Original Article

Oridonin Induces Apoptosis in Human Nasopharyngeal Carcinoma Cells Involving ROS Generation

(nasopharyngeal carcinoma / oridonin / apoptosis / reactive oxygen species)

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Abstract. Oridonin, an *ent*-kaurene diterpenoid isolated from the traditional Chinese herb *Rabdosia rubescens*, has been reported to be a potent cytotoxic agent against a wide array of cancer cells. However, its effect on human nasopharyngeal carcinoma (NPC) cells has not been well investigated. The present study aimed to explore the anti-tumour effect of oridonin in NPC cells and its underlying mechanisms. Cell viability was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay and colony formation assay. Apoptosis, mitochondrial membrane potential (MMP), reactive oxygen species (ROS), and expression of apoptosis-related proteins were analysed by flow cytometry with propidium iodide staining, JC-1 staining, DCFH-DA staining, and Western blot analysis, respectively. The results showed that oridonin concentration-dependently inhibited the cell viability, decreased the colony formation, and enhanced the apoptotic rate in NPC cells. Further, oridonin-induced apoptosis was mediated by the mitochondrial pathway in NPC cells, which was confirmed by the loss of MMP, downregulation of anti-apoptotic Bcl-2 family protein Mcl-1 and Bcl-2, upregulation of pro-apoptotic Bcl-2 family member Bax, and activation of caspase-3 and PARP. Notably, the augmented ROS generation played an essential role in oridonin-induced apoptosis in NPC cells, as the apoptosis-inducing effect was attenuated by ROS scavenger N-acetyl-L-cysteine. These results indicate that oridonin triggers apoptosis through the ROS-mediated mitochondrial pathway in NPC cells. This study supports oridonin to be an interesting candidate drug for the treatment of human NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is a highly prevalent head and neck cancer in Asia, especially in Southeast Asia and China, with an annual incidence rate of approximately 20–50 cases per 100,000 individuals (Jemal et al., 2011). A combination of radiotherapy and adjuvant chemotherapy is now the primary treatment for NPC. However, the 5-year survival rate is only 50–60 % due to distant metastasis, local recurrence, as well as development of drug resistance. Moreover, there is almost no effective therapy for those who are resistant to radiotherapy and have recurrence. Therefore, an alternative approach using less toxic compounds is of high value in NPC treatment.

Plants supply a wide range of compounds possessing medicinal properties and being used by humans from the ancient times to modern days. Natural products have been increasingly applied as anti-tumour agents in recent decades, and have played vital roles in the design and development of more than 60 % of the anti-tumour drugs in clinical application (Kingston, 2011; Newman and Cragg, 2012). Many cytotoxic chemotherapeutic drugs currently used, such as taxol, vincristine and etoposide, are originally obtained from herbs. These drugs are still applied as the first line treatment for many cancers. Meanwhile, there are many drugs of plant origin that are still under laboratory evaluations or clinical

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Abbreviations: BCA – bicinchoninic acid, DCFH-DA – 2',7'-dichlorofluorescein diacetate, DMSO – dimethyl sulphoxide, FADD – Fas-associated death domain, FBS – foetal bovine serum, JC-1 – 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide, MMP – mitochondrial membrane potential, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, NAC – N-acetyl-L-cysteine, NPC – nasopharyngeal carcinoma, PI – propidium iodide, PVDF – polyvinylidene fluoride, ROS – reactive oxygen species, SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

