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Original paper

DNA damage induced by oridonin involves cell cycle arrest at G2/M phase in human MCF-7 cells

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Aim of the study: To study the mechanisms of inhibition of cell growth and induction of DNA damage in oridonin-treated MCF-7 human breast cancer cells.

Material and methods: The cytotoxicity of oridonin-treated MCF-7 cells was measured by MTT assay. Cell cycle phase distribution was analyzed by flow cytometry. P-ATM, P-CHK2, γH2AX and P-P53 protein expressions were detected by Western blot analysis. The expression of r-h2ax and P-ATM was also detected by immunofluorescence staining. The degree of cellular damage of oridonin-induced MCF-7 human breast cancer cells was confirmed by the comet assay analysis of DNA fragmentation.

Results: Oridonin inhibited cell growth in a time- and dose-dependent manner. The IC50 values at 48 and 72 hours were 78.3 and 31.62 µmol/l, respectively. Oridonin induced G2/M phase arrest in MCF-7 cells. MCF-7 cells treated with oridonin showed significant DNA damage as shown by an increase in olive tail moment (OTM). The protein expression levels of P-ATM, P-CHK2, γH2AX and P-P53 were increased significantly in a dose-dependent manner.

Conclusions: DNA damage provokes p53-mediated G2/M cell cycle arrest in oridonin-induced MCF-7 cells through the mechanism of CHK2 activation by activated ATM protein kinase, which is induced by oridonin.

Key words: oridonin, ATM, CHK2, cell cycle, p53, apoptosis.

Introduction

Breast cancer is a leading cause of morbidity and mortality in women, in both developed and developing countries [1]. Chemotherapy is the most common treatment for cancer. Unfortunately, many of the chemotherapeutic drugs are non-specific and cause severe side effects. Therefore, searching for new alternative strategies for the prevention and treatment of breast cancer is essential. Natural products, including plants, microorganisms and marines, have been considered as valuable sources for anticancer drug discovery [2]. Oridonin is extracted and purified from a traditional Chinese herb, *Rabdosia rubescens*, a member of the salvia family. Oridonin (Fig. 1) has various biological, pharmaceutical, and physiological properties and functions [3, 4]. Oridonin has been studied extensively in China and has been shown to possess anti-tumor activity against murine sarcoma and leukemia cells [5]. It has been reported that oridonin was found to induce growth inhibition and apoptosis of the human breast cancer cell line MCF-7 [6]. However, a systematic analysis of the mechanisms of oridonin's anti-tumor activity in MCF-7 has not been undertaken.

The DNA damage response (DDR), caused by a variety of stimuli, arrests the cell cycle to allow damage repair or direct cell apoptosis. An imbalance between DNA damage and DNA repair activities may affect cell viability. After DNA damage, the cell cycle is arrested at the transition from the G1 to S phase or from the G2 to M phase of the cell cycle. Increasing evidence indicates a central role for p53 in mediating cell cycle arrest or apoptosis [7]. One of the early events initiated by double-stranded DNA breaks (DSBs) is the phosphorylation of serine 139 in the SQE motif located on the tail of histone H2AX (γH2AX) by ATM/ATR kinases and the subsequent rapid formation of γH2AX foci at the DSB sites [8]. These foci are essential in facilitating the assembly of repair factors on damaged DNA sites and also aid in the activation of DNA damage checkpoints [9]. The loss or reduction of H2AX could facilitate tumorigenesis [10]. Taken together, a large body of evidence demonstrates that γH2AX foci play an essential role in the cellular DNA damage response. In addition, to facilitate the formation of γH2AX nuclear foci, ataxia-telangiectasia mutated (ATM) also induces cell cycle arrest through phosphorylation of its targets, p53 and CHK2 [11]. CHK2 then phosphorylates CDC25C on serine 216 (S216), which prevents CDC25C from dephosphorylating tyrosine 15 and threonine 14 of CDC2

(or CDK1), resulting in cell cycle arrest at the G2 phase [12]. In the present study, we demonstrated that activated p53 contributed to oridonin-induced cell cycle arrest in human breast MCF-7 cells, and we further investigated the mechanisms of cell growth inhibition and DNA damage repair in oridonin-treated MCF-7 human breast cancer cell.

