (20). As shown in Fig. 2E, procaspase 3, procaspase 9, and PARP were significantly cleaved in the co-treatment of oridonin and $As_2O_3$, but cleavage of procaspase 3 and PARP were hardly detected in any single agent treatment.

*Oridonin elicits elevation of cellular ROS level.* We next assessed the ROS production in the variously treated Bel7402 cells using DCFDA fluorescence dye. As shown in Fig. 3A and B, exposure of cells to comparatively low concentration of $As_2O_3$ (8 $\mu$M) hardly elicited any elevation of cellular ROS level. But low dose of oridonin alone (5 $\mu$g/ml) induced remarkable ROS level elevation and co-treatment with oridonin and $As_2O_3$ dramatically augmented this ROS level elevation.

Glutathione (GSH) is a major cellular reductant and has been shown to be crucial for regulation of cell proliferation, cell cycle progression and apoptosis (21). So, we analyzed the GSH level by using CMF fluorescence whose intensity is proportional to the intracellular GSH level. As shown in Fig. 3C and D, one population of CMF fluorescence negative cells (~20%) were induced by comparably low dose of oridonin, which indicated that treatment with oridonin caused depletion of cellular GSH. Besides, combination of oridonin and $As_2O_3$ decreased further the GSH level (~30%). Treatment with 8 $\mu$M $As_2O_3$ alone causes no obvious depletion of GSH when compared with control. In addition, in combination treatment of oridonin and $As_2O_3$, the negative CMF fluorescence cells partly showed PI positive staining (~10%) (Fig. 3E), which indicated that some cells showing GSH depletion were not viable. Although oridonin alone decreased GSH level, the PI staining mostly was negative, these data indicated that oridonin alone could not induce cell death.

*Co-treatment with oridonin and $As_2O_3$ causes accelerated loss of mitochondrial membrane potential.* Excessive ROS production may decrease in the mitochondrial membrane potential and impairment of the mitochondrial respiratory chain (22). We used DiOC6(3) to measure the mitochondrial membrane potential. As shown in Fig. 4A and B, the two-drug combination caused much more reduction of DiOC6 fluorescence compared with single agent treatment. This indicates that the effect of oridonin in enhancing $As_2O_3$ induced apoptosis involves mitochondrial damage. Loss of mitochondrial membrane potential resulted in release of cytochrome C from mitochondrial to cytosol and initiates apoptosis. As expected, co-treatment with oridonin and $As_2O_3$ caused cytochrome C release, and this relocation was inhibited by NAC (Fig. 4C).