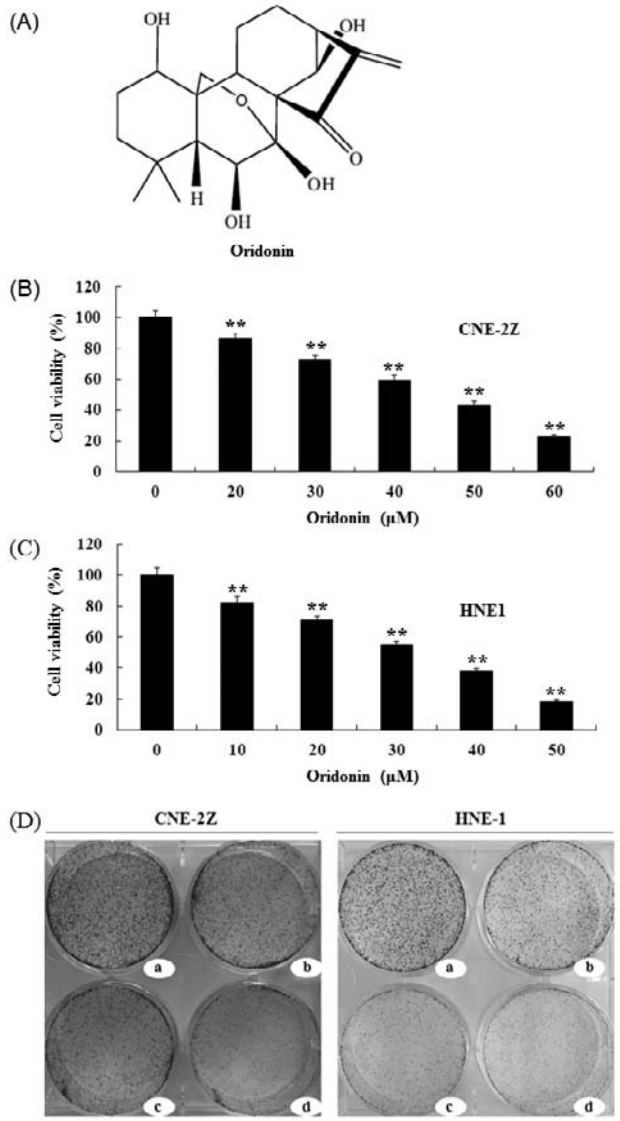

Fig. 1. Inhibitory effects of oridonin on the viability of NPC cells

(**A**) Chemical structure of oridonin. (**B**, **C**) The cells were treated with DMSO or oridonin (20, 30, 40, 50, 60 μmol/l in CNE-2Z cells, and 10, 20, 30, 40, 50 μmol/l in HNE-1 cells) for 48 h. Cell viability was determined by the MTT assay. **P < 0.01 *vs* control. (**D**) The cells were treated with: **a**) DMSO, **b**) 3 μmol/l oridonin, **c**) 4 μmol/l oridonin, **d**) 5 μmol/l oridonin in CNE-2Z cells, and **a**) DMSO, **b**) 2 μmol/l oridonin, **c**) 3 μmol/l oridonin, **d**) 4 μmol/l oridonin in HNE-1 cells for 7 days, then the colonies were analysed by crystal violet staining.

trials (Mondal et al., 2012). Oridonin is an *ent*-kaurene diterpenoid isolated from *Rabdosia rubescens* (Dong Ling Cao in Chinese) (structure shown in Fig. 1A), a traditional Chinese herb that is widely used to treat inflammatory diseases and cancers (Li et al., 2011). Previous *in vitro* and *in vivo* studies have demonstrated that oridonin displays strong anti-tumour activities against leukaemia, liver cancer, cervical cancer, epidermal squamous cell carcinoma, and other malignant diseases (Liu et al., 2012; Zhao and Chen, 2014). Accumulating studies have illustrated that the anticancer mechanisms are mainly correlated with the induction of apoptosis, autophagy and cell cycle arrest, inhibition of migration and invasion, as well as generation of intracellular reactive oxygen species (ROS) (Qi et al., 2012; Wang et al., 2013; Li et al., 2015; Shi et al., 2016; Xu et al., 2016). Due to its excellent bioactivities, low toxicity and promising anticancer profile, oridonin has become an attractive candidate compound for cancer therapy (Li et al., 2016). However, its effect on NPC cells has not been well investigated.

In the present study, we investigated the effects of oridonin on the proliferation and apoptosis in NPC cells. In an effort to explore the mechanism by which oridonin induced apoptosis, we determined its effect on mitochondrial membrane potential (MMP), expression of apoptosis-related proteins and production of ROS in NPC cells.

Material and Methods

Reagents and antibodies

RPMI-1640 medium and foetal bovine serum (FBS) were from Gibco (Grand Island, NY). Oridonin was purchased from Merck (Kenilworth, NJ). N-acetyl-L-cysteine (NAC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and propidium iodide (PI) were obtained from Sigma (Saint Louis, MO). The 2',7'-dichlorofluorescein diacetate (DCFH-DA) kit and the 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide (JC-1) kit were obtained from Beyotime (Wuhan, China). The anti-PARP, caspase-3 antibodies were obtained from Abcam (Cambridge, UK). The anti-Mcl-1, Bcl-2, Bax and β-actin antibodies were purchased from ProteinTech (Chicago, IL). Dimethyl sulphoxide (DMSO) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was from BioSharp (Hefei, China).

Cell culture

Human nasopharyngeal carcinoma cell lines CNE-2Z and HNE-1 were obtained from Shanghai Cell Bank (Shanghai, China). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. These cells were maintained in a humidified 5% CO_2 containing atmosphere at 37 °C.

Cytotoxicity assay

CNE-2Z and HNE-1 cells were plated in 96-well plates at a volume of 100 μl per well (6×10^4 cells/ml) and incubated overnight to allow the cells to adhere tightly. The medium was replaced with fresh medium the next day. The cells were treated with DMSO or various concentrations of oridonin and incubated for 48 h in an incubator. After incubation, 15 μl of MTT solution (5 mg/ml) was added to each well for an additional 4 h.

