

Full Paper

Oridonin Induced Autophagy in Human Cervical Carcinoma HeLa Cells Through Ras, JNK, and P38 Regulation

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Abstract. In this study, we investigated autophagy induced by oridonin in HeLa cells. HeLa cells were exposed to oridonin, and the fluorescent changes, autophagic levels, and protein expressions were evaluated. Oridonin induced autophagy in HeLa cells in vitro in a dose- and time-dependent manner. Oridonin-treated HeLa cells, which had been prelabeled with the autophagosome-specific dye monodansylcadaverine (MDC), recruited more MDC-positive particles and had a significantly higher fluorescent density; and simultaneously, expressions of autophagy-related proteins, MAP-LC3 and Beclin 1, were increased by oridonin. In oridonin-induced HeLa cells, pretreatment with 3-methyladenine (3-MA, the specific inhibitor of autophagy) dose-dependently decreased the autophagic ratio accompanied with downregulation of the protein expressions of MAP-LC3 and Beclin 1. Furthermore, when a Ras inhibitor was applied, the autophagic levels were augmented, whereas P38 and JNK inhibitors decreased the autophagic ratio significantly, indicating that this oridonin-induced autophagic process was negatively regulated by Ras, but positively regulated by P38 and JNK MAPKs. Raf-1 and ERK1/2 had no obvious correlation to these signaling pathways.

Keywords: oridonin, autophagy, Ras, P38 MAPK, JNK MAPK

Introduction

Diterpenoids are important compounds that are known to demonstrate various bioactivities. Oridonin, an *ent*-kaurane diterpenoid isolated from different sources such as *Isodon trichocarpus*, *Isodon Japonicus*, and *Isodon shikokianus*, has various pharmacological and physiological effects such as anti-inflammation, anti-bacterial, and anti-tumor effects. It has been used for the treatment of human cancers (1–3), especially esophageal carcinoma and prostate carcinoma. Previous studies have reported that oridonin has inhibitory effects on the cell growth of leukemia and hepatoma cancer both in vitro and in vivo (4, 5). Up to now, there have been few reports on the autophagy-inducing effect of oridonin or on its functional mechanisms.

Autophagy is an intracellular protein transport pathway common to all eukaryotic cells (6). It is a well-known mechanism whereby cells degrade parts of their own intracellular constituents, including cytoplasm and organelles (7–10). The initial step of the autophagic process is the formation of a double membrane-bound vacuole (autophagosome) derived from a part of the endoplasmic reticulum (11) or from the cytoplasmic lipid pool called the phagophore (12). The autophagosomes next receive hydrolases by fusion with lysosomes and late endosomes to form an autolysosome.

Beclin 1, the mammalian functional homolog of yeast Apg6, induces autophagy in cultured breast cancer cells and inhibits their tumorigenicity. In fact, human Beclin 1 is mono-allelically deleted in 40%–70% of breast and ovarian cancers (13). This is the first mammalian “autophagic gene” reported, and it has the properties of a tumor suppressor gene. Whether or not the expression of Beclin 1 is altered in cancers other than breast and ovarian carcinoma is currently not known. The protein

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Beclin 1 is able to shuttle between the nucleus and the cytoplasm (14). Its role in the nucleus is unknown but it seems that nuclear Beclin 1 does not control autophagy and does not inhibit tumorigenicity of breast carcinoma cells. These data suggest that the tumor suppressor of Beclin 1 may be correlated with its implicated control of autophagy.

Microtubule-associated proteins light chain 3 (MAP-LC3), a homolog of Apg8p, is essential for autophagy and associated to the autophagosome membranes after processing. Two forms of LC3, the cytosolic LC3-I and the membrane-bound LC3-II, are produced post-translationally. LC3-I is formed by the removal of the C-terminal 22 amino acids from newly synthesized LC3, followed by the conversion of a fraction of LC3-I into LC3-II (15, 16). Therefore, under the condition of induced autophagy, LC-3 was significantly accumulated.

In this study, we demonstrated oridonin-induced autophagy in human cervical carcinoma HeLa cells. 3-MA and inhibitors of P38 and JNK MAPKs decreased, whereas an inhibitor of Ras enhanced the sensitivity of oridonin-challenged HeLa cells to autophagy. Therefore, Ras, P38 MAPK, and JNK MAPK, but not Raf-1 and ERK1/2, were tightly involved in oridonin-induced autophagy in HeLa cells.

Materials and Methods

Chemical reagents

Oridonin was obtained from the Kunming Institute of Botany, The Chinese Academy of Sciences (Kunming, China). The purity of oridonin isolated from *Rabdosia rubescens* was confirmed by HPLC to be higher than 99%. Oridonin was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution. The DMSO concentration was kept below 0.1% in all the cell cultures and did not exert any detectable effect on cell growth or cell death.

Fetal bovine serum (FBS) was purchased from TBD Biotechnology Development (Tianjin, China); monodansylcadaverine (MDC), 3-methyladenine (3-MA), Raf-1 inhibitor GW5074, Ras inhibitor Manumycin A, P38 inhibitor SB203580, JNK inhibitor SP600125, and ERK1/2 inhibitor PD98059 were purchased from Sigma Chemical (St. Louis, MO, USA); rabbit polyclonal antibodies against P38, p-P38, JNK, p-JNK, ERK1/2, p-ERK, MAP-LC3, and Beclin 1 and horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit or goat-anti-mouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The cervical carcinoma HeLa cell line was obtained

from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (GIBCO, Gaithersburg, MD, USA) supplemented with 10% FBS and 0.03% L-glutamine (GIBCO), and maintained at 37°C with 5% CO₂ in a humidified atmosphere.