Material and methods

Material

Oridonin was obtained from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from TBD Biotechnology Development (Tianjin, China); Thiazolyl blue (MTT) was from Sino-American Biotechnology (Beijing, China); polyclonal antibodies against r-h2ax, phosphorylated ATM, phosphorylated CHK2 and phosphorylated P53, horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit or goat-anti-mouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell lines and cell culture

The human breast cancer MCF-7 cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in RPMI 1640 medium (GIBCO, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 0.03% L-glutamine (GIBCO, Gaithersburg, MD, USA) and maintained at 37°C with 5% CO_2 in a humidified atmosphere.

Cell growth inhibition assay

The cytotoxic effect of oridonin on human breast cancer MCF-7 cells was measured by MTT assay. MCF-7 cells were cultured in RPMI 1640 medium containing 10% FBS incubated with oridonin at the concentrations of 0, 10, 20, 40, 80, 160 µmol/l. After treatment with different concentrations of oridonin for 24, 48 and 72 hours, MTT was added to each well at a final concentration of 0.4 mg/ml and further incubated for 3 h at 37°C. The MTT solution in medium was then removed. To solubilize formazan crystals formed in viable cells, 100 µl of dimethyl sulfoxide (DMSO) was added to each well before the absorbance at 492 nm was measured using a microplate ELISA reader (Model 550, Bio-Rad, USA). The IC50 value was expressed as the concentration of oridonin that inhibited the growth of cells by 50%. IC50 values of each sample were collected from three replicates. The mean and standard deviations were calculated from the value of the three replicates. Values for the IC50 assay were obtained.

Measurement of DNA damage by the comet assay

Oridonin-induced DNA damage was determined using the comet assay. Cells were treated with oridonin (80 and 160 µmol/l) for 48 hours in complete medium, and the comet assay procedure in this study was a modification of the method described earlier [13]. Briefly, after treatment with oridonin, the cells were harvested and resuspended in ice-cold PBS. Approximately 1×10^4 cells in a volume of 100 µl of 0.5% (w/v) low melting point agarose were pipetted onto frosted microscope slides and allowed to solidify under a coverslip, which was then carefully removed. The slides were

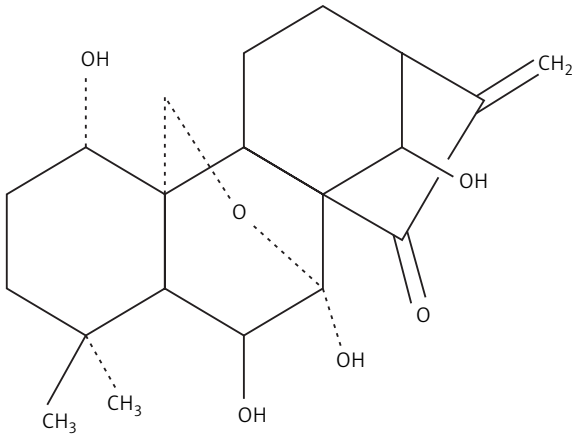


Fig. 1. Chemical structure of oridonin

immersed in ice-cold lysis solution containing 2.5 mol/l NaCl, 10 mmol/l Tris, 100 mmol/l Na_2-EDTA, and 1% (w/v) N-lauroylsarcosine, adjusted to pH 10.0, with 1.0% Triton X-100 added immediately before use. After 2 hours at 4°C, the slides were removed from the lysis solution, drained, and placed in a horizontal gel electrophoresis tank filled with buffer [0.3 mol/l NaOH, 1 mmol/l EDTA (pH 13)] and subjected to electrophoresis for 20 minutes at 300 mA. Slides were transferred to neutralization solution (0.4 mol/l Tris-HCl, pH 7.5) for 3–5 minutes, washed and stained with 75 µl ethidium bromide (20 µg/ml) for 5 minutes. After a final wash in double-distilled water, the gels were covered with glass cover slips. Slides were viewed using a fluorescence microscope (Olympus BX60F-3, Olympus Optical, Japan). The image analysis software (CASP, Poland) provides a full range of densitometric and geometric parameters describing the complete comet, as well as the head and tail portions. For each sample, the tail lengths (in micrometers) of a minimum of 50 comets were analyzed. The length of the comet was quantified as the distance from the center of the cell nucleus to the tip of the tail in pixel units. The tail moment, expressed in arbitrary units, was calculated by multiplying the percent of DNA (fluorescence) in the tail by the length of the tail.