Then the medium from each well was removed and replaced by 150 µl of DMSO to dissolve the violet blue crystals. The cytotoxicity of oridonin was quantified by measuring the absorbance at 570 nm using a spectrophotometer.

Colony formation assay

Cells were seeded in 6-well plates (1×10^4 cells/well) and allowed to adhere overnight, then treated with DMSO or oridonin in the growth medium for 7 days. The colonies were washed twice with PBS, fixed with 4% paraformaldehyde for 20 min at –20 °C, stained with 2% crystal violet for 10 min at room temperature, washed with distilled water, dried at room temperature, and photographed by a camera.

Apoptosis analysis

Cells were incubated with DMSO or different concentrations of oridonin for 48 h. The cells were harvested, washed with PBS, subjected to hypotonic solution (50 µg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100) staining. Cells permeable to PI were then evaluated using a BD Accuri C6 flow cytometer (Becton Dickinson and Co., Piscataway, NJ). Apoptosis was evaluated according to the fraction of cells with sub-G1 DNA content.

MMP analysis

To detect MMP in the different treatment groups, cells were seeded in 6-well plates and treated with DMSO or oridonin for 24 h. Then, the cells were subjected to JC-1 staining according to the manufacturer's protocol and detected using a fluorescence microscope (Olympus, Tokyo, Japan).

Intracellular ROS detection

Cells were seeded in 6-well plates and treated with DMSO or oridonin for 5 h. A fluorescent DCFH-DA kit was used to determine the intracellular ROS levels. Briefly, cells were incubated with DCFH-DA (10 µmol/l) for 20 min at 37 °C in the dark, washed with serum-free medium three times, and detected by flow cytometry. To remove the oridonin-induced production of ROS, ROS scavenger NAC (5 mmol/l) was incubated with the cells for 1 h before the addition of DMSO or oridonin, and ROS generation was measured.

Western blot analysis

The expression of apoptotic signalling proteins was analysed by the Western blot technique. Cells were treated with DMSO or oridonin for 24 h, harvested and homogenized in RIPA buffer for 30 min on ice, and centrifuged at 12,000 rpm (Microfluge 22R Centrifuge, Beckman Coulter, Brea, CA) for 30 min at 4 °C. The protein concentrations were determined by the bicinchoninic acid (BCA) assay. Equivalent amounts of protein from each sample were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were then blocked with 5% skim milk, incubated overnight with respective primary antibodies at 4 °C, followed by incubation with secondary antibodies. The protein bands were detected by an enhanced chemiluminescence substrate and imaged in a Bio-Rad gel imaging equipment (Bio-Rad, Hercules, CA).

Statistical analysis

All experiments were repeated at least three times. Data are presented as the mean ± SD. SPSS software (version 16.0) was used for statistical analysis of this study. Statistical significance was evaluated by performing one-way analysis of variance (ANOVA) followed by LSD test. P-values of less than 0.05 were considered to indicate statistically significant differences.

Results

Effects of oridonin on cell viability in NPC cells

Two NPC cell lines, i.e., CNE-2Z and HNE-1, were treated with different concentrations of oridonin for 48 h, and cell viability was determined by the MTT assay. As presented in Fig. 1B and C, oridonin significantly decreased the cell viability of NPC cells in a concentration-dependent manner. The calculated IC_{50} value was 42.3 and 28.0 µmol/l for CNE-2Z and HNE-1 cells, respectively. As detected by the colony formation assay, oridonin at lower concentrations also displayed obvious cytotoxicity for NPC cells (Fig. 1D).

Oridonin induces apoptosis in NPC cells

It has been well defined that most of the currently applied chemotherapeutic drugs exert their anti-tumour activity by inducing a programmed cell death called apoptosis in tumour cells (Dean et al., 2012). To determine whether the inhibition effect of oridonin on cell viability observed in NPC cells was relevant to apoptosis, PI staining combined with flow cytometric analysis was conducted to detect the influence of oridonin on apoptosis. After treatment for 48 h, oridonin induced obvious apoptosis in both the CNE-2Z and HNE-1 cell lines compared with DMSO treatment ($P < 0.01$). The apoptotic rate was 1.4 ± 0.6 %, 14.7 ± 2.5 %, 30.3 ± 2.9 %, and 41.4 ± 3.1 % following 48 h of treatment with 0, 30, 40, and 50 µmol/l oridonin, respectively, in CNE-2Z cells. Meanwhile, it was 1.0 ± 0.7 %, 18.1 ± 1.9 %, 34.7 ± 2.2 %, and 45.8 ± 2.4 % following 48 h of treatment with 0, 20, 30, and 40 µmol/l oridonin, respectively, in HNE-1 cells (Fig. 2A and B).

