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Jaridonin-induced G2/M phase arrest in human esophageal cancer cells is caused by reactive oxygen species-dependent Cdc2-tyr15 phosphorylation via ATM–Chk1/2–Cdc25C pathway

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Abstract

Jaridonin, a novel diterpenoid from *Isodon rubescens*, has been shown previously to inhibit proliferation of esophageal squamous cancer cells (ESCC) through G2/M phase cell cycle arrest. However, the involved mechanism is not fully understood. In this study, we found that the cell cycle arrest by Jaridonin was associated with the increased expression of phosphorylation of ATM at Ser1981 and Cdc2 at Tyr15. Jaridonin also resulted in enhanced phosphorylation of Cdc25C via the activation of checkpoint kinases Chk1 and Chk2, as well as in increased phospho-H2A.X (Ser139), which is known to be phosphorylated by ATM in response to DNA damage. Furthermore, Jaridonin-mediated alterations in cell cycle arrest were significantly attenuated in the presence of NAC, implicating the involvement of ROS in Jaridonin's effects. On the other hand, addition of ATM inhibitors reversed Jaridonin-related activation of ATM and Chk1/2 as well as phosphorylation of Cdc25C, Cdc2 and H2A.X and G2/M phase arrest. In conclusion, these findings identified that Jaridonin-induced cell cycle arrest in human esophageal cancer cells is associated with ROS-mediated activation of ATM–Chk1/2–Cdc25C pathway.

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Introduction

Esophageal cancer is a relatively rare form of cancer with remarkable geographic variation in incidence (Parkin et al., 2005; Stoner et al., 2007). Some areas in the world have a much higher incidence than others (Wei et al., 2005, 2011). For example, Linzhou (formerly Linxian) and its nearby cities in Henan Province, China were reported to be the highest incidence areas for esophageal squamous cell cancer (ESCC): the average rates for men and women were estimated to be about 161 and 103 per 100,000, respectively (Wang et al., 1997; Yang, 1980; Lu et al., 1988). Until now, primary modalities for treatment of ESCC have included surgery alone and chemotherapy plus radiation therapy. However, the overall 5-year survival rate in ESCC patients undergoing definitive treatments remains very low, only ranging from 5% to 30% (American Cancer Society, 2012). Therefore, there is currently an urgent need for developing new agents for treatment of patients with ESCC.

It has been estimated that the natural world has 10 times more chemical diversity than synthetic compounds (RTI International News Release, 2005). Therefore, natural products (e.g. plant products and herbs) are rich resources for identifying new lead compounds for anti-cancer agents (Normile, 2003). Recently, we isolated a new ent-kaurene diterpenoid, named Jaridonin, from *Isodon rubescens* ("Donglingcao" in Chinese), which is a perennial herb of the *Isodon* genus belonging to the Labiatae family (Liu et al., 2011). The herb has been used as a folk, botanical medicine for the treatment of cancer (especially, esophageal cancer) and inflammatory disease by local people in Henan Province, China (Sun et al., 2006). Since 1977, the standard extract of this herb has also been developed as a drug product for treating sore throats and inflammation in China (Zou et al., 2011). The major constituents of *I. rubescens* are diterpenoids (representative compound: Oridonin and Ponicidin, Fig. 1A), and they have been reported to have anti-cancer effects against several cancers. For instance, Oridonin targets AML1-ETO fusion protein and shows potent antitumor activity with low adverse effects on t (8; 21) leukemia in vitro and in vivo, t(8; 21) is the most frequent of chromosomal translocations and creates a fusion protein, AML1–ETO in human leukemia (Zhou et al., 2007); Eriocalyxin B, a oridonin analogue, remarkably decreases the xenograft tumor size and prolongs the survival time in murine t(8; 21) leukemia models (Wang et al., 2007); and Inflexinol, a novel kaurane diterpene compound, also inhibits colon cancer cell growth in vitro and in vivo (Ban et al., 2009). We have also shown previously that Jaridonin significantly induces apoptosis of esophageal cancer cells by activating mitochondrial apoptotic pathway and inhibits proliferation of human esophageal cancer cells by causing cell cycle arrest (Ma et al., 2013). However, the involved mechanism of cell cycle arrest is not fully understood. In this study, on one hand, we documented that Jaridonin was more potent inducing cell cycle arrest in esophageal cancer cells than oridonin and ponicidin; on the other hand, we investigated the mechanism of Jaridonin-induced cell cycle arrest using EC9706 and EC109 cells as a model. Our results provide first evidence for the generation of reactive oxygen species (ROS) causing activation of ATM checkpoint signaling as a central mechanism of Jaridonin-induced G2/M phase arrest and growth inhibition in human esophageal cancer cells.

Materials and methods

Reagents and antibody

The primary antibodies for Chk1/2, Cdc2, p-Cdc2 (Tyr15), p-Cdk2 (Thr160), p-Cdc25C (Ser216) and p-H2A.X (Ser139) were purchased from Signal way antibody Inc. (Pearland, TX, USA). The antibodies against GAPDH were from Good HERE Biotech Inc. (Hangzhou, China). Antibodies for CDK2 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies for p-ATM (Ser1981), p-Chk1 (Ser345) and p-Chk2 (Thr68) were from Cell Signaling Technology (Beverly, MA). The horseradish peroxidase and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were obtained from Zhongshan Golden Bridge Biotech Inc. (Beijing, China). GSH Assay Kit, the ROS detection kit and N-acetyl-L-cysteine (NAC) were all purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Enhanced chemiluminescence detection reagents were from Pierce Biotechnology, Inc. (Rockford, IL). KU-55933 was purchased from Selleck Chemicals (Houston, TX, USA). Propidium iodide (PI) and caffeine were from Sigma (St. Louis, USA).

Cell culture conditions and compounds

Human esophageal cancer cell lines EC9706, EC109 were purchased from China Center for Type Culture Collection (CCTCC, Shanghai, China). All cell lines used in this study were within 20 passages after receipt. The cell lines were tested and authenticated by CCTCC. The human esophageal carcinoma EC9706 cell line has been proven to be esophageal carcinoma of the fungating type, which is poorly-differentiated squamous cell carcinoma (Hou et al., 2007; Li et al., 2009; Wang et al., 2006). EC109 cell line is well-differentiated (Hou et al., 2007). The normal human esophageal epithelial cells (HEECs) were obtained from Wuhan PriCells Biomedical Technology Co., Ltd. (Wuhan, China). Immunocytochemistry demonstrated the expression of cytokeratin, confirming the epithelial origin of the cells. All cell lines were cultured in RPMI 1640 medium, containing 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. Cells were incubated at 37 °C in a humidified atmosphere, containing 5% CO_2. Pure Jaridonin, oridonin and ponicidin were isolated from *I. rubescens* in our laboratory. 99.9% purity Jaridonin was used. The chemical structures are shown in Fig. 1A and were confirmed by NMR, MS and IR data as well as X-ray spectra. Purities were determined by HPLC and were all above 98%. Jaridonin, oridonin and ponicidin were dissolved in dimethyl sulfoxide (DMSO), aliquoted, and stored at −80 °C. The concentration of DMSO in culture medium was kept below 0.1% (*v/v*), a concentration known not to affect cell proliferation.

Trypan blue dye exclusion assay

Cells were plated at a density of 8×10^3/well in six-well plates in a complete culture medium. After attaching overnight, the cells were refreshed with fresh medium, and either untreated or treated as indicated in the figure legends. Twenty-four hours later, both floating and adherent cells were collected and suspended in 10 ml of PBS. The cells were then mixed with 2 ml of 0.4% trypan blue solution, and live (unstained) and dead (stained) cells were counted under an inverted microscope.

Flow cytometry for cell cycle analysis

Cells were treated with compounds at the indicated concentrations, trypsinized, washed in PBS, and fixed in ice cold 70% ethanol overnight. The fixed cells were washed twice with PBS, resuspended in hypotonic buffer (0.5% Triton X-100, 0.5 mg/ml RNase and 100 μg/ml PI in PBS), incubated at 37 °C for 30 min. DNA contents/cells were analyzed in Accuri C6 flow cytometer (Becton, Dickinson & Co.; Franklin Lakes, NJ), with a total of 10,000 cells tested. The histograms of DNA distribution were modeled as a sum of G0/G1, S and G2/M phase, using FlowJo software.