Cell cycle analysis

Cell cycle analysis was carried out by FACScan flow cytometer using a standard protocol. Briefly, MCF-7 cells were harvested by centrifugation, washed with PBS, and fixed with cold 70% ethanol overnight. The fixed cells were then stained with PI solution consisting of 50 µg/ml PI, 20 µg/ml RNase A and 0.1% Triton X-100. After 1 h incubation in the dark, fluorescence-activated cells were sorted in a FACScan flow cytometer. The distribution of cells in the different cell-cycle phases was analyzed using Multicycle software (Phoenix Flow Systems, San Diego, CA). The percentage of cells in each phase of the cell cycle was determined at least in triplicate and expressed as mean ± SD.

Immunofluorescence staining

Cells were treated as defined in the figure legends. Immunofluorescent staining was carried out by fixing cells

Table 1. Effect of oridonin on inhibition rate of MCF-7 cells

Oridonin (μmol/l)	Inhibition rate (%)		
	24 hours	48 hours	72 hours
0	0	0	0
10	5.1 ±0.1	8.4 ±0.3	12.1 ±0.5
20	17.5 ±0.3	21.8 ±1.1*	31.6 ±1.7*
40	22.4 ±1.5*	37.9 ±2.3*	57.8 ±2.7*
80	35.8 ±2.1*	51.7 ±2.8*	68.9 ±3.3*
160	41.3 ±2.7*	66.5 ±3.1*	89.3 ±5.1*

**P < 0.05, compared with control*

with pre-chilled (–20°C) acetone-methanol for 15 min. The primary antibodies, anti-γH2AX (Upstate, 0.5 μg/ml) and anti-phospho-ATM (S1981) (Cell Signaling, 1 : 100), were then added to the slides at 4°C overnight. After washing, secondary antibodies, FITC-Donkey anti-rabbit IgG (1 : 200, Jackson Immuno Research Lab) and Rhodamine-Donkey anti-mouse IgG (1 : 200, Jackson Immuno Research Lab) were applied for 1 h at room temperature. The slide was then covered with VECTASHIELD mounting medium with DAPI (VECTOR Lab Inc., Burlingame, CA94010). Images were taken with a fluorescent microscope (Carl Zeiss, Axiovert 200).

Cell lysis and Western blot

Cells were lysed in a buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM NaF, 1 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml leupeptin and 10 μg/ml aprotinin. Fifty μg of total cell lysate was separated on SDS-PAGE gel and transferred onto Immobilon-P membranes (Millipore). Membranes were blocked with 5% skim milk and then incubated with the indicated antibodies at room temperature for 1 h. Signals were detected using an ECL Western Blotting Kit (Amersham). Primary antibodies and concentrations used were: anti-phospho-p53(S15) (Cell Signaling, 1 : 1000); anti-γH2AX (1 : 1000, Upstate), anti-phospho-ATM (S1981) (Rockland, 1 : 500), anti-CHK2 (Cell Signaling, 1 : 1000) and anti-actin (Santa Cruz, 1 : 1000).

Table 2. Effect of oridonin on DNA damage

Groups	Percent of cells with tails (%)	Tail length (μm)	Tail moment
control	7.15 ±1.04	8.71 ±1.18	1.62 ±0.53
oridonin 80 (μmol/l)	49.25 ±2.76*	29.38 ±1.37*	5.72 ±1.84*
oridonin 160 (μmol/l)	73.53 ±3.16**	60.42 ±3.25**	15.76 ±0.87**

**P <0.05, **P < 0.01, compared with control*

Statistical analysis

All the grouped data were statistically evaluated with SPSS/13.0 software. Statistical analysis was performed by one-way analysis of variance (ANOVA). *P*-values of less than 0.05 were considered to be significant. All the results were expressed as the mean ± SD.