Observation of fluorescent changes

HeLa cells (3×10^5) in RPMI 1640 containing 10% FBS were seeded into 6-well culture plates and cultured for 24 h. 3-MA at 2 mmol/L was added 1 h before 64 μ mol/L-oridonin treatment. After 24 h, the cells were cultured with 0.05 mmol/L MDC at 37°C for 60 min, and the cellular fluorescent changes were observed at 24 h using an OLYMPUS IX70 inverted microscope (Olympus, Tokyo) equipped with a filter system (excitation filter: 356 nm, barrier filter: 545 nm). Images were obtained with a CCD camera (Media Cybernetics, Bethesda, MD, USA) and processed using the Sports Advance image collective system (Media Cybernetics).

Quantification of MDC labeling cells by flowcytometric analysis

In brief, after fixed times, cells were harvested by trypsin and rinsed with PBS. For measuring autophagic ratio, the cell pellets were suspended with 0.05 mmol/L MDC at 37°C for 60 min as described previously (17). The samples were analyzed by flow cytometry (Becton Dickinson FACScan, CA, USA) using the Cell Quest software (BD Biosciences, San Jose, CA, USA) to determine the percentage of cells undergoing autophagy which recruited MDC-positive particles.

HeLa cells (2×10^6) were treated with 2 mmol/L 3-MA 1 h before treatment with oridonin at 0, 16, 32, 64 and 128 μ mol/L at 6, 12, 24, 36 and 48 h to confirm the relationship between autophagy and the dose or time of oridonin treatment.

To confirm the suppressive effect of 3-MA on autophagy, we investigated various concentrations of 3-MA (2, 4, 6 and 8 mmol/L) introduced before 64 μ mol/L oridonin treatment at 24 h.

To determine effects of Ras, Raf-1, ERK1/2, P38, and JNK on oridonin-stimulated autophagy in HeLa cells (1×10^6), we evaluated the autophagic ratios in cells treated with the inhibitors of these five proteins compared with cells treated with oridonin alone after 24 h.

Western blot analysis

HeLa cells (2×10^6) were preincubated with or without 2 mmol/L 3-MA, 1 μ mol/L PD98059, 10 nmol/L GW5074, 3 μ mol/L manumycin A, 1 μ mol/L SP600125, or 1 μ mol/L SB600125 for 1 h and then treated with

64 $\mu\text{mol/L}$ oridonin for 24 h. Both adherent and floating cells were collected and Western blot was performed as previously described (18). Briefly, HeLa cells were washed with ice-cold PBS and solubilized with lysis buffer (1% SDS, 1 mmol/L phenylmethyl-sulfonyl-fluoride (PMSF), 1 mmol/L EDTA, 2 mmol/L leupeptin, and 1 mmol/L aprotinin). Protein concentration was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The protein lysates were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were soaked in blocking buffer (5% skim milk) and then incubated with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies overnight. The color was developed with dimino-benzidine (DAB).

Statistical analysis

All data and results presented were confirmed in at least three independent experiments, unless otherwise indicated. The data are expressed as means \pm S.D. Statistical comparisons were made by Student's *t*-test. $P < 0.05$ was considered significant.

Results

Oridonin induced autophagy in HeLa cells in a dose- and time-dependent manner

Oridonin induced HeLa cell autophagy in a time- and concentration-dependent manner. Oridonin from 32 – 128 $\mu\text{mol/L}$ exerted potent autophagic effect on HeLa cells. After 36 h oridonin treatment, the autophagic ratio had a significant upward trend (Fig. 1).

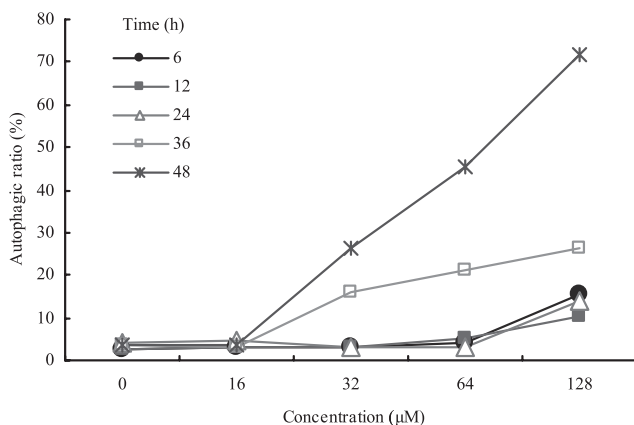


Fig. 1. Effects of oridonin on cell growth and autophagy in HeLa cells. Time and dose curve of cell autophagic ratio by oridonin treatment. HeLa cells were treated with oridonin at various doses for 6, 12, 24, 36 and 48 h. The autophagic levels were tested with flowcytometric analysis that could guarantee the results' precision, so there were no error bars in Fig. 1.

MDC recruited to oridonin-challenged HeLa cells

The oridonin-treated group showed higher fluorescent density and more MDC-labeled particles in HeLa cells compared with the control group (Fig. 2A), indicating that oridonin increased MDC recruitment to autophagosomes in the cytoplasm of the cells. Moreover, the flow cytometric analysis indicated that the percentage of MDC-positive cells by oridonin treatment was larger than that in the control, and the proportion of autophagic cells increased from 10.27% in the control group to 28.4% in the oridonin-treated group (Fig. 2B). All these results suggested that HeLa cells underwent autophagy when stimulated by 64 $\mu\text{mol/L}$ oridonin.