Involvement of the mitochondrial pathway in oridonin-induced apoptosis

Mitochondria are vital organelles that are involved in the release of apoptotic signals during the mitochondrial pathway for the execution of apoptosis (Ohta, 2003). Mitochondrial dysfunction leads to the loss of MMP and

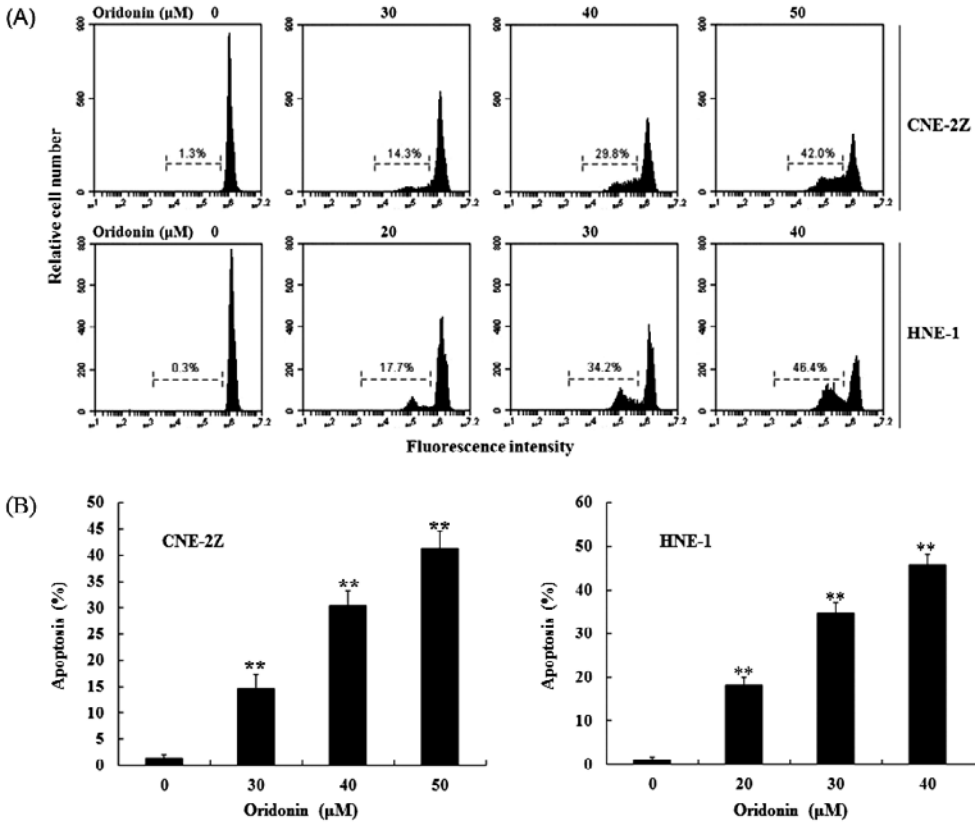

Fig. 2. Oridonin induces apoptosis in NPC cells.
(**A**, **B**) The cells were treated with DMSO or oridonin (30, 40, 50 μmol/l in CNE-2Z cells, and 20, 30, 40 μmol/l in HNE-1 cells) for 48 h, stained with PI and analysed by flow cytometry. **P < 0.01 *vs* control.

release of cytochrome c from the mitochondria into the cytosol (Kakkar and Singh, 2007), which is an important factor involved in apoptotic cell death. In order to confirm whether oridonin induced apoptosis by triggering the mitochondrial pathway, the NPC cells were treated with various concentrations of oridonin for 24 h, and MMP was detected by JC-1 staining coupled with flow cytometry. This assay is based on the principle that red fluorescence will be present in areas with high MMP, while green fluorescence will be prevalent in areas with low MMP. As shown in Fig. 3A, green fluorescence increased markedly as the concentration of oridonin enhanced, indicating loss of MMP occurring in NPC cells.

Members of the Bcl-2 family of proteins are known as pivotal regulators of the mitochondrial apoptosis that is initiated by permeabilization of the mitochondrial outer membrane upon disrupting the balance between pro-apoptotic and pro-survival cues in the cells (Bodur et al., 2016). To elucidate the molecular mechanisms participating in oridonin-induced apoptosis, the expression of Bcl-2 family proteins was analysed by Western blot analysis after treatment with different concentrations of oridonin in NPC cells. As we could observe in Fig. 3B, the expression of anti-apoptotic proteins Mcl-1 and Bcl-2 was downregulated upon oridonin exposure, while the expression of pro-apoptotic member Bax was upregulated, which confirmed that the mitochondrial pathway was involved in oridonin-induced apoptosis. To further characterize the process of apoptosis, the activation of caspase-3 and PARP was assessed. As shown in Fig. 3B, the cleavage of caspase-3 and PARP increased in the oridonin treatment group compared to the control group, which indicated the occurrence of apoptosis.