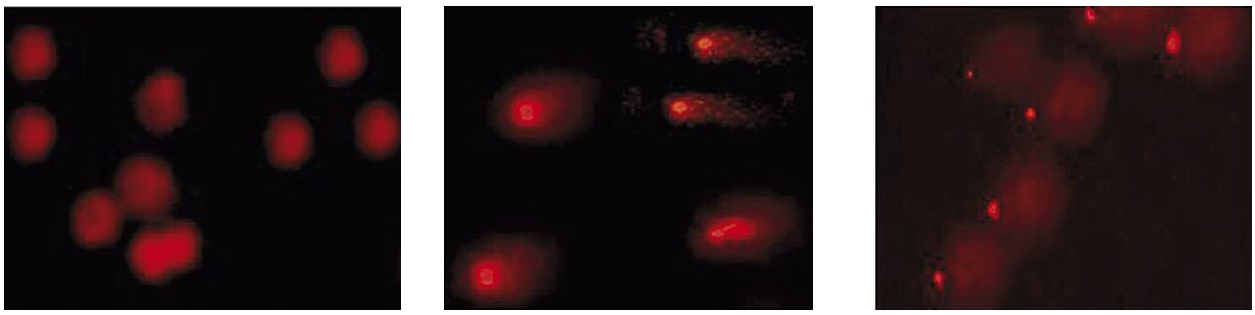
Results

Cytotoxicity of oridonin on MCF-7 cells

Oridonin induced MCF-7 cell death in a time- and dose-dependent manner. Oridonin from 20 to 160 μmol/l showed inhibitory effects on MCF-7 cell growth. By 48 h after 80 μmol/l oridonin treatment, the cell death rate reached almost 50% (Table 1). The IC50 value of MCF-7 cells, treated with oridonin for 48 h, was 78.3 μmol/l. Therefore, 48 h incubation with oridonin appears sufficient for the half induction of cell death.

Effect of oridonin on DNA damage

We determined the effect of oridonin on cellular DNA damage using the comet assay, which was used as a biomarker of apoptosis. As shown in Fig. 2 and Table 2, treatment of MCF-7 cells with oridonin (80 and 160 μmol/l) for 48 hours resulted in significant DNA damage ($p < 0.01$) compared with cells that were not treated with oridonin. The DNA damaging effect in terms of DNA fragmentation was determined by measuring the tail length and tail moment of the comet under a microscope and casp software. The data represented at least 50 DNA-damaged cells in each treatment group. Mean comet tail moment was based on analysis of 100 comet images per slide after treatment with various concentrations of oridonin.



control | oridonin (80 μmol/l) | oridonin (160 μmol/l)

Fig. 2. Effect of oridonin on DNA damage. DNA damage as detected using an alkaline comet assay 48 h after oridonin treatment in MCF-7 cells. Photomicrographs of damaged DNA after the various oridonin treatments showing an increase in the tail length and tail moment