3-MA exerted suppressive effects on oridonin-induced autophagy

When the specific autophagic inhibitor 3-MA was applied, both the fluorescent density and MDC-labeled particles induced by oridonin decreased (Fig. 3A). The percentage of MDC-positive cells decreased compared with the group treated with oridonin alone; the autophagic ratio dropped from 28.4% in the oridonin-treated group to 11.86% in the 3-MA and oridonin-treated group (Fig. 3B).

To further confirm the negative regulatory effects of 3-MA on oridonin-induced autophagy, the ratios of different doses of 3-MA in inhibition of autophagy at 24 h after oridonin addition were examined. The autophagic ratio of the oridonin-treated group (21.08%) was significantly higher than that of the control (5.09%), but when 3-MA was introduced, the autophagic percentage was sharply reduced from 21.08% to 5.86% – 20.33% (Fig. 3C). Accordingly, compared with the oridonin-alone treated group, every group treated with a combination of oridonin and 3-MA showed inhibition of autophagy and the inhibition ratios were dependent on the dose of 3-MA from 3.558% – 72.201% (Fig. 3D).

Ras, P38, and JNK participated in autophagic process

It was reported that a Ras mutant that selectively activated the Raf-1/MEK/ERK1/2 MAPKs cascade stimulated autophagy in human colon cancer cells, whereas another Ras mutation selectively activating the class I PI3K pathway inhibited the autophagic process (19, 20); however, the functions of P38 and JNK MAPKs in this pathway remain to be clarified. To investigate the influences of these kinases on oridonin-induced autophagy in HeLa cells, inhibitors of Ras, Raf-1, ERK1/2, P38, and JNK were applied. As shown in Fig. 4A, the Ras inhibitor Manumycin A augmented autophagy more significantly, indicating that Ras was a negative regulator of autophagy under these conditions. On the other hand, the P38 inhibitor SB203580 and

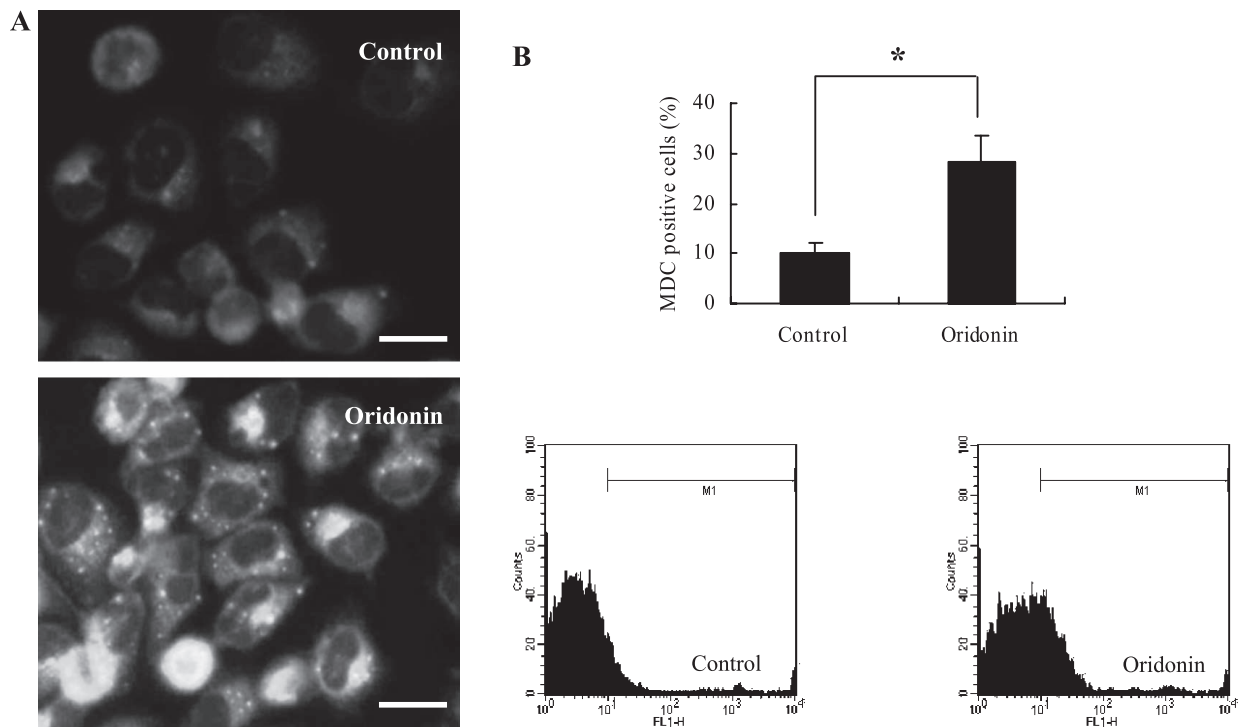


Fig. 2. Oridonin induced fluorescent changes of HeLa cells. A: Cellular fluorescent density was examined in the absence and in the presence of oridonin ($\times 400$ magnification, Bar: $30\mu\text{m}$); cells were labeled with MDC. B: Columns created from the flowcytometric histogram of the control and oridonin group. * $P < 0.05$.

JNK inhibitor SP600125 downregulated this autophagic process, suggesting that both P38 and JNK promoted oridonin-induced autophagy in HeLa cells.