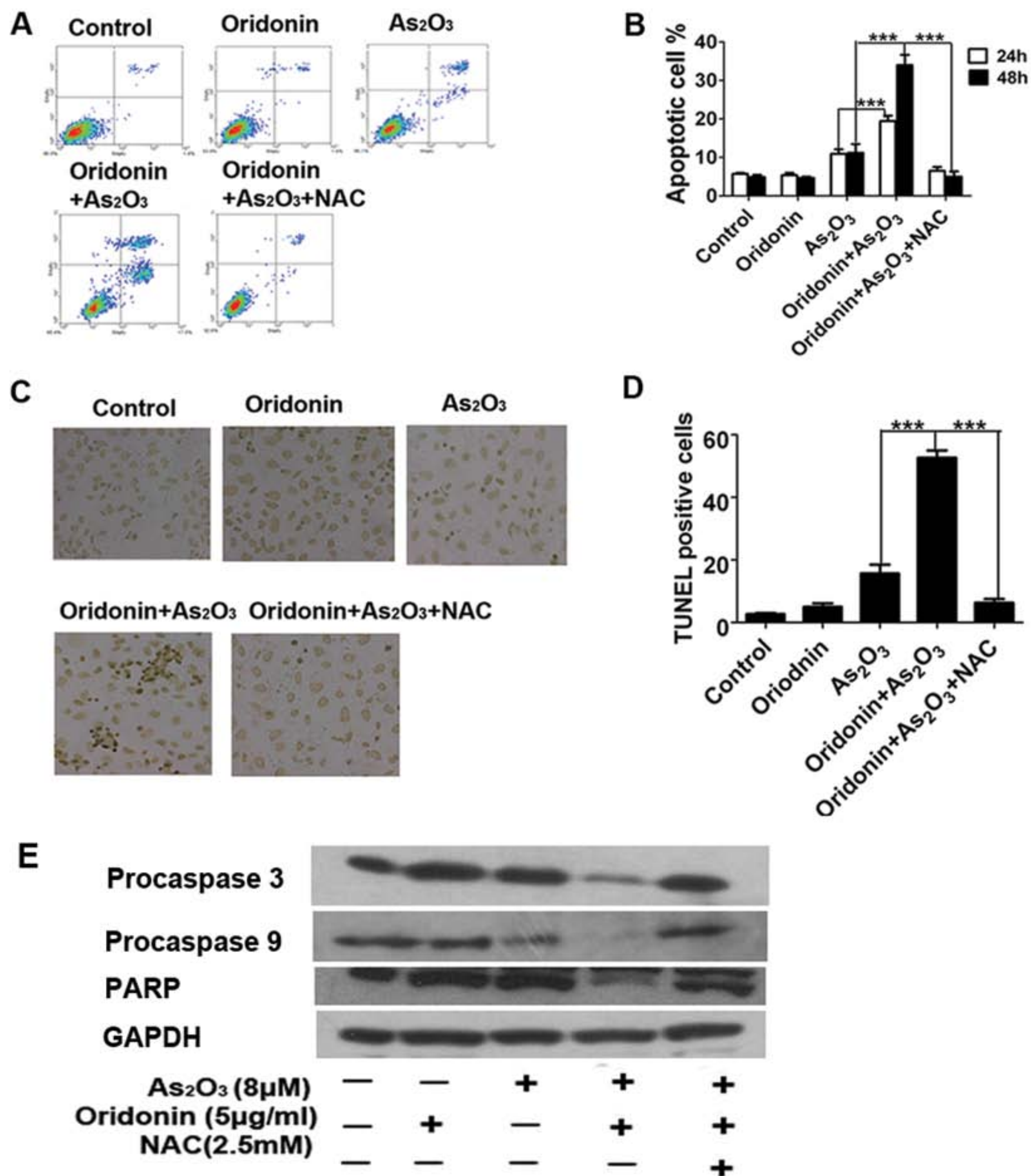


Figure 2. Apoptosis analysis by annexin V/PI stains (A and B) and TdT-mediated dUTP nick-end labeling (TUNEL) assays (C and D). Bel7402 cells were treated with 5 $\mu$g/ml oridonin, 8 $\mu$M $As_2O_3$, the combination of two drugs, or the combination of two drugs plus 2.5 mM NAC for 24 or 48 h. Annexin V/PI stains was analyzed by flow cytometry, density plots for 48-h treatment (A) and the percentage of annexin V positive cells was counted (B). Cells treated as mentioned above for 24 h were fixed for TUNEL assay as described in Materials and methods. Detection by microscope magnification x200) (C), the number of TUNEL positive cells was quantified (D). Representative experiments, n=3, mean ± SD. $^{***}p<0.001$. (E) Cleavage of procaspase 3, procaspase 9 and PARP. After treatment, Bel7402 cells were lysed and subjected to Western blot analysis.

It has been shown that translocation of Bax from cytosol to mitochondrial could cause loss of mitochondrial membrane potential (23). We investigated whether the combination of oridonin and $As_2O_3$ induces translocation of Bax. As shown in Fig. 4C, co-treatment with oridonin and $As_2O_3$ caused remarkable increase of Bax in mitochondria and reduction in the cytosol.

Because excessive ROS could damage cellular components and trigger intracellular $Ca^{2+}$ overload (24), we used Fluo4 to monitor intracellular $Ca^{2+}$ level. As shown in Fig. 4D and E, treatment with $As_2O_3$ alone increased the intracellular $Ca^{2+}$ level and oridonin enhanced $As_2O_3$-induced intracellular $Ca^{2+}$ overload. Although it has been known that elevation of cytosolic-free calcium concentration could play a role as a signal for the activation of apoptosis, pre-treatment of Bel7402 cells with 25 $\mu$M BAPTA/AM, an intracellular calcium chelator, could not block this synergistic cytotoxicity induced by combination of $As_2O_3$ and oridonin (data not shown).