Western blotting analysis

Cells were washed with cold PBS and lysed in cell lysis buffer (25 mM Tris·HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) for 30 min on ice. Total protein contents were measured by Protein Assay Reagent (Beyotime Institute of Biotechnology, Haimen, China). Clarified protein lysates (30–80 μg) were electrophoretically resolved on denaturing SDS-polyacrylamide gel (8–12%), and then transferred to nitrocellulose membranes. The membranes were blocked with 5% non fat milk in PBST buffer (PBS, PH = 7.3 and 0.05% Tween-20) and incubated overnight at 4 °C with the primary antibodies, then probed with horseradish peroxidase conjugated secondary antibodies. Visualization of bands by an enhanced chemiluminescence kit was performed according to the manufacturer's instructions. The intensities of bands were determined by the Image-Pro Plus software. For each protein, densitometric values from controls were arbitrarily converted to 1.0, and values of samples from other groups were normalized accordingly and expressed as fold changes. GAPDH were used as loading controls.

Measurement of ROS levels

Intracellular levels of hydrogen peroxide or superoxide were determined using the redox-sensitive probes 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) or dihydroethidium (DHE) (Meeran et al., 2008; Watanabe et al., 2006). Control and treated cells were washed twice with PBS, and then incubated with 10 μM DCFH-DA or DHE for 30 min at 37 °C. Cells were washed three times with serum free RPMI1640 medium to remove extracellular fluorescent dye. The fluorescence signal was determined by a flow cytometer.

Reduced glutathione measurement

The intracellular reduced glutathione levels were measured using the GSH and GSSG Assay Kit (Beyotime, PR China). Briefly, EC9706 cells were treated with Jaridonin at the indicated concentrations for 8 hours. Cells were harvested and re-suspended in 20 μL cell medium, and were counted using hemocytometer. Ten microliters of cells was mixed with 30 μL 5% metaphosphoric acid, then frozen and thawed twice using liquid nitrogen and 37 °C water. The samples were centrifuged and the supernatant was used for GSH and GSSG assays. The intracellular levels of reduced glutathione were measured by measuring the absorbance at 412 nm using a microplate reader. The standard curve was also generated using the GSH standard provided with the kit. Equal number of cells was lysed as per manufacturer's instructions provided with the kit, and total protein contents were measured by Protein Assay Reagent (Beyotime, Haimen, China).

Statistics

Data are expressed as mean ± standard deviation (SD). The significance of the difference between different groups was determined with analysis of variance (ANOVA) and Student *t* test. The differences were considered significant at *P* < 0.05.

Results

Jaridonin caused G2/M phase cell cycle arrest in EC9706 cells

Fig. 1 depicts representative histograms for cell cycle distribution in EC9706 cells following a 12 h exposure to 0.1% dimethyl sulfoxide (DMSO) or 40 μM Jaridonin, oridonin or ponicidin. Neither oridonin nor ponicidin treatment had any appreciable effect on G2/M cells. As can be seen in Fig. 2, a 12 h exposure of EC9706 cells to growth suppressive concentrations of Jaridonin (20 and 40 μM) resulted in a statistically significant increase in G2/M fraction that was accompanied by a decrease in G0/G1 and S phase cells. For example, the percentage of G2/M fraction was increased by about 3- and 5-fold on treatment of EC9706 cells with 20 and 40 μM Jaridonin, respectively, when compared with DMSO-treated control. These results indicated that the inhibitory effect of Jaridonin against proliferation of EC9706 cells correlated with G2/M phase cell cycle arrest.

Effect of Jaridonin on cell cycle regulatory proteins

Based on an observed G2/M phase arrest in EC9706 cells by Jaridonin, we next assessed the levels of cell cycle regulators associated with this effect. Jaridonin treatment (10, 20 and 40 μM for 12 h) induced protein expression of Chk1 and Chk2 (Fig. 3). Furthermore, Cdc2, CDK2 and Cdc25C were inhibited due to phosphorylation on Tyr15, Thr160 and Ser216, respectively, following Jaridonin treatment with a slight decrease in total Cdc2 and CDK2 level (Fig. 3). Overall, these results suggested the possible involvement of Chk1/2, Cdc25C and Cdc2 in Jaridonin-induced G2/M phase arrest in EC9706 cells.,

Jaridonin-induced G2/M phase arrest involves Cdc2 (Tyr15) phosphorylation

Since we observed that cells with Jaridonin-dependent G2/M phase arrest were not able to complete mitosis under normal culture conditions, we synchronized the cells by serum starvation for 24 h and released them using medium containing serum without or with Jaridonin, and cell cycle analysis was done as a function of time from 0 to 24 h. As shown in Figs. 4A and B, control cells entered S and G2/Mphase after 4 h of the release, and passed through G0/G1, S and G2/M phases of the next cell cycle (Fig. 4A); however, Jaridonin-treated cells arrested and remained in G2/M phase even after 24 h of the treatment (Fig. 4B). Jaridonin caused a time-dependent increase in the G2/M phase cell population from 16% after 8 h to 43% after 24 h of treatment, compared with control 6.4% (Fig. 4B). These results demonstrate that Jaridonin induces an irreversible G2/M phase arrest under both unsynchronized and synchronized conditions. Next we investigated molecular mechanisms underlying this effect by assessing the phosphorylation of Cdc2 (Tyr15) under synchronized conditions. Immunoblot analysis for phospho-Cdc2 (Tyr15) showed an increased accumulation after 8 h of release and a peak after 12 h and obviously declined thereafter in control cells (Fig. 4C); however, Jaridonin treatment caused an increase in phospho-Cdc2

(Tyr15) after 8 h, which became robust at 16 h and remained elevated for up to 24 h (Fig. 4C). Together, these results showed that Jaridonin-induced G2/M phase arrest involves Cdc2 (Tyr15) phosphorylation, and therefore, we further elucidated the role of this pathway in Jaridonin efficacy in EC9706 cells.

Jaridonin activates ATM kinase

We have shown previously that Jaridonin causes DNA damage of EC9706 and EC109 human esophageal cancer cells (Ma et al., 2013). ATM is nuclear kinase identified as being activated in response to DNA damage/genotoxic stress in eukaryotic cells (Kastan and Lim, 2000; Shiloh, 2003). Based on the results described above and earlier reports, we hypothesized that Jaridonin activates ATM kinases. Jaridonin treatment (10, 20 and 40 μM) of cells for 12 h resulted in a strong increase in the levels of p-ATM protein, which showed that ATM was activated. This is further supported by the observation that Jaridonin treatment of cells for 12 h showed increased serine 139 phosphorylation of histone H2A.X as a main target for ATM(Burma et al., 2001) (Fig. 5A). Together, these findings suggest that Jaridonin causes DNA damage leading to ATM activation followed by serine 139 phosphorylation of H2A.X.

Jaridonin causes phosphorylation of Cdc25C and Cdc2, as well as G2/M phase arrest via ATM-dependent Chk1/Chk2 pathway