It was interesting that both SP600125 and SB203580, like PD98059, enhanced HeLa sensitivity to cell death in the oridonin-applied system at 24 h (Fig. 4B), indicating that JNK and P38 contributed to cell death. These unexpected results were probably due to oridonin increasing both gene expressions and phosphorylation of P38 and JNK MAPKs, while 3-MA pretreatment attenuated the gene expressions of these two MAPKs rather than their phosphorylation (Fig. 5: B and C). The present data allowed us to deduce that non-phosphorylated P38 and JNK MAPKs might facilitate autophagy to antagonize cell death, and the influences of SP600125 and SB203580 on MAP-LC3 and Beclin 1 protein expressions presented consistent results to further confirm our hypothesis (Fig. 6: C and D).

P38 and JNK MAPKs contributed to, but ERK1/2 had no change in oridonin-induced autophagy

Under the condition of oridonin administration, 3-MA introduction played pivotal roles on both MAPKs expressions and activations. Oridonin and 3-MA exerted no effects on ERK1/2 expression, but p-ERK expression was decreased by oridonin application, whereas 3-MA

preincubation had no influence on oridonin-suppressed p-ERK expression (Fig. 5A). Oridonin enhanced both of P38 and JNK gene expressions and phosphorylations. However, combined application of oridonin and 3-MA decreased P38 and JNK gene expressions compared with oridonin alone treatment, but seemed had no obvious effects on their phosphorylation (Fig. 5: B and C).

Both MAP-LC3 and Beclin 1 protein expressions were altered by 3-MA, Ras, P38, and JNK in oridonin-stimulated HeLa cells

Since MAP-LC3 and Beclin 1 are two of the autophagic markers in mammalian cells, to investigate influences of MAPKs on these two proteins expression, western blotting to analyze proteins alternation was carried out. With oridonin treatment, LC3-II and Beclin 1 protein expressions were both enhanced, and the Ras inhibitor Manumycin A further augmented expression of both proteins. However, 3-MA, P38 inhibitor SB203580, and JNK inhibitor SP600125 downregulated the expression of LC3-II and Beclin 1 (Fig. 6). All the results allowed us to confirm that 3-MA and Ras were negative regulators, whereas P38 and JNK were positive regulators of oridonin-induced autophagy in HeLa cells.