*Enhanced activation of MAPKs by co-treatment with oridonin and $As_2O_3$.* Mitogen-activated protein kinases (MAPKs) are activated by a wide variety of cellular stimuli and involved in the regulation of most cellular processes. To investigate a potential involvement of MAPKs in combination treatment induced cell death, we analyzed the activation status of p38, ERK, JNK. As shown in Fig. 5, combination treatment of cells with oridonin and

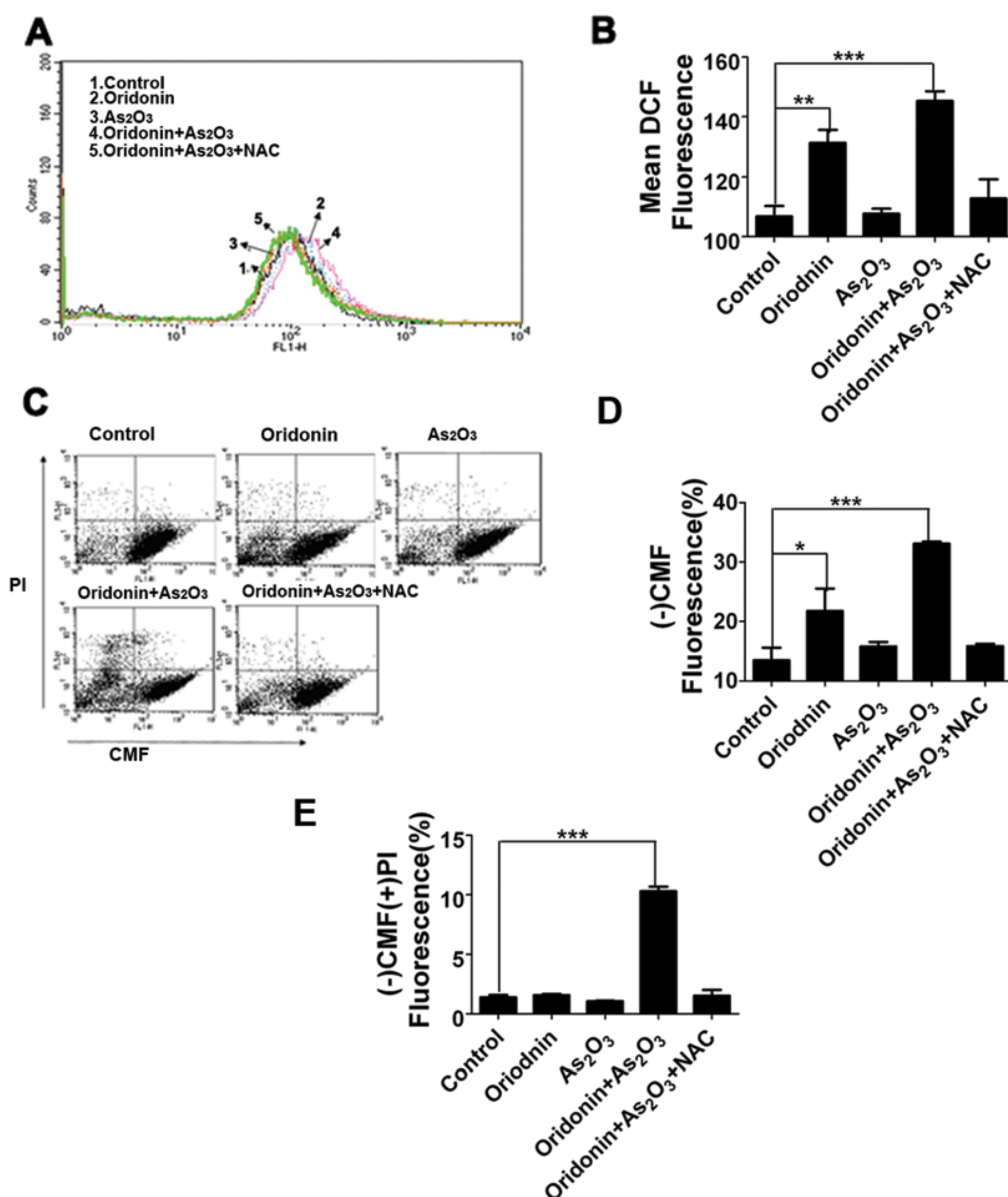


Figure 3. Detection of intracellular ROS and GSH level. Bel7402 cells were treated with 5 $\mu$g/ml oridonin, 8 $\mu$M $As_2O_3$, the two-drug combination, or the combination plus 2.5 mM NAC for 20 h. DCF fluorescence was measured by flow cytometry (A) and the mean fluorescence of DCF is shown (B). CMF and PI double staining was analyzed by flow cytometry in dot plots (C). The percentage of CMF fluorescence negative cells (D) and CMF fluorescence negative and PI fluorescence positive cells (E) are shown. Representative experiments, n=3, mean ± SD. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