ROS generation is involved in oridonin-induced apoptosis

The intracellular redox state is an essential factor affecting apoptosis by changing the equilibrium between the cell survival and death progression. ROS is a mediator of intracellular signals and plays a critical role in causing apoptotic cell death (Clutton, 1997; Fang et al., 2016). To explore the underlying molecular mechanisms of oridonin-induced apoptosis in NPC cells, we determined ROS generation by DCFH-DA staining coupled with flow cytometry. As presented in Fig. 4A and B, oridonin exposure resulted in a concentration-dependent ROS accumulation in NPC cells compared to the control cells. Next, we tested whether ROS inhibition affected oridonin-induced apoptosis. Pre-treatment of NPC cells with 5 mmol/l NAC for 1 h effectively blocked the oridonin-induced formation (Fig. 5A and B). In addition, the PI staining assay revealed that the inhibition of ROS

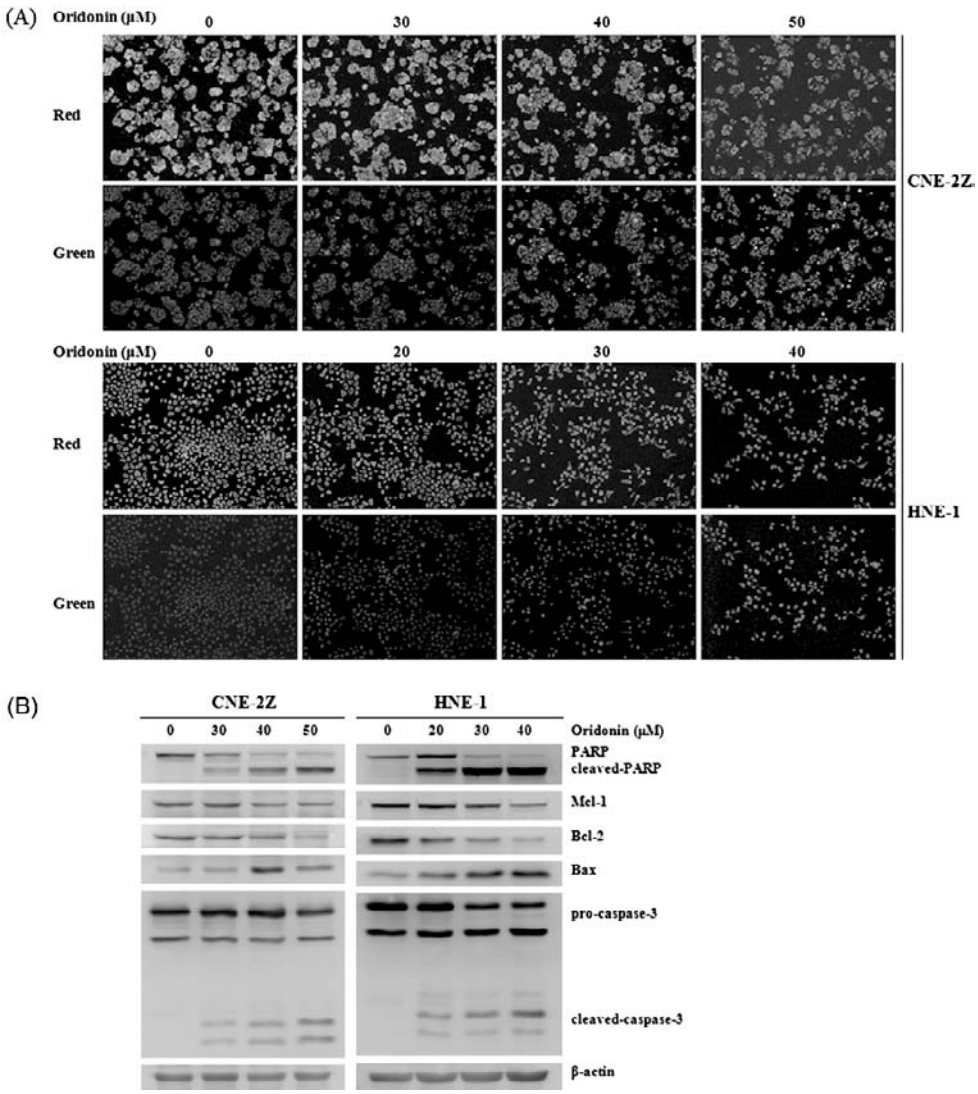

Fig. 3. Oridonin regulates MMP and expression of apoptosis-related proteins in NPC cells.
(**A**) The cells were treated with DMSO or oridonin (30, 40, 50 μmol/l in CNE-2Z cells, and 20, 30, 40 μmol/l in HNE-1 cells) for 24 h, then MMP was measured by JC-1 staining. (**B**) The cells were treated with DMSO or oridonin (30, 40, 50 μmol/l in CNE-2Z cells, and 20, 30, 40 μmol/l in HNE-1 cells) for 24 h. Whole-cell lysates were subjected to examination of the protein levels of PARP, Mcl-1, Bcl-2, Bax and caspase-3 by Western blot analysis. β-Actin was used as the loading control.