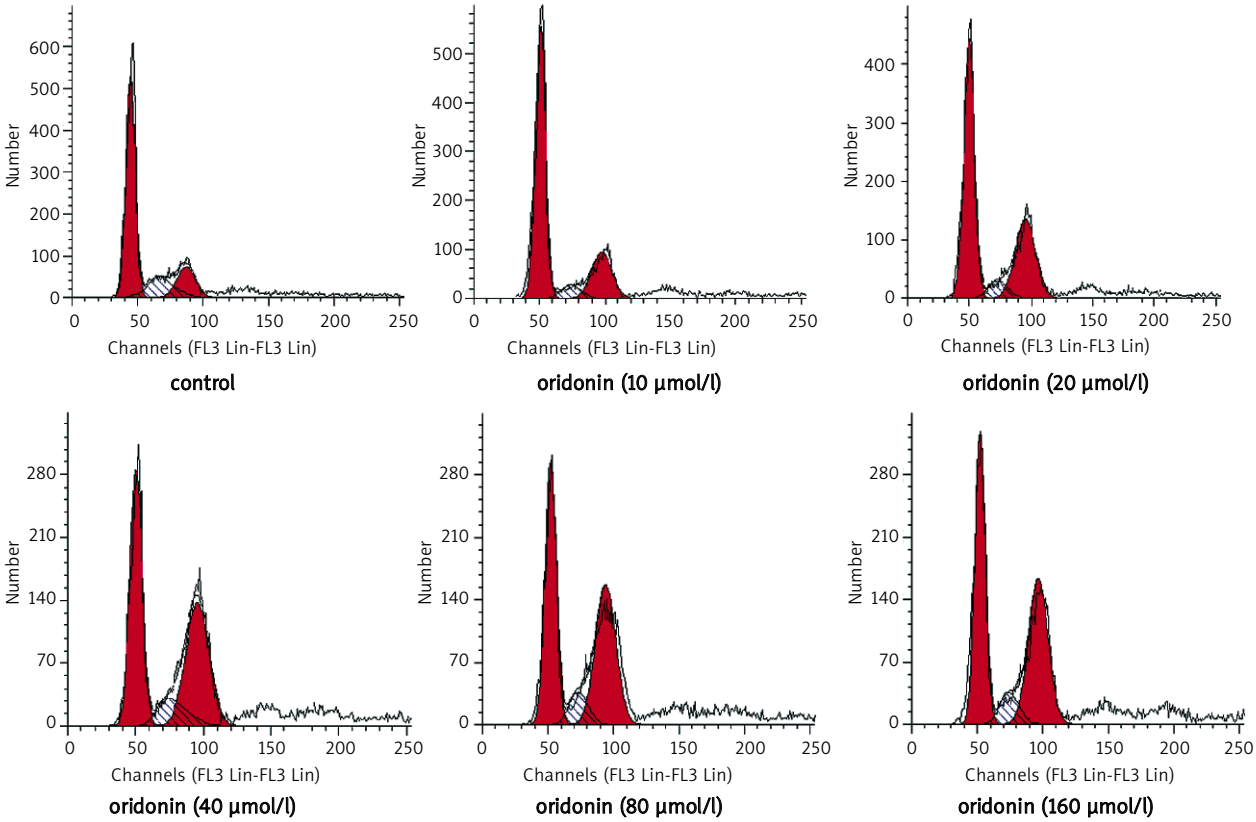


Fig. 3. Effects of oridonin on cell cycle distribution in cultured MCF-7 cells. Flow cytometric analysis of the DNA content in control and oridonin-treated groups (10, 20, 40, 80 and 160 μmol/l) for 48 h

Effect of oridonin on cell cycle distribution

To determine whether oridonin regulates the cell cycle, the distribution of MCF-7 cells in various compartments of the cell cycle was analyzed by flow cytometry after oridonin treatment for 48 h. The percentage of cells in each phase of the cell cycle in control and treated groups is summarized in Fig. 3 and Table 3. Compared with the control, an accumulation of MCF-7 cells in the G2/M phase was noted in the treatment group with various oridonin concentrations.

Table 3. Effects of oridonin on cell cycle analysis

Groups	G0/G1(%)	S (%)	G2/M (%)
control	67.43	16.32	16.25
oridonin (10 μmol/l)	64.32	13.95	21.73
oridonin (20 μmol/l)	61.73	8.62	29.65*
oridonin (40 μmol/l)	50.65	8.5	40.85**
oridonin (80 μmol/l)	32.65	13.57	53.78**
oridonin (160 μmol/l)	28.11	9.72	62.17**

*Analysis of DNA in MCF-7 cells. The percentage of each cell cycle was determined by flow cytometry. Compared with the control, oridonin could induce G2/M arrest in MCF-7 cells.*p < 0.05, **p < 0.01, compared with control*

Effect of oridonin on the formation of ATM S1981 phosphorylation and γH2AX nuclear foci in MCF-7 cells

To explore the G2/M arrest induced by oridonin, we observed co-localization between phosphor-S1981 ATM foci and those of γH2AX foci in MCF-7 cells treated with oridonin. Furthermore, a higher number of Ctrl MCF-7 cells exhibited γH2AX nuclear foci when treated with increasing doses of oridonin (Fig. 4).