$As_2O_3$ resulted in dramatic increase of the phosphorylated form of p38, ERK1, JNK1/2, and the activation of these kinases could be blocked by the anti-oxidization agent NAC. These results suggested that combination treatment with oridonin and $As_2O_3$ induced ROS stress-dependent MAPKs activation. Pre-treatment of cells with SB203580, SP600125, U0126, respectively as p38, JNK, ERK specific inhibitor, failed to shown any obvious changes in combination treatment-induced cytotoxicity effects (data not shown).

*Inhibition of Akt, XIAP expression and NF-κB activity.* To explore pro-survival signaling pathways in this combination strategy, we investigated akt, X-linked inhibitor of apoptosis (XIAP) expression and NF-κB activity in Bel7402 cells. As shown in Fig. 6A, co-treatment with oridonin and $As_2O_3$ significantly decreased Akt, X-linked inhibitor of apoptosis (XIAP) expression and NAC could also block this effect. Besides, Akt phosphorylation (p-Akt) which represents the activated form was not detected in Bel7402 cells (data not shown). The activation of NF-κB was evaluated using NF-κB driven luciferase reporter assay and co-treatment with oridonin and $As_2O_3$ significantly reduced the activity of NF-κB compared with administration of $As_2O_3$ alone (Fig. 6B).

*Enhanced antitumor activity in vivo of the two-drug combination.* To evaluate the anti-tumor efficacy *in vivo*, we used the subcutaneous murine hepa1-6 tumor model and intraperitoneal administration of drugs (3 mg/kg $As_2O_3$, 10 mg/kg oridonin or

Figure 4. Measurement of mitochondria membrane potential and cytoplasmic calcium. Bel7402 cells were treated with 5 $\mu$g/ml oridonin, 8 $\mu$M $As_2O_3$, the two-drug combination, or the combination plus 2.5 mM NAC for 20 h. The histograms (A) and mean fluorescence intensity (B) for DiOC6 are shown. Bax and cytochrome C relocation were detected by Western bloting, mitochondrial and cytosol fractions were denoted as M and C respectively (C). The histograms (D) and mean fluorescence intensity (E) of Fluo 4 dye was used for labeling intracellular calcium spikes. Representative experiments, n=3, mean ± SD. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

their combination) to tumor bearing mice. These comparable low doses of oridonin and $As_2O_3$ have been used in previous combination therapy studies (25,26). Consistence with the synergistic pro-apoptosis effect *in vitro*, the two-drug combination displayed significantly enhanced antitumor activity compared with that induced by oridonin or $As_2O_3$ alone (Fig. 7A and B).Besides, the body weight loss of mice in the two-drug combination was not significant when compared with that in $As_2O_3$ single agent treatment (Fig. 7C and D).

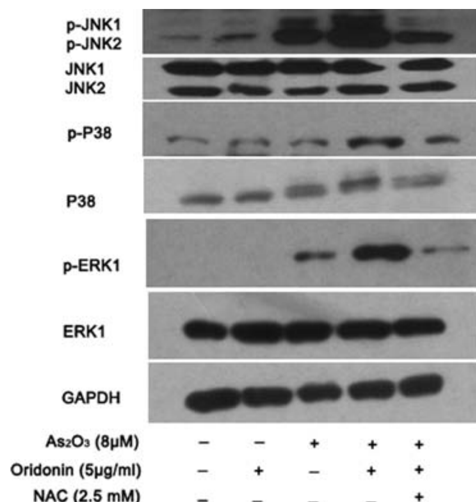


Figure 5. Detection of JNK, p38 and ERK activation. Bel7402 cells were treated with 5 $\mu$g/ml oridonin, 8 $\mu$M $As_2O_3$, the two-drug combination, or the combination plus 2.5 mM NAC for 20 h, the total cell extracts were subjected to Western blot analysis.