by NAC markedly attenuated the number of oridonin-induced apoptotic cells (Fig. 5C and D). Collectively, these results suggested that ROS generation was involved in oridonin-induced apoptosis in NPC cells.

Discussion

Dysregulation of proliferation and apoptosis are tightly correlated to the development and progression of most cancers. In this study, we demonstrated that oridonin significantly decreased the cell viability in CNE-2Z and HNE-1 cells in a concentration-dependent manner. Further, the result of the PI staining assay indicated that the inhibition effect of oridonin on cell viability in NPC cells was linked to apoptosis. Oridonin treatment resulted in obvious apoptosis in NPC cells as compared with DMSO treatment.

Currently, induction of apoptosis as a targeted therapy plays a critical role in the development of anticancer agents (Bai and Wang, 2014). Apoptosis, the best defined form of programmed cell death, is a tightly controlled cell suicide mechanism that is vital for eukaryotic development and elimination of harmful or unwanted cells. The process of apoptosis is mainly triggered by two pathways in mammalian cells, the intrinsic mitochondrial pathway and the extrinsic death receptor pathway (Hengartner, 2000). In the death receptor pathway, caspase-8 and caspase-10 are activated following recruitment of the Fas-associated death domain (FADD) protein to initiate the caspase cascade, resulting in acti-

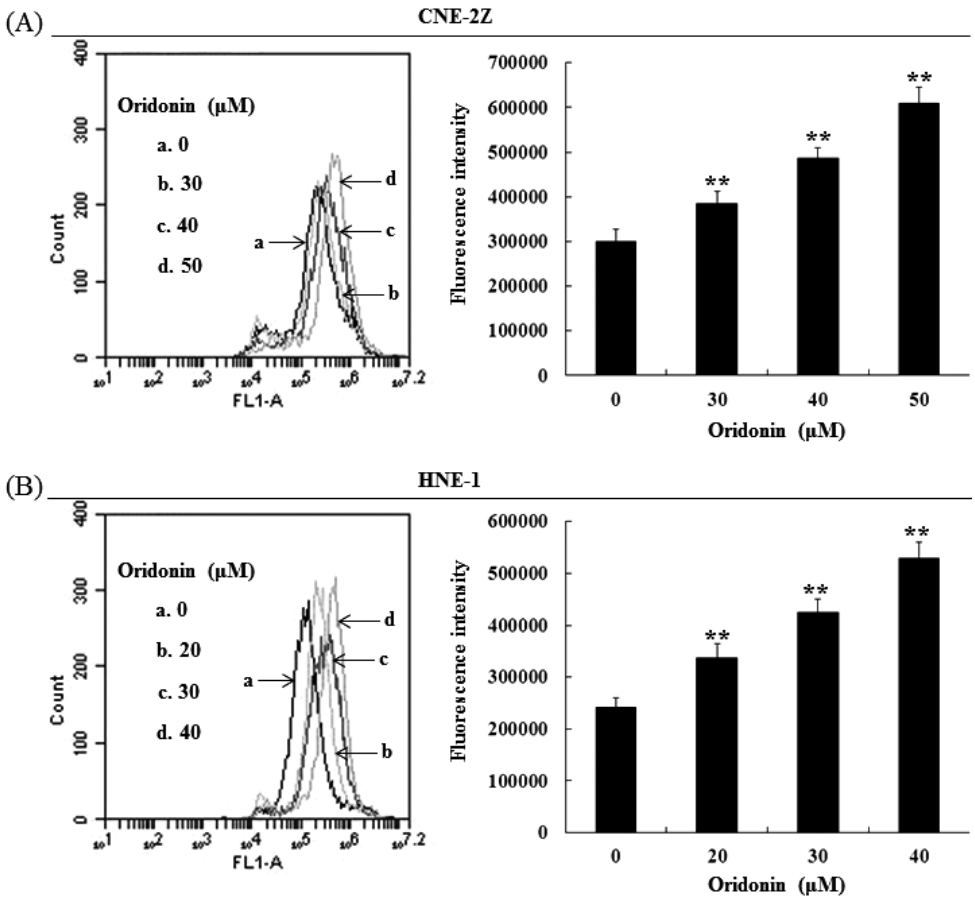

Fig. 4. Oridonin enhances ROS generation in NPC cells.
(**A**) CNE-2Z cells were treated with DMSO or oridonin (30, 40, 50 μmol/l) for 5 h, and the intracellular ROS formation was determined by DCFH-DA staining. **P < 0.01 *vs* control. (**B**) HNE-1 cells were treated with DMSO or oridonin (20, 30, 40 μmol/l) for 5 h, and the intracellular ROS formation was determined by DCFH-DA staining. **P < 0.01 *vs* control.