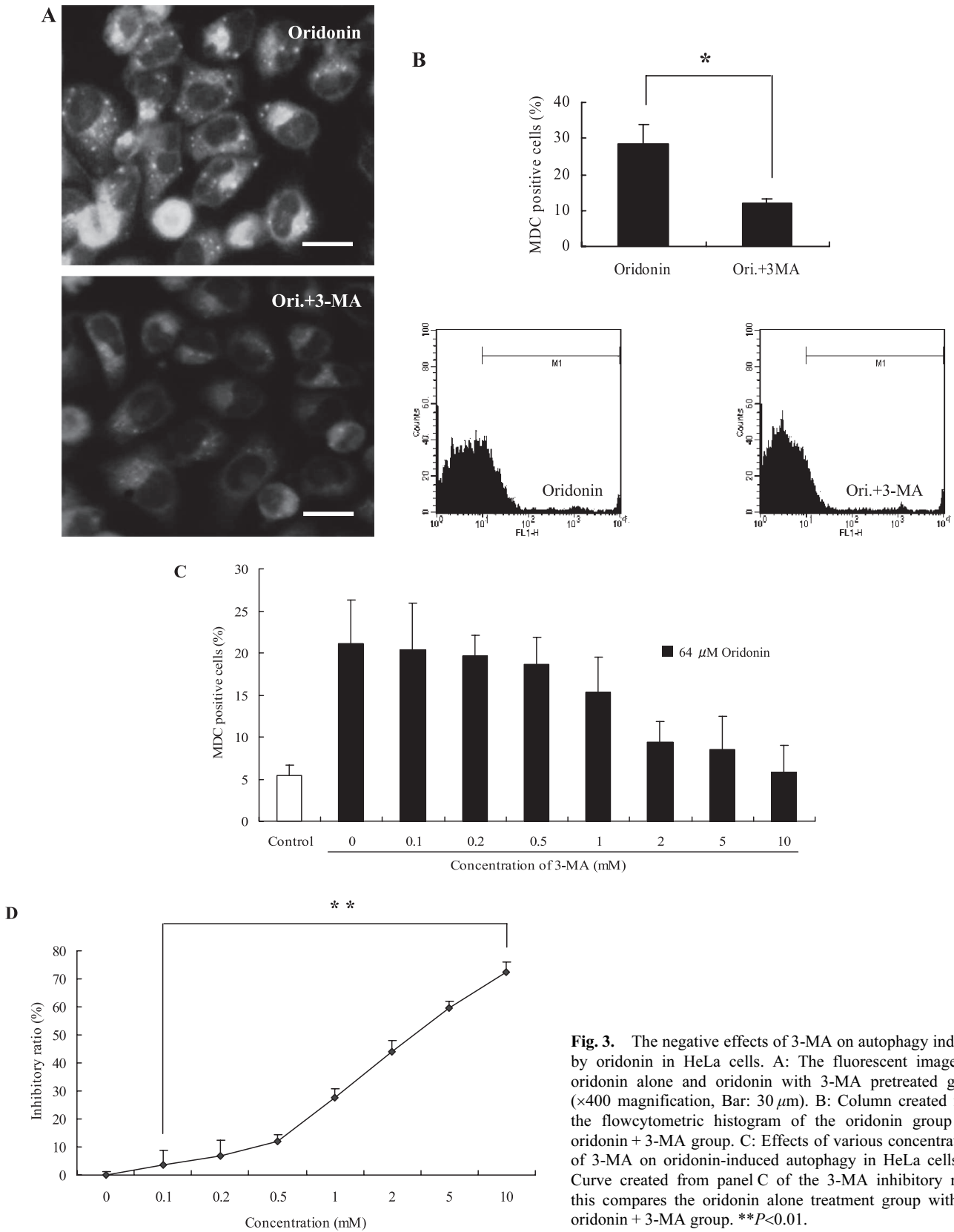


Fig. 3. The negative effects of 3-MA on autophagy induced by oridonin in HeLa cells. A: The fluorescent images of oridonin alone and oridonin with 3-MA pretreated group ($\times 400$ magnification, Bar: $30\ \mu\text{m}$). B: Column created from the flowcytometric histogram of the oridonin group and oridonin + 3-MA group. C: Effects of various concentrations of 3-MA on oridonin-induced autophagy in HeLa cells. D: Curve created from panel C of the 3-MA inhibitory ratio; this compares the oridonin alone treatment group with the oridonin + 3-MA group. $**P < 0.01$.

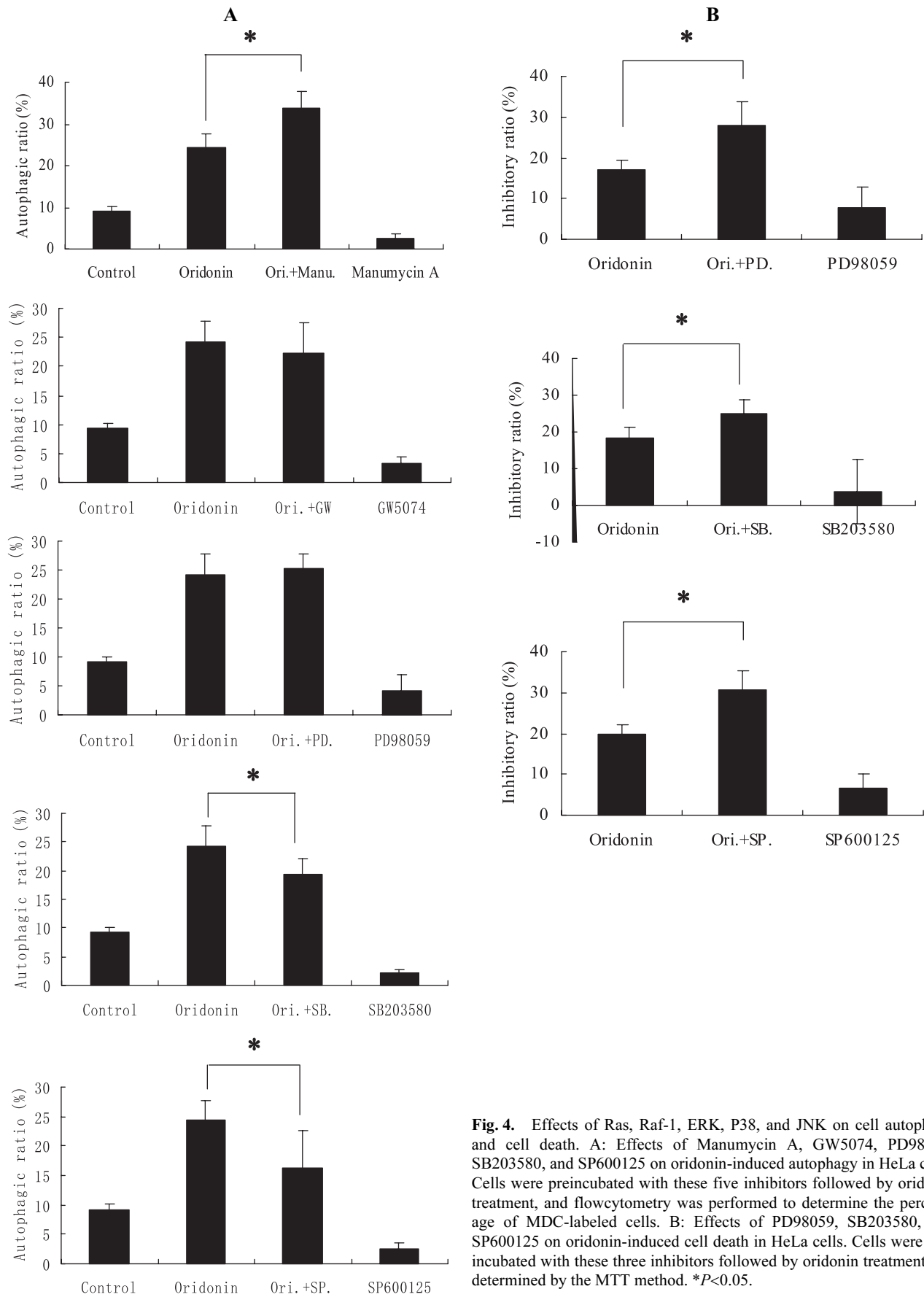


Fig. 4. Effects of Ras, Raf-1, ERK, P38, and JNK on cell autophagy and cell death. A: Effects of Manumycin A, GW5074, PD98059, SB203580, and SP600125 on oridonin-induced autophagy in HeLa cells. Cells were preincubated with these five inhibitors followed by oridonin treatment, and flowcytometry was performed to determine the percentage of MDC-labeled cells. B: Effects of PD98059, SB203580, and SP600125 on oridonin-induced cell death in HeLa cells. Cells were preincubated with these three inhibitors followed by oridonin treatment and determined by the MTT method. * $P<0.05$.