Since ATM kinases are known to activate Chk1 and Chk2 (Khanna et al., 2001), we next to examine whether the expression of p-Chk1 and p-Chk2 in response to Jaridonin is ATM dependent using caffeine, a known inhibitor of ATM kinases (Sarkaria et al., 1999). 10 mM caffeine pre-treatment of cells for 15 min blocked the Jaridonin-induced activation of ATM, H2A.X, Chk1 and Chk2 kinases (Fig. 5B). Chk1 and Chk2 kinases are known to phosphorylate Cdc25C phosphatase at Ser216 in response to DNA damage. Negative regulation of Cdc25C by phosphorylation at Ser216 is an important regulatory mechanism used by cells to block mitotic entry under normal cell cycle progression or after DNA damage (Sarkaria et al., 1999). The findings discussed above suggested that Jaridonin-induced ATM checkpoint signaling followed by Chk1/Chk2 activation possibly results in phosphorylation of the Cdc25C phosphatase at Ser216 and associated negative regulation, which in turn produces accumulation of inactive phosphorylated Cdc2 at Tyr15. Thus, the cell cycle progression is prevented by inhibition of the mitotic CDK. Consistent with this hypothesis, we observed that Jaridonin induces the phosphorylation of Cdc25C at Ser216 (Fig. 5B), which was inhibited by caffeine pre-treatment of cells (Fig. 5B), clearly suggesting the role of ATM-mediated Chk1/2 kinase pathway for inhibitory phosphorylation of Cdc25C (Ser216). As phospho-Cdc25C (Ser216) is impaired for dephosphorylation of Cdc2 (Tyr15), we observed a strong increase in phospho-Cdc2 (Tyr15) levels following Jaridonin treatment, which was also totally inhibited by caffeine (Fig. 5B), clearly suggesting the upstream involvement of ATM–Chk1/2–Cdc25C pathway in this effect. Next, we examined the biological relevance of these molecular alterations following caffeine and/or Jaridonin treatments. Consistent with the above results, Jaridonin induced a strong G2/M phase arrest, which was reversed by caffeine (10 mM) pre-treatment (Fig. 5C). To further confirm this observation, KU55933, a potent ATM inhibitor, was used again for examining this finding. Similar to caffeine, KU55933 (10 μM) pretreatment of cells almost

completely reversed Jaridonin induced G2/M phase arrest in EC9706 cells (Fig. 5D). Together, these results suggested that Jaridonin-induced G2/M phase arrest involves ATM checkpoint signaling.

Jaridonin-induced the accumulation of p-ATM and G2/M phase cell cycle arrest were linked to ROS generation

It has been shown previously that exposure of 293T cells to H_2O_2 causes activation of ATM (Guo et al., 2010). As the cell cycle arrest in Jaridonin treated EC9706 cells was associated with a increase in the protein level of p-ATM (Fig. 5A), we hypothesized that its increase in our model may be caused by ROS generation. We have shown previously that Jaridonin treatment caused ROS production in EC9706 cells and hydrogen peroxide is a major component of ROS induced by Jaridonin (Ma et al., 2013). In the present study, we firmly established that the Jaridonin-treated EC9706 cells exhibited a statistically significant increase in DCF fluorescence after 4 h (Fig. 6A), but ethidium bromide fluorescence was not observed any changes compared with control at both 4 and 8 h time points (Fig. 6B). However, the precise mechanism by which Jaridonin treatment causes ROS generation remains elusive. Consequently, we considered the possibility that Jaridonin-induced ROS production might be due to depletion of intracellular glutathione (GSH), which is an important intracellular anti-oxidant involved in maintaining intracellular reduction–oxidation balance (Burhans and Heintz, 2009). We explored this possibility by measuring GSH levels in EC9706 and EC109 cells following treatment with DMSO (control) or 40 μM Jaridonin for 8 h. Jaridonin treatment observably affects intracellular GSH level in either EC9706 or EC109 cell line (Fig. 6C, data related to EC109 not shown). Further, pretreatment of EC9706 cells with 5 mM NAC abrogated Jaridonin-induced decrease in the intracellular levels of reduced glutathione (Fig. 6C). These results indicated that ROS production in Jaridonin-treated EC9706/EC109 cells was caused by depletion of GSH levels.

To further examine the association of ROS generation with cell cycle arrest, we exposed EC9706 cells to Jaridonin in the absence or presence of NAC, a known antioxidant, prior to analysis of cell cycle distribution. As can be seen in Fig. 7A, the G2/M phase cell cycle arrest was observed in Jaridonin-treated (20 and 40 μM, 12 h) EC9706 cells when compared with controls. On the other hand, the G2/M phase arrest was reversed almost to control levels in EC9706 cultures cotreated with NAC(5 mM) and Jaridonin. 5 mM NAC treatment alone did not affect cell cycle distribution in EC9706 cells. Furthermore, the Jaridonin-mediated increase in the phosphorylation of ATM and Cdc2 was attenuated in the presence of 5 mM NAC (Fig. 7B). These results indicated that Jaridonin-mediated increase in p-ATM protein level and Tyr15 phosphorylation of Cdc2 was likely initiated by ROS.

Jaridonin selectively induces cell growth, G2/M phase arrest and ROS generation in esophageal squamous cancer cells but not normal cells

Next, we raised two questions whether the positive correlation between Jaridonin-induced cell cycle arrest and the alteration of ROS in EC9706 was restricted to this cell line due to its unique characteristics, and whether Jaridonin-mediated inhibition of EC9706 cell viability and cell cycle progression were selective for cancer cells. We addressed this question by

determining the effect of Jaridonin treatment on cell cycle distribution as well as ROS level using EC109 esophageal cancer cells and human esophageal epithelial cells (HEECs). As can be seen in Fig. 8A, when compared with control, treatment of EC109 cells with 20 and 40 μM Jaridonin resulted in an approximate 7- and 9-fold increase in G2/M fraction, respectively, which was relatively more pronounced in this cell line compared with EC9706 (Fig. 2). In addition, 20 μM Jaridonin-treated EC109 cells also revealed a marked alteration in ROS level (Fig. 8B). Collectively, these results indicated that the positive correlation between Jaridonin-mediated ROS generation and cell cycle arrest is not unique to the EC9706 cell line.

The viability of HEECs, assessed by trypan blue dye exclusion assay, was not significantly affected by Jaridonin treatment even at concentrations (e.g. 10, 20 and 40 μM) that were highly cytotoxic to the EC109 cell line (Fig. 8C). In agreement with these results, Jaridonin treatment did not alter cell cycle distribution in HEECs cultures even at 40 μM (Fig. 8D), a concentration that caused statistically significant enrichment of the G2/M fraction in EC9706 and EC109 cells. And, 20 μM Jaridonin treatment did not alter ROS level in HEECs (Fig. 8E). These results indicated that the effects of Jaridonin were selective for cancer cells.

Discussion

Jaridonin, a novel ent-kaurene deterpenoid, was isolated from extracts of Jiyuan *I. rubescens*. Our preliminary data have shown no or low in vivo toxicity of Jaridonin in animals (data not shown). Further experiments are ongoing to evaluate the in vivo anti-ESCC efficacy of this compound using xenograft models in nude mice. These results may provide insight for further developing this novel ent-kaurene diterpenoid compound as a new chemotherapeutic agent for treatment of ESCC. The present study indicates that Jaridonin treatment inhibits G2/M progression in ESCC cells, and that one of the plausible mechanisms accounting for the activity of Jaridonin in ESCC cells occurs through ROS-dependent activation of ATM, phosphorylation of Cdc2 and G2/M phase arrest. On the other hand, HEECs cells are highly resistant to growth inhibition as well as cell cycle arrest by Jaridonin (Figs. 8C and D) even at concentrations that are highly cytotoxic to the ESCC cells. These results suggest that Jaridonin may selectively target cancer cells but spare normal cells, which is a highly desirable property of potential cancer preventive and therapeutic agents. Jaridonin is relatively more effective than oridonin and ponicidin in G2/M arrest phase of ESCC cells. However, it remains to be determined whether this structure–activity relationship is unique to the ESCC cells or applicable to other types of cancer cells.

Cancer is frequently considered to be a disease of the cell cycle (Park and Lee, 2003). Cell cycle de-regulation resulting in uncontrolled cell proliferation is one of the most frequent alterations that occur during tumor development. Therefore, cell cycle blockade is regarded as an effective strategy for eliminating cancer cells (Buolamwini, 2000). Among the major regulated cell cycle checkpoints, the G2/M checkpoint is known to maintain chromosomal integrity by allowing cells to repair DNA damage before entering mitosis. In response to DNA damage, molecular sensors such as ataxia telangiectasia mutated (ATM) can be activated, which initiate signal transduction pathways that lead to cell cycle arrest and allow