vation of the downstream apoptotic effectors (Bodmer et al., 2000), while in the mitochondrial pathway, cytochrome c is released from the mitochondria into the cytosol in response to various apoptotic stimuli. The release of cytochrome c mediates cleavage of caspase-9, which contributes to the activation of effector caspases such as caspase-3 (Hacker and Paschen, 2007; Scatena et al., 2007). The effector caspases cleave a set of vital proteins such as PARP and eventually lead to apoptosis (Liu et al., 1996). To explore the role of mitochondria in oridonin-induced apoptosis, we analysed perturbations in MMP under oridonin treatment. This demonstrated that changes in NPC cells associated with apoptosis were accompanied by the loss of MMP. The mitochondrial apoptotic pathway was therefore speculated to be involved in oridonin-induced apoptosis.

The Bcl-2 family, a well-known family of apoptosis-regulating proteins, plays a primary role in the mitochondrial apoptotic pathway (Volkmann et al., 2014). Bcl-2, Bcl-xL and Mcl-1 are typical anti-apoptotic members that block the release of cytochrome c from the mitochondria and thus inhibit apoptosis. In contrast, Bax is a pro-apoptotic member that triggers cytochrome c leaking from the mitochondria and activates caspase-9 (Martinou and Youle, 2011; Ola et al., 2011). The balance between anti-apoptotic and pro-apoptotic members influences the occurrence of apoptosis, and is correlated with the success rate of chemotherapy in cancer patients (Czabotar et al., 2014) . The expression of Mcl-1, Bcl-2 and Bax was examined in the present study. The results indicated that Bax was upregulated, while Mcl-1and Bcl-2 were downregulated in NPC cells following oridonin treatment. In addition, caspase-3 cleavage and PARP cleavage both increased in the oridonin treatment group. These data confirmed that apoptosis occurred in NPC cells following exposure to oridonin, and the mitochondrial pathway was involved in this process.

ROS are generated by mitochondria and in other cellular processes, and can oxidize a wide range of cell components, including proteins, lipids and DNA, thus damaging cell structures and disturbing biochemical functions. When the antioxidant mechanisms are overwhelmed by excessive ROS accumulation and subsequent oxidative stress occurs, cell damage and cell death are mediated (Nathan, 2003). ROS play an essential role in intracellular signalling, and recent evidence has re-