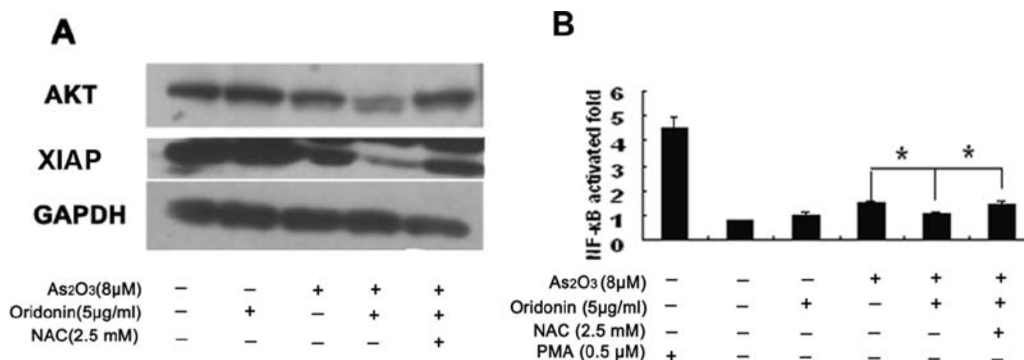


Figure 6. Analysis of Akt and XIAP protein expression (A) and NF-κB activation (B). Bel7402 cells were treated with 5 $\mu$g/ml oridonin, 8 $\mu$M $As_2O_3$, the two-drug combination, or the combination plus 2.5 mM NAC for 24 h, total cell extracts were subjected to Western bloting for Akt, XIAP and p65. In the luciferase assay, cells were treated as described in Materials and methods, PMA was used as positive control. Representative experiments, n=3, mean ± SD. *p<0.05.

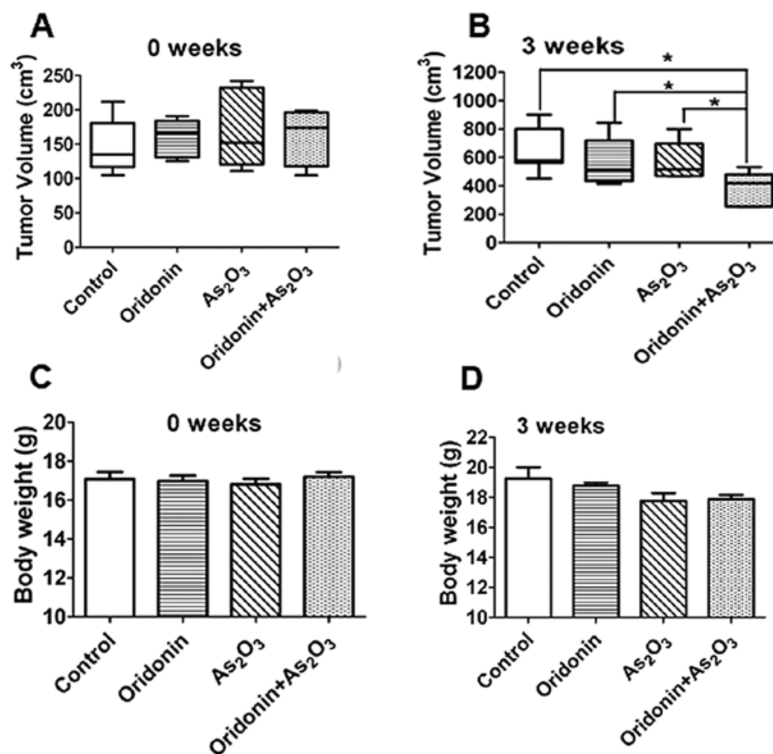


Figure 7. Evaluation of antitumor activity of $As_2O_3$, oridonin or the two-drug combination in murine HCC model. Hepa1-6 tumor bearing mice received daily injection of 200 $\mu$l 0.9% NaCl, equal volume of $As_2O_3$ at dose of 3 mg/kg, oridonin at dose of 10 mg/kg or the combination of the two drugs for 3 weeks. The tumor volume (A and B) and body weight (C and D) at 0 or 3 weeks after administration were measured. Each bar represents the mean ± SD. *p<0.05 (n=7 mice).