Effects of oridonin on the protein expression of P-ATM, P-CHK2, γH2AX and P-P53

To further investigate the molecular mechanisms underlying oridonin inhibition of cell growth and induction of DNA damage, we examined the expression of P-ATM, P-CHK2, γH2AX and P-P53 in MCF-7 cells. Immunoblotting analysis revealed that the expression of these proteins was increased in oridonin treatment cells in a dose-dependent manner (Fig. 5).

Discussion

In the present study, we found that oridonin can inhibit the growth of human breast cancer MCF-7 cells, which was consistent with the previous study. We further investigated the mechanism of how oridonin inhibits cell growth, and found that oridonin treatment not only induced G2/M arrest in MCF-7 cells, but also caused significant DNA damage, shown in the comet assay.

The DNA damage checkpoints are biochemical pathways that delay or arrest cell cycle progression in response to genomic DNA damage. In response to DNA damage, cells

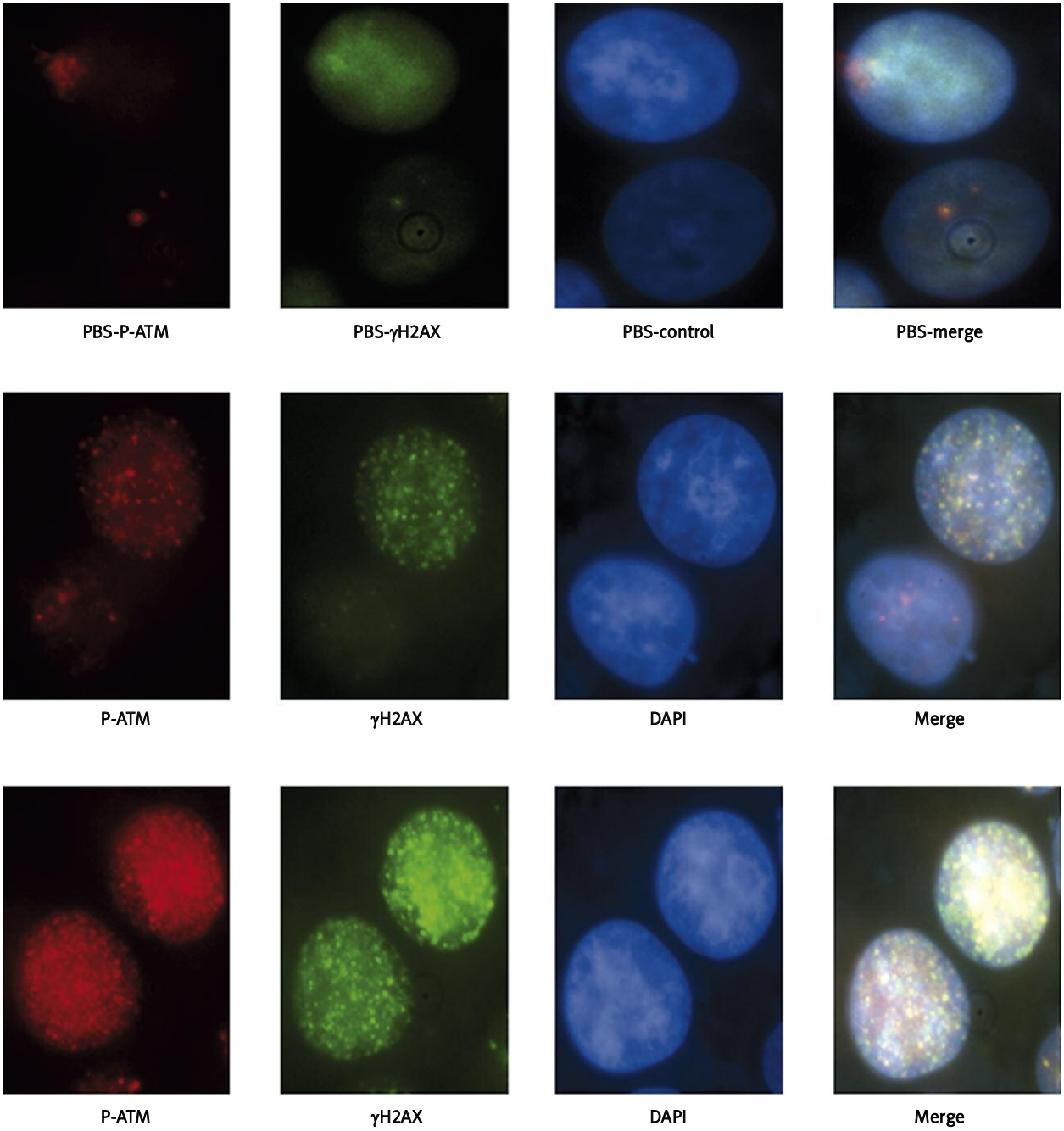


Fig. 4. Formation of ATM S1981 phosphorylation and γH2AX nuclear foci in oridonin-treated MCF-7 cells. MCF-7 cells were treated with oridonin for 8 h, followed by double immunofluorescent staining for ATM S1981 phosphorylation (red) and γH2AX (green). Nuclei were counter-stained with DAPI (blue). At least 200 cells in four randomly selected fields were counted to determine the percentage of cells positive for active ATM or γH2AX nuclear foci. Experiments were repeated three times

would activate ataxia-telangiectasia mutated (ATM), one of the sensor kinases, which in turn phosphorylates multiple downstream substrates, including the effector kinase CHK2 [14], resulting in cell-cycle checkpoint initiation and/or apoptosis. In our study, we found that oridonin treatment not only induced G2/M arrest in MCF-7 cells, but also caused significant DNA damage, shown in the comet assay. A number of studies have revealed that different molecular mechanisms correspond to DNA damage checkpoints at different phases [15], and ATM/CHK2 signaling pathways especially play important roles [16]. Markers of a constitutively active DNA damage response have been described in many types of malignant lesions in different tissues [17]. Therefore, the major kinases of the signaling pathways in MCF-7 cells treated by oridonin were analyzed. Tumor suppressor p53 participates in multiple cellular activities including the cell-cycle checkpoints and DNA repair, playing a critical role in maintaining genome integrity and stability [18]. P53 can also trigger cell apoptosis, which is associated with treatment failure in childhood acute lymphoblastic leukemia [19]. However, more than half of human tumors display p53 mutation or deficiency. Therefore, the DNA damage checkpoints in tumor cells may be different from those