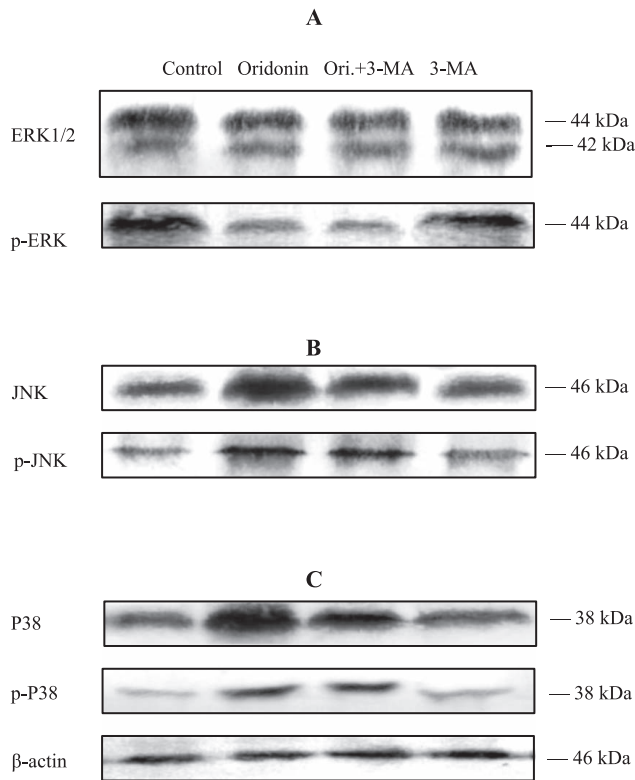


Fig. 5. Protein expressions of oridonin-stimulated ERK (A), JNK (B), and P38 (C) MAPKs with or without 3-MA treatment.

Discussion

Normal cell growth and development requires a well-controlled balance between protein synthesis and organelle biogenesis versus protein degradation and organelle turnover. One of the major pathways for degradation of cellular constituents is autophagy. Autophagy is a highly regulated and multi-step form of proteolysis that appears to have been conserved throughout evolution. Studies decades ago demonstrated that autophagy involves the rearrangement of subcellular membranes for the purpose of sequestering cytoplasmic cargo, followed by fusion to a lysosome, in order to degrade and recycle intracellular components (21 – 23).

Autophagy is a dynamic process consisting of the formation and fusion of membrane compartments. Therefore, it is essential to identify protein components of autophagic membrane in order to unravel the mechanism of the phenomenon. As a universal Atg8 homologue in autophagosomal membranes, microtubule-associated protein light chain 3 (MAP-LC3) is a useful reporter for autophagy (15). LC3 undergoes ubiquitin-like post-translational modifications that target it to the isolation membrane of the autophagosome, which makes it a general marker for autophagic membranes, which is essential for the dynamic process of autophagosome formation. Pro-LC3 is processed to its cytosolic form, 18-kDa LC3-I, which is modified to a membrane-bound

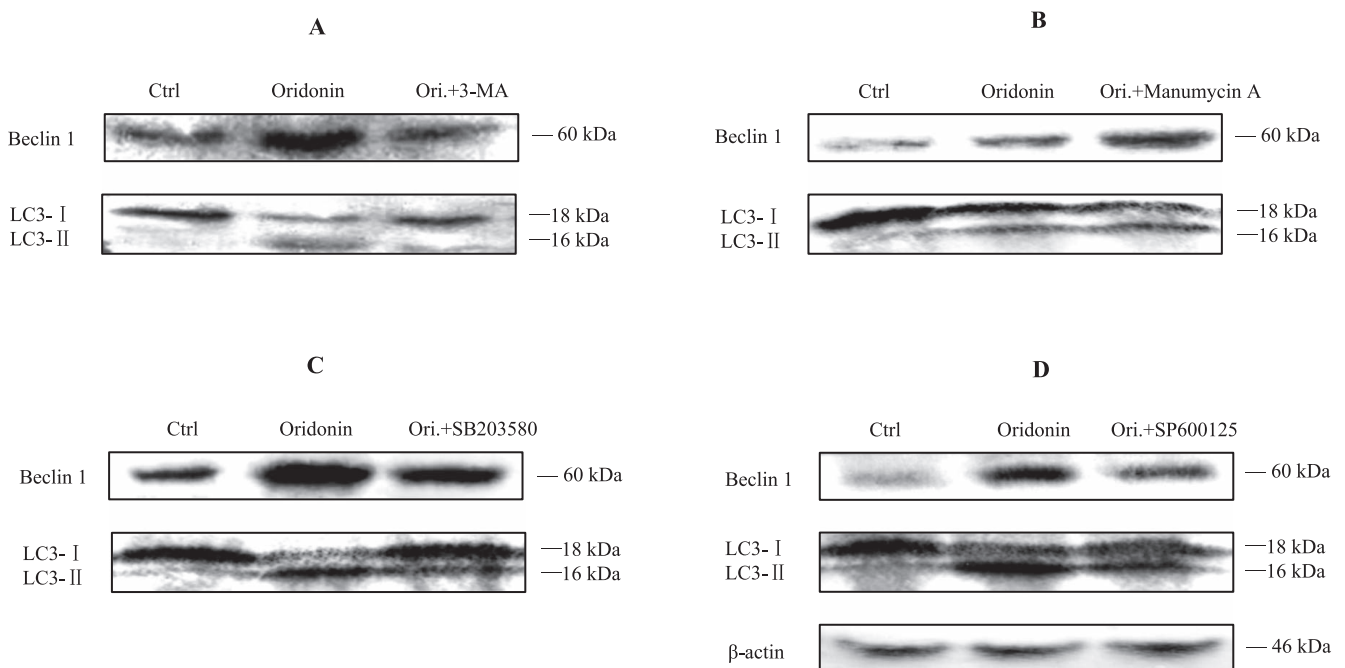


Fig. 6. Protein expressions of MAP-LC3 and Beclin 1 in oridonin-challenged HeLa cells. A: Effect of 3-MA, B: influence of Manumycin A, C: role of SB203580, and D: function of SP600125.