time to correct the damage (Khanna et al., 2001). Activated ATM results activation of Chk1 and Chk2 by phosphorylation, and stabilizes p53 by phosphorylation (Al Rashid et al., 2011). Phosphorylation of Cdc25C, which is controlled by Chk1 and Chk2 activation, is involved in the G2/M transition (Bartek and Lukas, 2003). At the onset of mitosis, Cdc2 cyclin-dependent kinase and Cyclin B complexes are activated by Cdc25C-mediated dephosphorylation of the inhibitory sites on Cdc2 (Nurse, 1990). Consistent with these reports, our previous study has shown that Jaridonin could induce ROS-dependent DNA damage and increase p53 level dramatically (Ma et al., 2013). Our present study identifies Jaridonin activity in ESCC cells via ATM checkpoint signaling-dependent G2/M phase arrest. We also showed that ATM activation by Jaridonin induces phosphorylation of Chk1 (Ser345) and Chk2 (Ser516) followed by that of Cdc25C (Ser216) and Cdc2 (Tyr15). Inhibition of ATM kinases by their specific inhibitor caffeine abrogated the phosphorylation of Chk1 and Chk2 as well as Cdc25C and Cdc2. Inhibition of ATM kinases using caffeine treatment also reversed Jaridonin-induced G2/M phase arrest. Further, we also confirmed that even in the synchronized condition cells accumulate in the G2/M phase up to 24 h of Jaridonin treatment. One possible reason could be that Jaridonin-induced DNA damage is not getting repaired leaving the cells in G2/M phase, which is later removed by the cytotoxic effect of the compound. These results suggest that Jaridonin induces irreparable DNA damage through ATM-dependent signaling causing G2/M phase arrest in EC9706 cells. The results of our study are also in accord with some other naturally occurring cancer preventive agents. In recent studies it has been shown that gallic acid or Jaceosidin-induced G2/Marrest in human cancer cells was associated with a rapid and sustained phosphorylation of Cdc25C at Ser216 via activation of Chk1/2 (Agarwal et al., 2006; Cho et al., 2011).

The present study also indicates, for the first time, that the Jaridonin-induced ATM-dependent signaling causing G2/M phase arrest in ESCC cells is initiated by ROS generation. This conclusion is based on the following observations: (a) Jaridonin treatment causes ROS generation in both EC9706 and EC109 cells, (b) the Jaridonin-induced increase of p-ATM and phosphorylation of Cdc2 is attenuated in the presence of NAC, and (c) the cell cycle arrest caused by Jaridonin is also abolished on cotreatment with NAC.

In summary, this study provides a mechanistic basis for Jaridonin efficacy selectively in human esophageal cancer cells, but not in normal HEECs. Our data indicate that Jaridonin efficacy in EC97096 and EC109 cell lines is via ROS-dependent DNA damage and G2/M phase arrest involving ATM–Chk1/2–Cdc25C–Cdc2 pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

ROS	Reactive oxygen species
NAC	N-acetyl-L-cysteine
DHE	Dihydroethidium
DCFH-DA	2′,′-Dichlorodihydrofluorescein diacetate
ESCC	Esophageal squamous cell cancer
ATM	Ataxia telangiectasia mutated kinase
Cdc25C	Cell division cycle 25C
Cdc2	Cyclin-dependent kinase 1

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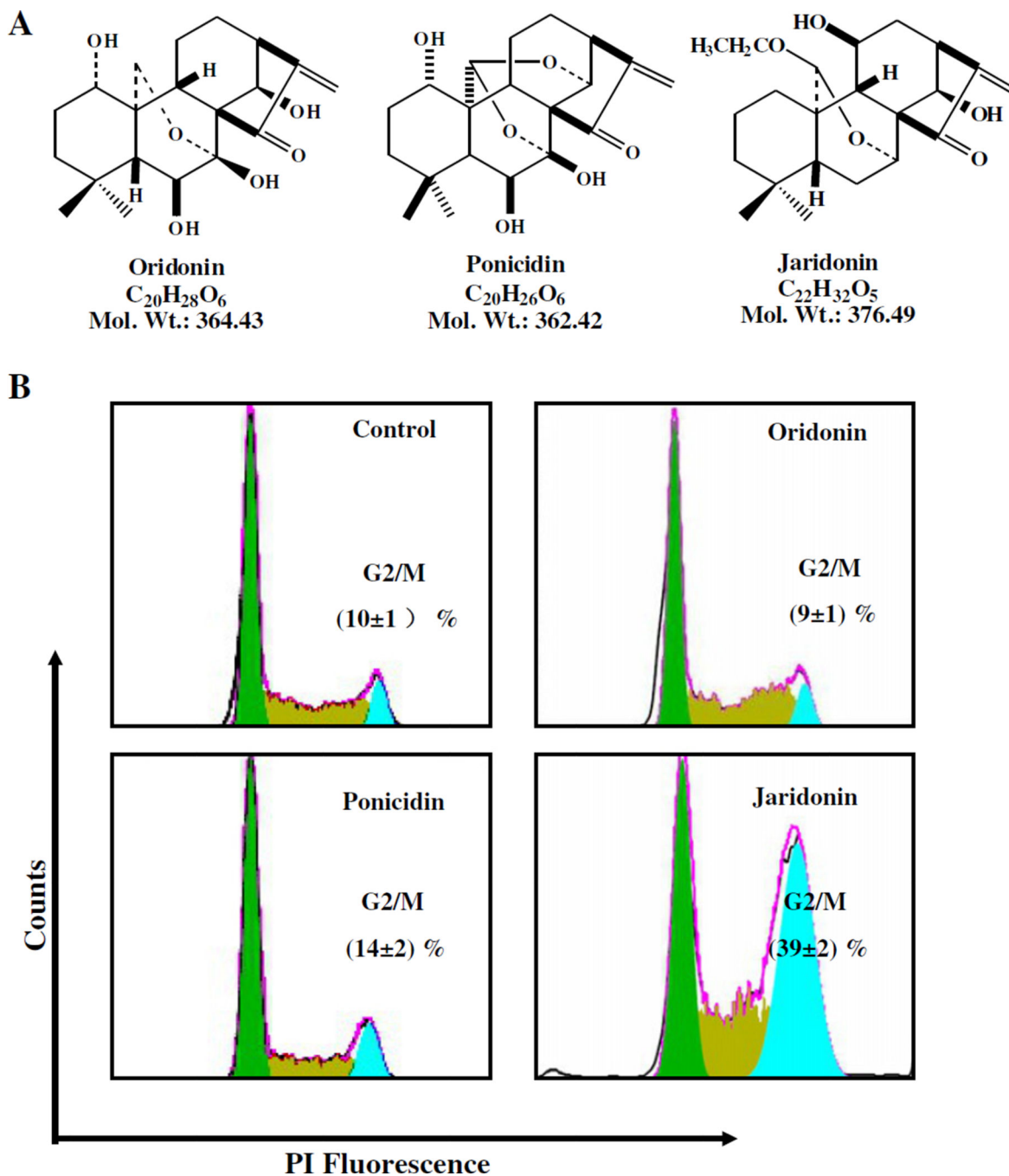
Fig. 1.
(A) Chemical structures of Jaridonin, oridonin and ponicidin. (B) Representative histograms depicting cell cycle distribution in EC9706 cultures treated with 0.1% DMSO (control) or 40 μM oridonin, ponicidin or Jaridonin for 12 h. Similar results were observed in three independent experiments.
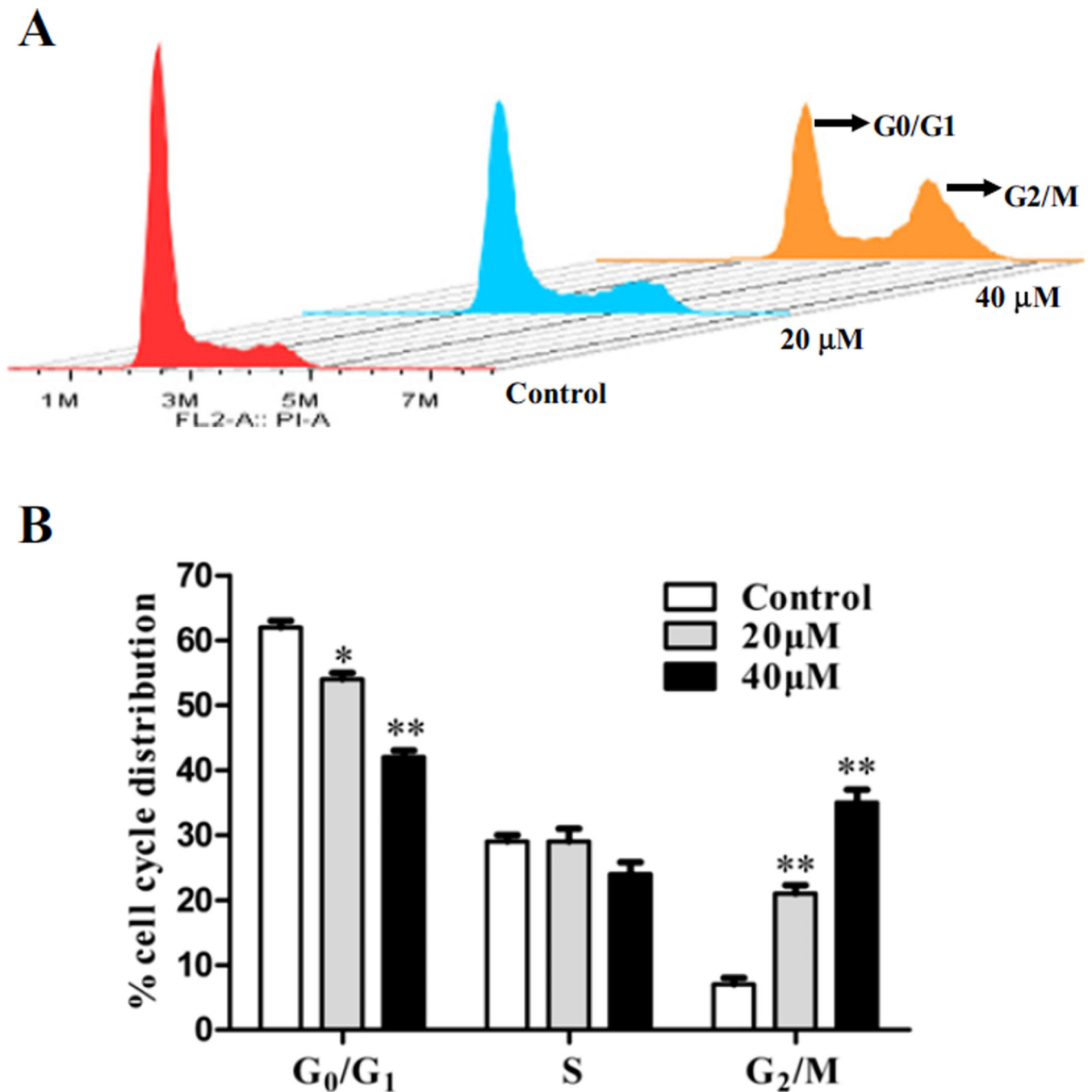
Fig. 2.
Jaridonin induces G2/M phase arrest in the cell cycle progression of EC9706 cells. Cells were cultured as described in Materials and methods and treated with either 0.1% DMSO (control) or varying concentrations of Jaridonin for 12 h. At the end of these treatments, cells were collected and incubated with PI solution at room temperature (RT) for 1 h in dark and subjected to FACS analysis as detailed in materials and methods. (A) Representative histograms depicting cell cycle distribution in EC9706 cultures treated with 20 or 40 μM