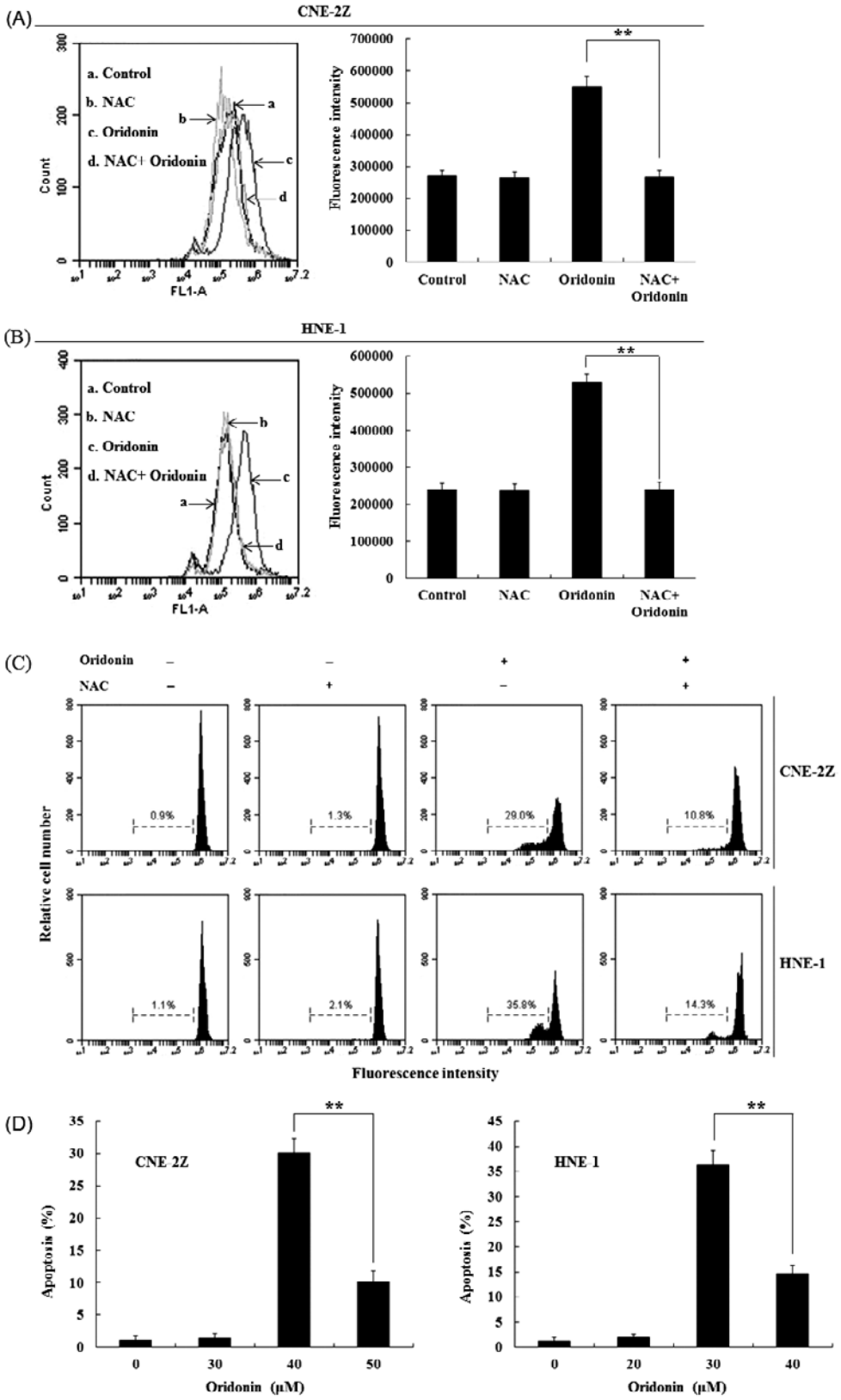

Fig. 5. Inhibition of ROS generation by NAC attenuates oridonin-induced apoptosis in NPC cells.
(**A**) CNE-2Z cells were pre-treated with 5 mmol/l NAC for 1 h, then incubated with 40 μmol/l oridonin for 5 h, and the intracellular ROS formation was determined by DCFH-DA staining. **P < 0.01. (**B**) HNE-1 cells were pre-treated with 5 mmol/l NAC for 1 h, then incubated with 30 μmol/l oridonin for 5 h, and the intracellular ROS formation was determined by DCFH-DA staining. **P < 0.01. (**C**, **D**) The cells were pre-incubated with 5 mmol/l NAC for 1 h, then treated with oridonin (40 μmol/l in CNE-2Z cells, and 30 μmol/l in HNE-1 cells) for 48 h. Apoptosis was identified by flow cytometry with PI staining. **P < 0.01.

vealed their participation in apoptosis (Jolly et al., 1997; Lin et al., 2010). They trigger apoptosis by causing different cellular stresses, including mitochondrial dysfunction, DNA damage and microtubule disruption mediated by a variety of signal transducers (Mates et al., 2012; Zhang et al., 2015). Several apoptotic regulators are redox-sensitive, such as cytochrome c, Bcl-2, and caspases, and their functions are primarily regulated by cellular ROS (Trachootham et al., 2008). A previous study has demonstrated that ROS-mediated DNA damage and JNK pathway activation were involved in oridonin-induced apoptosis in diffuse large B cell lymphoma (Xu et al., 2016). In our study, we found that oridonin treatment dramatically enhanced the generation of ROS in NPC cells, accompanied by the reduction of MMP, downregulation of anti-apoptotic proteins Mcl-1 and Bcl-2, upregulation of pro-apoptotic protein Bax, and cleavage of caspase-3 and PARP. In addition, we found that NAC, a ROS scavenger, effectively attenuated oridonin-induced apoptosis. These data implied that oridonin triggered apoptosis through the ROS-mediated mitochondrial pathway in NPC cells.

In summary, the results of the present study demonstrated that oridonin induced significant apoptosis in NPC cells, which was mediated by accumulation of ROS, loss of MMP, regulation of Bcl-2 family protein expression, and activation of caspase-3 and PARP. Notably, the augmented ROS generation played an important role in oridonin-induced mitochondrial apoptosis in NPC cells. Therefore, oridonin has the potential to be an interesting candidate drug for the treatment of human NPC.

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Pei Zhang and Su-Rong Zhao contributed equally to the study.

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