form, 16-kDa LC3-II. LC3-II is localized to autophagosomes and pre-autophagosomes, making this protein an autophagosomal marker. Following the fusion of autophagosomes with lysosomes, intra-autophagosomal LC3-II is degraded by lysosomal hydrolytic enzymes (24). We described here that under the conditions of oridonin-induced autophagy, LC3-II was abundantly expressed through LC3-I conversion.

The yeast Apg6 gene has been shown to complement its yeast deletion strains by restoring their ability to form autophagosomes or to perform autophagic activity (25). Beclin 1, the mammalian orthologue of the yeast Apg6 gene, can stimulate autophagy when overexpressed in mammalian cells and inhibit growth and tumor genesis of these cells (26). Levine and co-workers not only confirmed the involvement of beclin in mammalian autophagy, but also demonstrated that beclin acted as a suppressor of tumorigenesis (13). Beclin 1 forming a complex with the class III phosphatidylinositol (PtdIns) 3-kinase (PI3K) also participates in autophagosome formation, mediating the localization of other autophagy proteins to the preautophagosomal membrane (27). Based on the data in this study, Beclin 1 protein expression was provoked by oridonin treatment, further confirming the prevailing concept that this protein was tightly correlated to the autophagy process under the condition of oridonin treatment.

It was thought that autophagy induced by oridonin in HeLa cells could be suppressed under the condition of 3-MA introduction, and the inhibitory effect of 3-MA is dose-dependent, which was consistent with the general opinion that 3-MA is a specific inhibitor of the autophagic pathway.

The activation of the Ras/Raf-1/ERK1/2 pathway is able to promote different, and some times opposite, cellular processes. This wide variety of responses elicited by the activation of a single pathway has been shown to rely on the timing and strength of this activation (28). As a consequence, there must be a balance between activation and inhibition of the Ras/Raf/ERK1/2 signaling pathway to ensure an appropriate signaling output. Although the nature of this regulation has not been examined in detail, a recent study has found that Ras has a dual effect on autophagy; when it activates Class I PI3K, autophagy is inhibited, but when it activates the Raf/ERK1/2 cascade, autophagy is stimulated (29). The studies presented here are all consistent with Ras having a negative role in the control of this autophagic transport process, with elevated levels of Ras activity being found to essentially block the autophagy process. Ras decreased the sensitivity to autophagy as described before (29, 30). Raf-1 failed to exert influence on autophagy in this

system, which was consistent with the research of Pattingre's team (18). ERK1/2 alternation was the same as Raf-1, indicating that Ras displayed its suppressive role in oridonin-induced autophagy independent of the classical Ras/Raf-1/ERK1/2 pathway, but might be tightly related to some other signaling pathways, such as the Ras/PKA and Ras/Class I PI3K pathways. Further work will be necessary to identify the influences of these signals on this autophagic process.

Previous research showed conflicting data about the function of P38 MAPK in autophagy. Corcelle et al. reported that abrogation of P38 by SB203580 is sufficient to interfere with the normal autophagic maturation step (31). However, Prick et al. concluded that Hog1, the analogue of mammalian P38 MAPK, plays a negative role in the stabilization machinery of nitrogen-deprivation-induced autophagy in yeast cells during ambient osmolarity changes. This could be an analogy to the P38 MAPK pathway in mammalian cells, where osmosensing towards P38 MAPK is required for autophagy regulation by hypo-osmotic or amino acid-induced cell swelling (32). Here, we demonstrated that P38 was a contributive factor to oridonin-induced autophagy. Till now, the viewpoints about the role of JNK MAPK in autophagy seemed more consistent: studies of monocytes manipulated by RNAi technology indicate that JNK kinase contributes to a signal transduction pathway that induces both autophagy and cell death (33). In IRE1 (inositol-requiring enzyme 1 α , one of the transmembrane protein sensors for unfolded protein response)-deficient cells or cells treated with JNK inhibitor, the autophagy induced by ER stress was inhibited, indicating that the IRE1-JNK pathway is required for autophagy activation after ER stress (34). Our results showing that JNK positively controlled autophagy were consistent with those previous works. These findings underscored the critical role played by JNK and P38 MAPKs in the tight control of the autophagy process.

Taken together, we proposed that oridonin potently induced autophagy in HeLa cells through promotion of P38, JNK, MAP-LC3, and Beclin 1, but suppression of Ras.

References

- 1 Han QB, Li ML, Li SH, Mou YK, Lin ZW, Sun HD. Ent-kaurane diterpenoids from *Isodon rubescens* var. *lushanensis*. *Chem Pharm Bull*. 2003;51:790-793.
- 2 Zhang JX, Han QB, Zhao AH, Sun HD. Diterpenoids from *Isodon japonica*. *Fitoterapia*. 2003;74:435-438.
- 3 Kezoe T, Chen SS, Tong XJ, Heber D, Taguchi H, Koeffler HP. Oridonin induces growth inhibition and apoptosis of a variety of human cancer cells. *Int J Oncol*. 2003;23:1187-1189.