Jaridonin for 12 h. (B) Data are presented as mean ± SD of triplicate samples. * $P < 0.05$; ** $P < 0.01$ as compared with control.
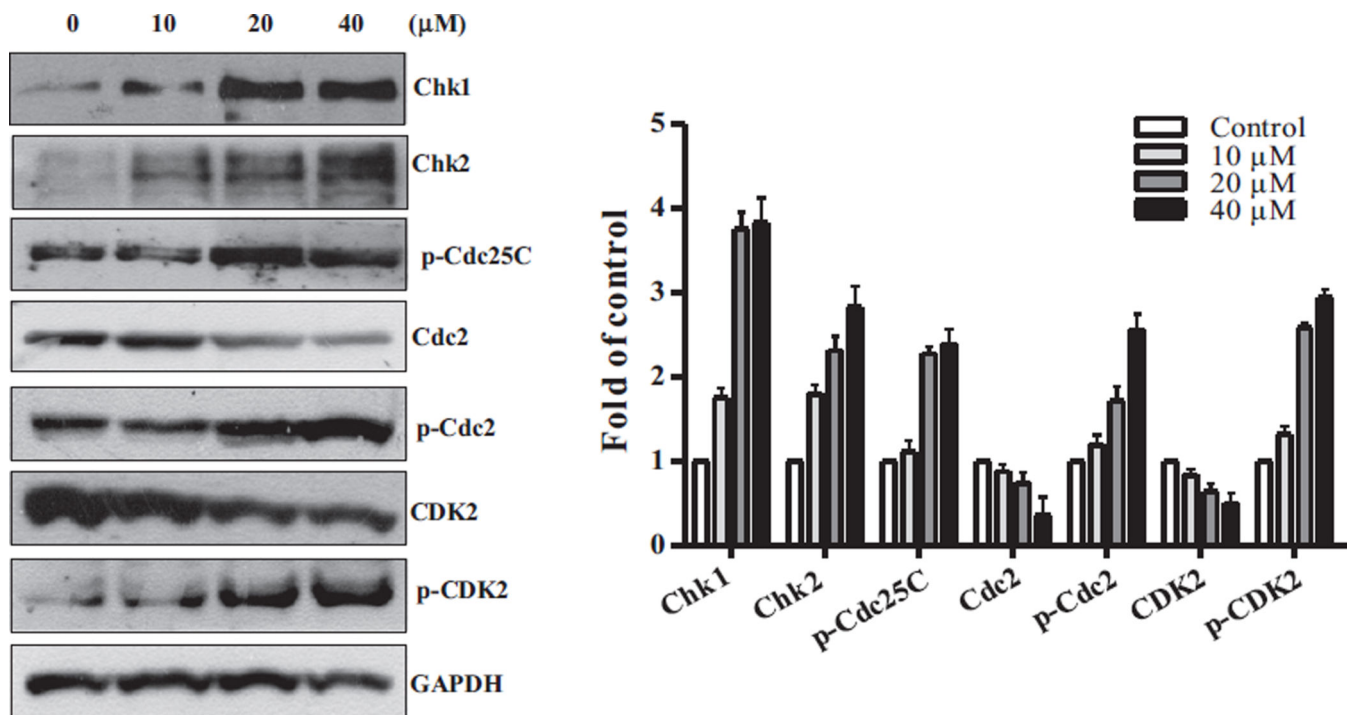
Fig. 3.
Effect of Jaridonin on cell cycle regulatory molecules in EC9706 cells. Cells were cultured as described in Materials and methods, and treated with either 0.1% DMSO alone (control) or varying concentrations of Jaridonin as labeled in the figure. At the end of the treatments, total cell lysates were prepared and subjected to SDS–PAGE followed by western blot. The results were representatives of three independent experiments. GAPDH was used as loading control.
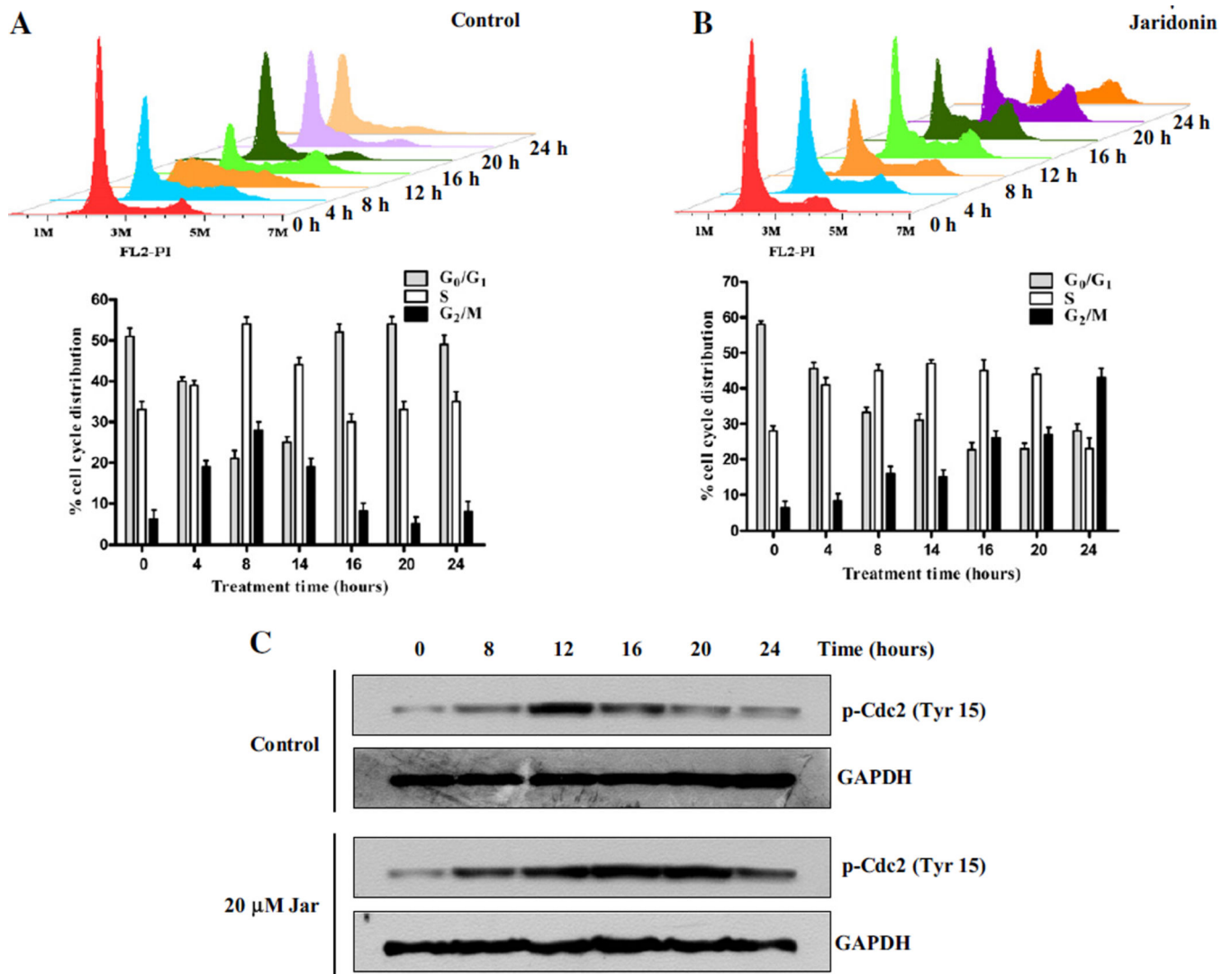
Fig. 4.
Jaridonin induces irreversible G2/Marrests and Cdc2 (Tyr15) phosphorylation in EC9706 cells. Cells were synchronized by serum starvation for 24 h and then released using media containing serum (A) with 0.1% DMSO (control) or (B) 20 μM Jaridonin and cell cycle analysis was done as a function of time from 0 to 24 h as indicated in the figure. (C) In similar treatments, cell lysates were prepared and subjected to SDS–PAGE followed by western blot.
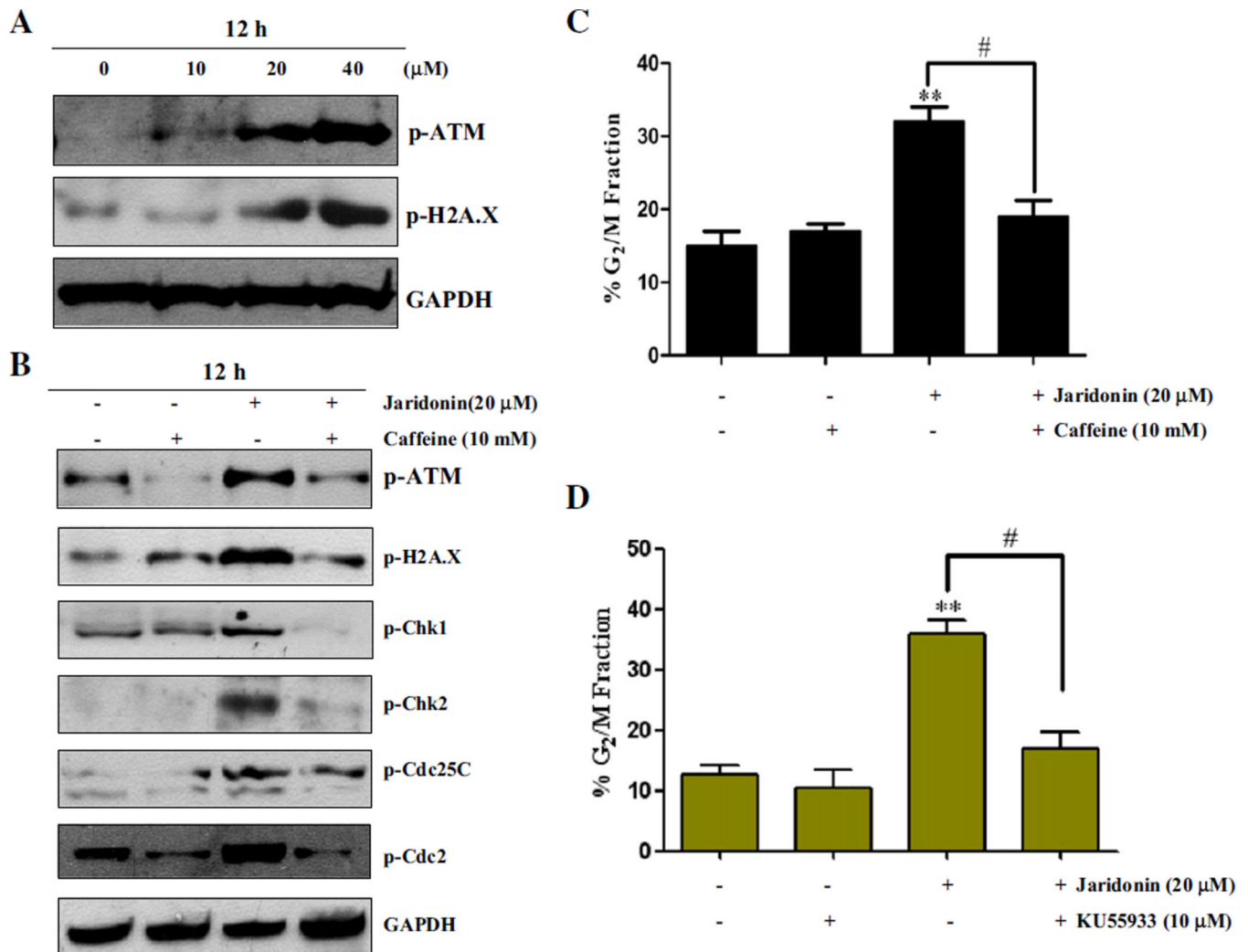
Fig. 5.
Jaridonin increases p-ATM protein expression, and activates ATM-dependent phosphorylation of Chk1/2, Cdc25 C and Cdc2, as well as G2/M arrest. EC9706 cells were cultured as described in materials and methods and treated with either 0.1% DMSO (control) or Jaridonin and/or caffeine (an inhibitor of ATM) for 12 h. Caffeine was added 15 min before Jaridonin, or in another