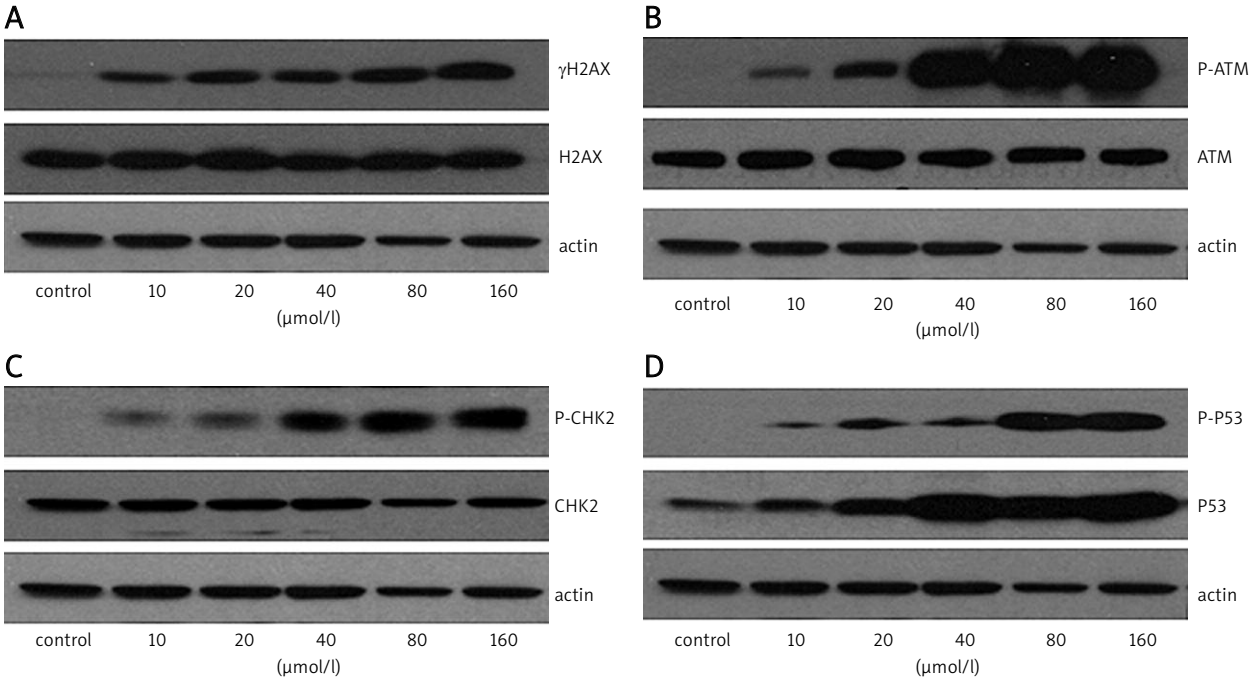


Fig. 5. Western blotting analysis of expression of γH2AX (A), P-ATM (B), P-CHK2 (C), and P-P53 (D) protein. The MCF-7 cells were treated with different concentrations of oridonin. Total cellular proteins were harvested and subjected to Western blotting analysis. β-actin was used as a loading control

in normal tissues. Study of the DNA damage checkpoints in tumor cells will not only help us to understand the regulatory mechanisms of a cell cycle, but also provide a potential theory for developing new tumor therapy.

In vertebrates, ATM and ATM- and Rad3-related (ATR), members of the phosphatidylinositol 3-kinase related protein family, are critical checkpoint regulators which work upstream of the DNA damage response pathway [20]. The checkpoint functions of ATR and ATM are mediated in part by a pair of checkpoint effector kinases termed CHK1 and CHK2 [21]. The histone H2AX is one of the targets for ATM-mediated phosphorylation [22]. Phosphorylated H2AX (γ-H2AX) was the gold standard for early detection of DNA damage. These molecules participate in the transmission of DNA damage signals to downstream molecules such as CHK2 to foci containing the site of damaged DNA. To consolidate the ATM activation, we demonstrated that oridonin clearly induced the nuclear foci of S1981-phosphorylated (phosphor-S1981) ATM in MCF-7 cells.

P53 is a tumor suppressor mainly involved in the transcriptional regulation of a large number of growth-arrest and apoptosis-related genes [23]. Upon genotoxic damage, p53 contributes to cell-cycle arrest at the G1/S and/or G2/M checkpoints through diverse mechanisms [24]. When analyzing the DNA damage signaling pathway induced by oridonin, for the first time, we found that treatment with oridonin induced G2/M arrest and DNA damage signaling involving γ-H2AX foci formation and CHK2 phosphorylation. The DNA damage signaling induced by oridonin was also mediated by ATM and the downstream signaling of ATM, which is involved in the phosphorylation of CHK2. It strongly suggests that ATM is necessary for this signaling pathway. Furthermore, the protein expression level of P-P53 increased significantly in a dose-dependent manner. In summary, the role of the ATM/CHK2/p53 pathway in MCF-7 cell suppression can be attributed to its influence on the induction of apoptosis as well as on the regulation of DNA damage dependent checkpoints.

In conclusion, oridonin may be a potentially promising agent of natural resource to treat breast cancer. DNA damage provoked p53-mediated G2/M cell cycle arrest in oridonin-induced MCF-7 cells. The mechanism was related to activation of the protein kinase ATM, which subsequently activated CHK2, leading to G2/M arrest.

The authors declare no conflict of interest.

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