- 4 Liu JJ, Huang RW, Lin DJ, Wu XY, Peng J, Pan XL, et al. Oridonin-induced apoptosis in leukemia K562 cells and its mechanism. *Neoplasma*. 2005;52:225–231.
- 5 Ikezoe T, Chen SS, Tong XJ, Heber D, Taguchi H, Koeffler HP. Oridonin induces growth inhibition and apoptosis of a variety of human cancer cells. *Int J Oncol*. 2003;23:1187–1189.
- 6 Klionsky DJ, Ohsumi Y. Vacuolar import of proteins and organelles from the cytoplasm. *Annu Rev Cell Dev Biol*. 1999;15:1–32.
- 7 Dunn WA Jr. Autophagy and related mechanisms of lysosome-mediated protein degradation. *Trends Cell Biol*. 1994;4:139–143.
- 8 Kim J, Klionsky DJ. Autophagy, cytoplasm-to-vacuole targeting pathway, and pexophagy in yeast and mammalian cells. *Annu Rev Biochem*. 2000;69:303–342.
- 9 Seglen PO, Bohley P. Autophagy and other vacuolar protein degradation mechanisms. *Experientia*. 1992;48:158–172.
- 10 Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. *Science*. 2000;290:1717–1721.
- 11 Dunn WA Jr. Studies on the mechanisms of autophagy: formation of the autophagic vacuole. *J Cell Biol*. 1991;110:1923–1933.
- 12 Fengsrud M, Roos N, Berg T, Liou W, Slot JW, Seglen PO. Ultrastructural and immunocytochemical characterization of autophagic vacuoles in isolated hepatocytes: Effects of vinblastine and asparagine on vacuole distributions. *Exp Cell Res*. 1995;221:504–519.
- 13 Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell*. 2004;6:463–477.
- 14 Ogier-Denis E, Codogno P. Autophagy: a barrier or an adaptive response to cancer. *Biochimica Biophysica Acta*. 2003;1603:113–128.
- 15 Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J*. 2000;19:5720–5728.
- 16 Paz Y, Elazar Z, Fass D. Structure of GATE-16, membrane transport modulator and mammalian ortholog of autophagy-cytosis factor Aut7p. *J Biol Chem*. 2000;275:25445–25450.
- 17 Biederbick A, Kern HF, Elsasser HP. Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. *Eur J Cell Biol*. 1995;66:3–14.
- 18 Suzuki K, Hino M, Kutsuna H, Hato F, Sakamoto C, Takahashi T, et al. Selective activation of P38 mitogen-activated protein kinase cascade in human neutrophils stimulated by IL-1 beta. *J Immunol*. 2001;167:5940–5947.
- 19 Pattingre S, Bauvy C, Codogno P. Amino acids interfere with the ERK1/2-dependent control of macroautophagy by controlling the activation of Raf-1 in human colon cancer HT-29 cells. *J Biol Chem*. 2003;278:16667–16674.
- 20 Pattingre S, De Vries L, Bauvy C, Chantret I, Cluzeaud F, Ogier-Denis E, et al. The G-protein regulator AGS3 controls an early event during macroautophagy in human intestinal HT-29 cells. *J Biol Chem*. 2003;278:20995–21002.
- 21 Keller JN. Autophagy and cell fate. *Intern J Biochem Cell Biol*. 2004;36:2363.
- 22 Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. *Science*. 2000;290:1717–1721.
- 23 Munafò DB, Colombo MI. A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. *J Cell Sci*. 2001;114:3619–3629.
- 24 Tanida I, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy. *The Intern J Biochem Cell Biol*. 2004;36:2503–2518.
- 25 Patel S, Caplan J, Dinesh-Kumar SP. Autophagy in the control of programmed cell death. *Curr Opin Plant Biol*. 2006;9:391–396.
- 26 Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature*. 1999;402:672–676.
- 27 Kihara A, Kabeya Y, Ohsumi Y, Yoshimori T. Beclin/phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. *EMBO Rep*. 2001;2:330–335.
- 28 Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*. 1995;80:179–185.
- 29 Furuta S, Hidaka E, Ogata A, Yokota S, Kamata T. Ras is involved in the negative control of autophagy through the class I PI3-kinase. *Oncogene*. 2004;23:3898–3904.
- 30 Budovskaya YV, Stephan JS, Reggiori F, Klionsky DJ, Herman PK. The Ras/PKA signalling pathway regulates an early step of autophagy process in *Saccharomyces cerevisiae*. *J Biol Chem*. 2004;279:20663–20671.
- 31 Corcelle E, Djerbi N, Mari M, Nebout M, Fiorini C, Fenichel P, et al. Control of the autophagy maturation step by the MAPK ERK and P38: lessons from environmental carcinogens. *Autophagy*. 2007;3:57–59.
- 32 Prick T, Thumm M, Kohrer K, Haussinger D, Vomdahl S. In yeast, loss of Hog1 leads to osmosensitivity of autophagy. *Biochem J*. 2006;394:153–161.
- 33 Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, et al. Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science*. 2004;304:1500–1502.
- 34 Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, et al. Autophagy is activated for cell survival after ER stress. *Mol Cell Biol*. 2006;26:9220–9231.