## Discussion

The recent phase II study of arsenic trioxide ($As_2O_3$) in patients with advanced hepatocellular carcinoma (HCC) showed that $As_2O_3$ was less active than sorafenib, an FDA approved drug for treatment of advanced HCC, and potential cardiotoxicity may hinder its further application (27). Numerous reports have demonstrated that combination treatment with $As_2O_3$ and other therapeutic agents could sensitize leukaemic or solid cancer cells to $As_2O_3$-induced apoptosis and overcome drug resistance in the treatment of various haematopoietic or solid malignancies (28-32). The goal of this study was to develop $As_2O_3$-based effective combination therapy against hepato-cellular carcinoma. We found that oridonin, a diterpenoid isolated from traditional Chinese medicine *Rabdosia rubescens*, in combination with $As_2O_3$ dramatically enhanced anti-tumor efficacy on human hepatoma cells *in vitro* and *in vivo* and this synergistic pro-apoptotic effect resulted from ROS mediated mitochondrial dysfunction and pro-survival signal inhibition.

It is known that ROS is a critical mediator for $As_2O_3$ induced apoptotic cell death (33), but treatment of Bel7402 cells with a relative low concentration of $As_2O_3$ (8 $\mu$M) induced no detectable ROS production. Cancer cells seem to have higher level of endogenous oxidative stress compared with normal cells, and the upregulation of antioxidant capacity in adaptation to intrinsic oxidative stress can confer drug resistance (34). In this study, we demonstrated that oridonin at a relative low dose,

which induced no detectable cells death, could decrease the GSH/GSSG ratio and elicit intracellular ROS level in Bel7402 cells. The increased ROS production induced by comparable low dose of oridonin did not reach the threshold to damage mitochondria leading to apoptosis, but it interrupted tumor intracellular redox status and abrogated the drug resistance of hepatoma cells to $As_2O_3$ treatment.

Accumulation of excessive ROS in leukemia cells in combined treatment with $As_2O_3$ and phytosphingosine led to disruption of the mitochondrial membrane potential, release of apoptotic factors, and resulted in apoptotic cell death (30). In this study, we also provided evidence that the elevation of intracellular ROS level induced by the oridonin-$As_2O_3$ combination treatment is essential for the loss of mitochondrial membrane potential and cytochrome C release. Complete inhibition of the two-drug combination treatment-induced apoptotic death by antioxidant NAC suggested that the increase of intracellular ROS level is critical for this synergic anti-tumor effect.

The involvement of members of MAPKs in stress induced apoptosis depends on the cell type, stimuli, and the latency of the activation (35). It has been shown that p38 plays a key role in combination treatment with phytosphingosine and $As_2O_3$-induced cell death in leukemic cells (30). For hepatoma cells, a previous study showed that $As_2O_3$-induced apoptosis is independent of MAPKs signaling pathways in HepG2 cells (36). Consistence with the study in HepG2 cells, p38, ERK1 and JNK1/2 were stimulated in response to combination treatment with oridonin and $As_2O_3$, but inhibition of their activation using a specific inhibitor failed to protect cells from death induced by the two-drug combination. Thus, MAPKs might not play important roles in executing proapoptotic activity in treatment of hepatoma with $As_2O_3$.

Overactivity of the cell survival signaling pathway appears to be a critical switch in progression and drug resistance of tumor cells (37). The pro-survival molecules such as Akt, XIAP and NF-κB were investigated in this study as they are key protecting factors against multiple chemotherapeutic treatment. Of note, single treatment with oridonin or $As_2O_3$ failed to decrease Akt and XIAP expression, but two-drug combination resulted in drastic downregulation of these two factors. Inhibition of NF-κB pathway has been reported by other studies and seems to play a role in synergic therapeutic effect induced by combination treatment with $As_2O_3$ and other agents (32). In this study we also found that NF-κB activation was significantly inhibited by oridonin and $As_2O_3$ combination treatment. This synergistic suppression of survival signaling factors may also attribute to the increase of intracellular ROS level, because NAC could block this effect.

In conclusion, the present results showed that combination treatment with oridonin and $As_2O_3$ synergize in the induction of apoptosis and antitumor efficacy in hepatocellular carcinoma cells and this synergistic anti-tumor effect results from mitochondrial apoptotic pathway by elevation of intracellular ROS level. This study facilitates the optimization of $As_2O_3$ therapy of HCC with improved anticancer efficacy and reduced adverse effects.

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