experiment KU55933 was added 1 h before Jaridonin. Cells were harvested and cell lysates were prepared and subjected to SDS–PAGE followed by western blot. Membranes were probed with (A) p-ATM and p-H2A.X, (B) anti-p-ATM, p-H2A.X, p-Chk1/2, p-Cdc25C, p-Cdc2 and GAPDH antibodies. (C) and (D) Similar treatments, cells were harvested and analyzed for cell cycle distribution as detailed in materials and methods. Data are presented as means ± SD of triplicate samples. ** $P < 0.01$ as compared with control; # $P < 0.05$ for Jaridonin versus Jaridonin + caffeine or KU55933.
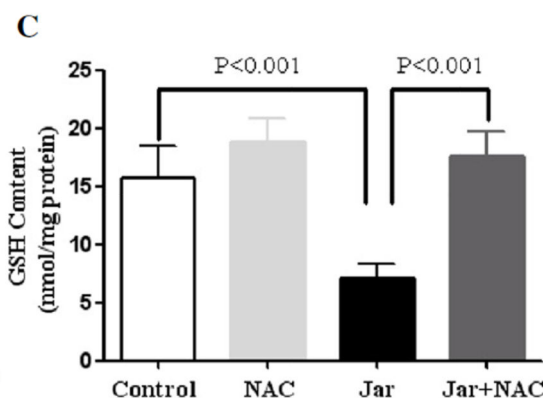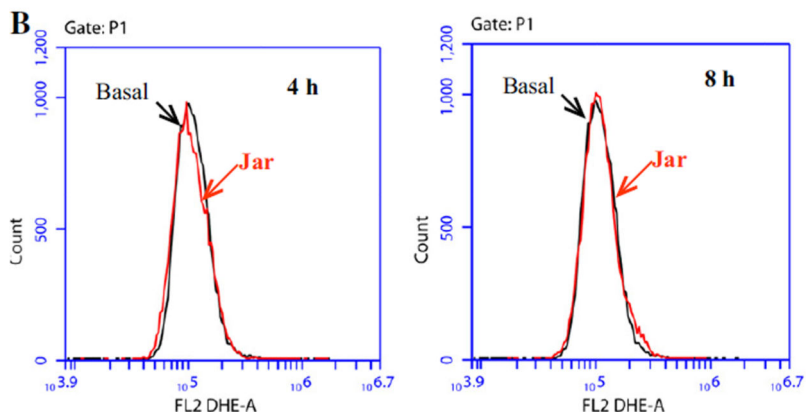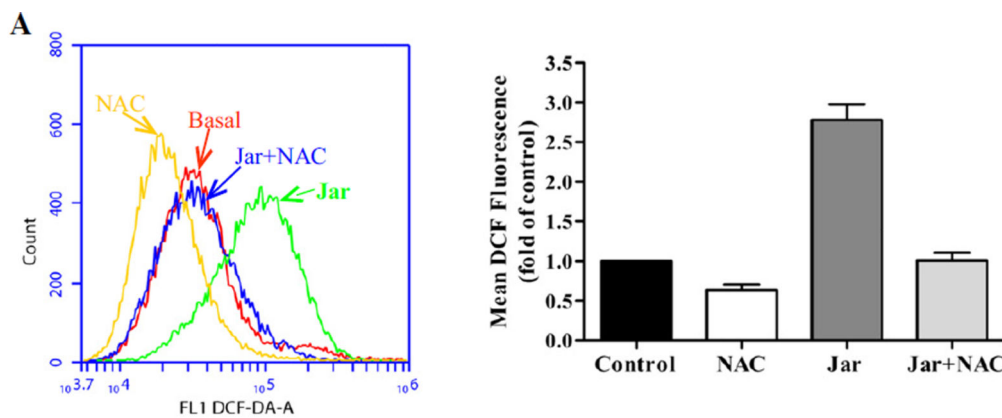
Fig. 6.
Jaridonin increases ROS generation in EC9706 cells through depletion of GSH levels. Cells were treated with 40 μM Jaridonin at indicated time, followed by incubation with 10 μM DCFH-DA or DHE for 30 minutes at 37 °C. The intracellular levels of ROS were determined by flow cytometer. (A) Flow cytometry analysis of percentages of DCF positive cells that were treated with either 0.1% DMSO (control) or 40 μM Jaridonin and/or NAC (5 mM) for 4 h. (B) Representative percentages of DHE positive cells detected by flow cytometry. (C) EC9706 cells with or without pretreatment with NAC (5 mM) for 2 h were treated with either 0.1% DMSO (control) or Jaridonin (40 μM) for 8 h. Cells were harvested and processed for estimation of reduced glutathione levels using the GSH and GSSG Assay Kit. Data are presented as means ± SD of triplicate samples. Jar, Jaridonin.

A

B
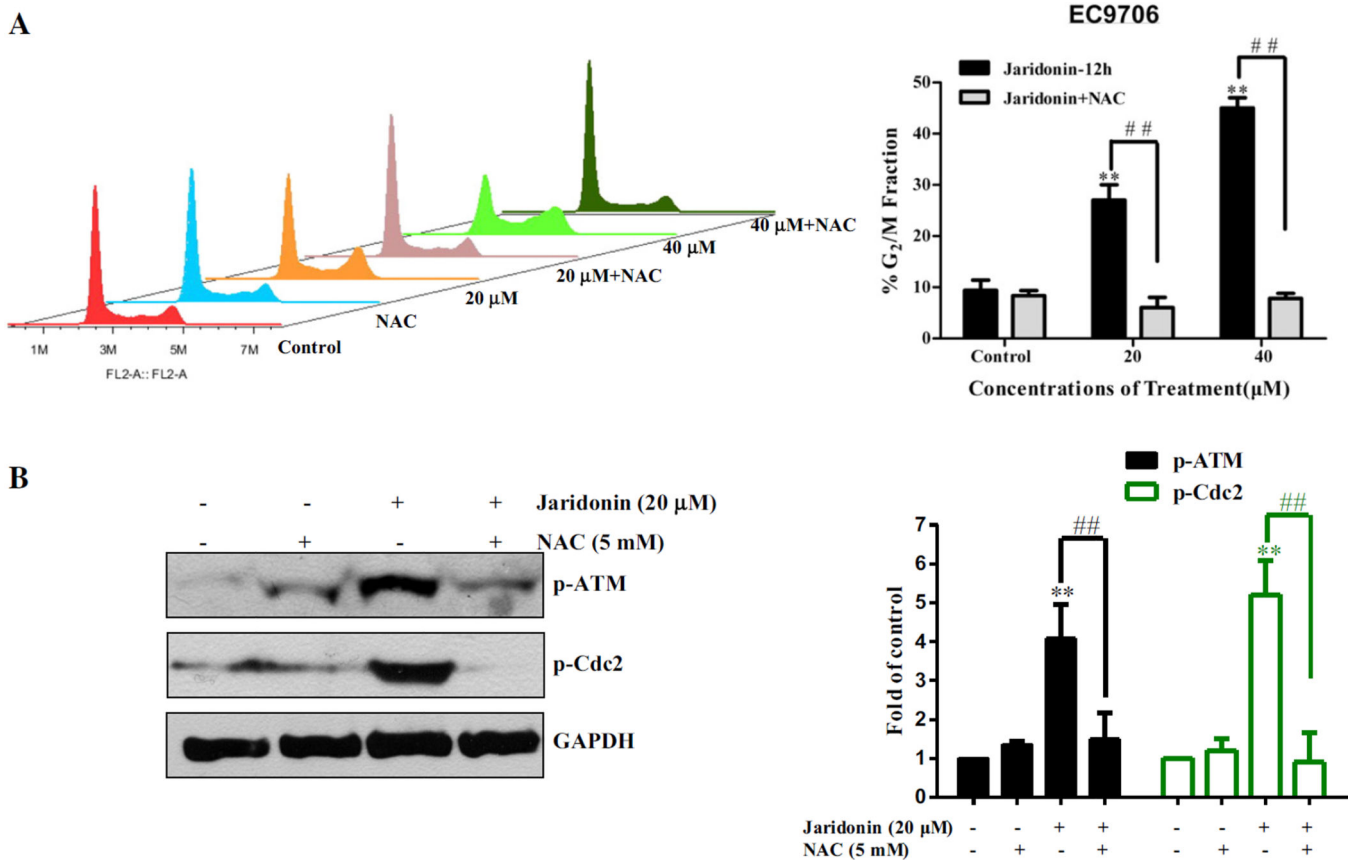
Fig. 7.
The effects of Jaridonin causing G2/M arrest, increasing ATM protein expression, and inducing phosphorylation of Cdc2 are reversed by NAC. (A) Percentage of G2/M fraction in EC9706 cultures treated for 12 h with 0.1% DMSO (control) or 20 μM or 40 μM Jaridonin in the absence or presence of 5 mM NAC. (B) Representative western immunoblotting depicting p-ATM and p-Cdc2 in EC9706 cultures treated with either 0.1% DMSO (control) or 20 μM Jaridonin and/or NAC (5 mM) for 12 h. Similar results were observed in three independent experiments. Data are presented as means ± SD of triplicate samples. ** $P < 0.01$ as compared with control; ## $P < 0.01$ for Jaridonin versus Jaridonin + NAC.
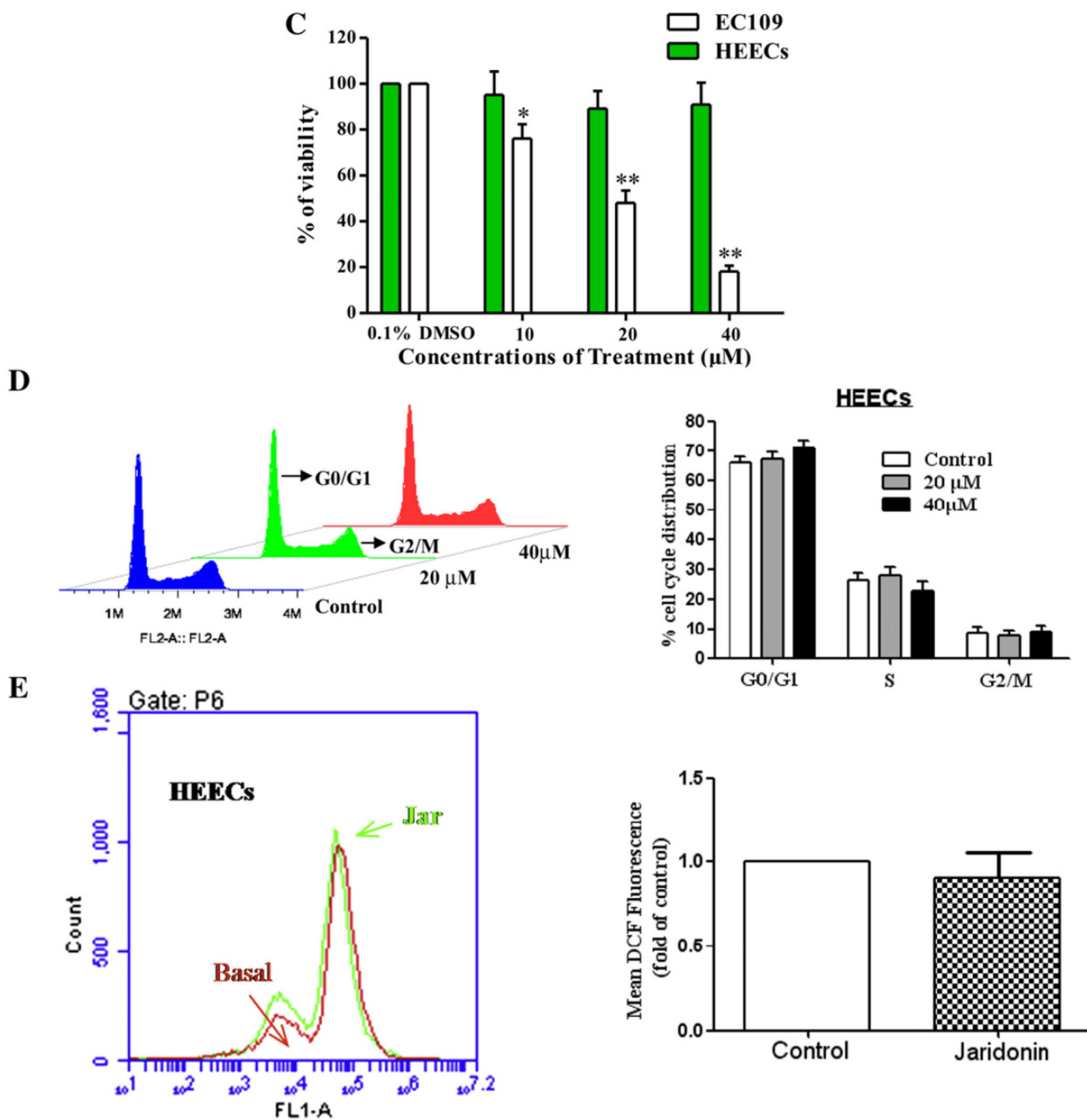
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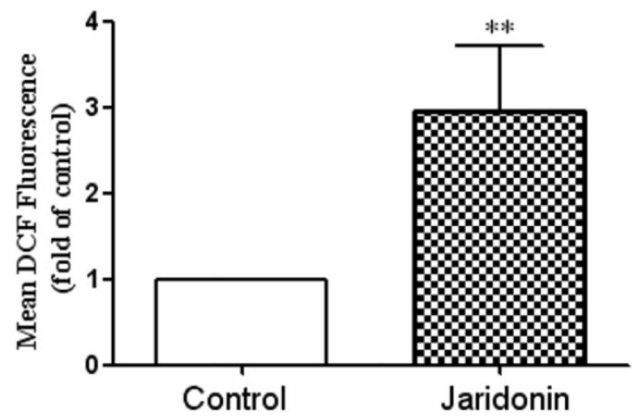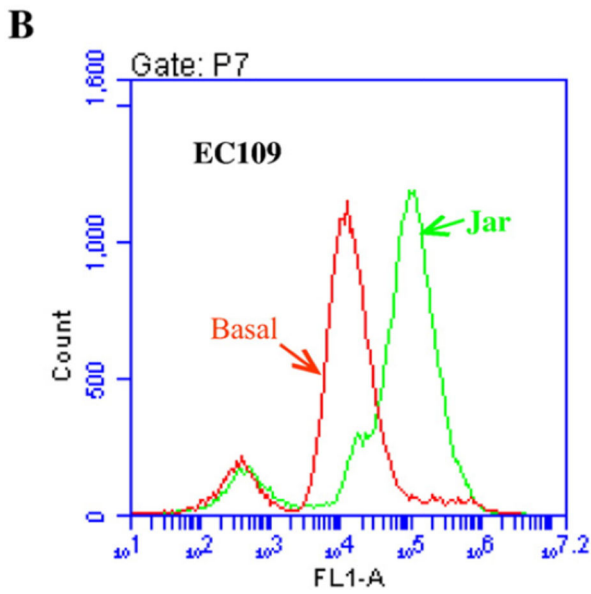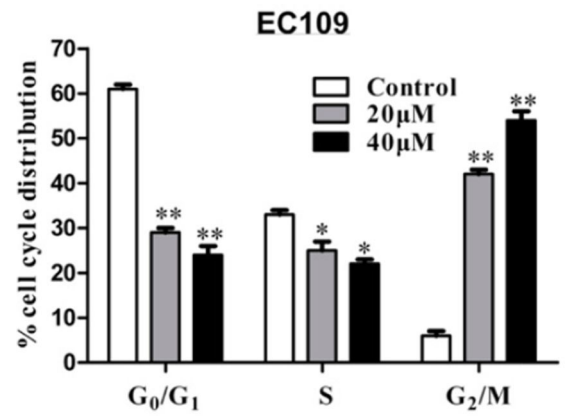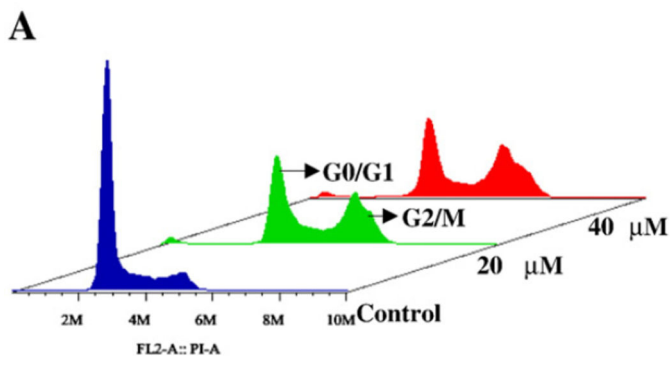
Fig. 8.
Effect of Jaridonin treatment on cell growth, cell cycle distribution and ROS generation in EC109 cells and normal HEECs. Cells were treated with either 0.1% DMSO (control) or varying concentrations of Jaridonin for 12 h. At the end of the treatments, cell cycle distribution was analysed by flow cytometry. The percentage of cell cycle distribution in EC109 and HEECs cells are shown in (A) and (D), respectively. (C) Survival rates of EC109 and HEECs are determined by trypan blue dye exclusion assay. Cells were treated with 0.1% DMSO or 40 μM Jaridonin for 24 h, followed by incubation with 10 μM DCFH-DA

for 30 minutes at 37 °C. The intracellular levels of ROS were determined by flow cytometer. Representative percentages of DCF positive cells and in EC109 and HEECs cells are shown in (B) and (E), respectively. Data are presented as means ± SD of triplicate samples.* $P <$ 0.05; ** $P < 0.01$ as compared with control.