Silibinin Activated p53 and Induced Autophagic Death in Human Fibrosarcoma HT1080 Cells *via* Reactive Oxygen Species-p38 and c-Jun N-Terminal Kinase Pathways

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Our previous research demonstrated that hepatic-protectant silibinin induced autophagy in human fibrosarcoma HT1080 cells through reactive oxygen species (ROS) pathway. Pifithrin-α (PFT-α), a specific inhibitor of p53, reduced autophagy and reversed silibinin's growth-inhibitory effect; besides, PFT-α decreased the activation of caspase-3, a crucial executor of apoptosis. Silibinin upregulated expression of p53/phosphorylated-p53 (p-p53) in a time-dependent manner. Catalase (scavenger of H_2O_2), superoxide dismutase (SOD) (scavenger of $O_2^{\cdot-}$), and SB203580 (inhibitor of p38) attenuated upregulation of p53 expression, suggesting that p53 might be partially regulated by ROS-p38 pathway. On the other hand, c-Jun N-terminal kinase (JNK) increased autophagic death in silibinin-treated cells, and JNK/p-JNK expression was upregulated by silibinin time-dependently. Inhibition of JNK by SP600125 did not influence generation of ROS. Scavengers of H_2O_2 or $O_2^{\cdot-}$ showed no effect on expression of JNK/p-JNK, indicating that JNK might not correlate with ROS in this process. However, activation of p53 was suppressed by SP600125; therefore the function of p53 was possibly controlled by JNK as well. Western blotting analysis showed that PFT-α reduced activation of extracellular regulated kinase1/2 (ERK1/2) and expression of protein kinase B (PKB, or Akt)/p-Akt. PD98059 (inhibitor of mitogen-activated protein kinase kinase (MEK)/ERK) and wortmannin (inhibitor of phosphoinositide 3-kinase (PI3K)/Akt) enhanced silibinin's cytotoxicity. Wortmannin augmented silibinin-induced autophagy, while PD98059 did not affect autophagic ratio. These results suggest that silibinin might induce p53-mediated autophagic cell death by activating ROS-p38 and JNK pathways, as well as inhibiting MEK/ERK and PI3K/Akt pathways.

Key words silibinin; autophagy; p53; c-Jun N-terminal kinase; p38; reactive oxygen species

Silibinin is a major active constituent of silymarin, a mixture of flavonolignans extracted from milk thistle (*Silybum marianum*). As a hepatic protectant and anti-inflammatory agent, silibinin (Fig. 1) is applied for liver disease.[1] Silibinin is considered relatively safe and there are only few reports about adverse reactions to this compoud.[2] The low-toxic property is one of the reasons that make silibinin an optimistic candidate anticarcinogen. Research has found that treatment with silibinin to some cancer cell lines leads to pro-oxidant instead of anti-oxidant effects.[3,4] It is reported that silibinin is not a good scavenger of $O_2^{\cdot-}$ and no reaction with H_2O_2 has been detected; however, silibinin reacts rapidly with $\cdot$OH in rat liver microsome.[5] Silibinin induces reactive oxygen species (ROS)-dependent activation of p38 in human fibrosarcoma HT1080 cells and glioma U87MG cells,[4] and we also demonstrated that the ROS-p38-NF-κB (nuclear transcription factor kappa B) pathway, which is attributed to silibinin-induced autophagy and cell death, was significantly suppressed by catalase (scavenger of H_2O_2[6]) and superoxide dismutase (SOD) (scavenger of $O_2^{\cdot-}$[7]).

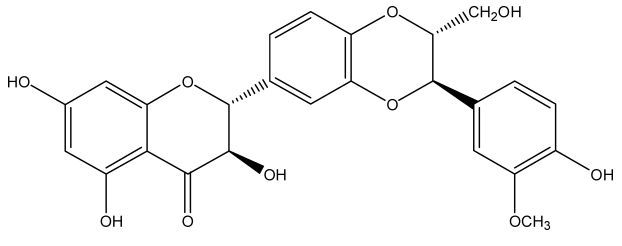

Fig. 1. Structure of Silibinin

Autophagy, which plays an intricate role in cancer and inflammation, is one of the crucial mechanisms of cellular stress adaptation response that maintains homeostasis. Autophagy-mediated stress survival is believed a beneficial factor in tumorigenesis, while inhibition of autophagy may cause persistent damage and chronic inflammation that subsequently drive tumor progression.[8]

Recent research reveals that autophagy is activated by p53, which is one of the best-characterized tumor suppressors involved in most tumorigenesis.[9] Accumulating evidence indicates that p53 regulates autophagy in a dual pattern: nuclear p53 acts as a transcription factor, which activates genes in charge of apoptosis, cell-cycle-arrest, and autophagy; on the other hand, cytoplasmic p53 represses autophagy *via* poorly characterized mechanisms.[10]

p38 and c-Jun N-terminal kinase (JNK) are both stress-activated members of mitogen-activated protein kinase (MAPK) family. p38 and JNK play essential roles in cell differentiation, growth, inhibition, and apoptosis. Evidence has demonstrated ROS-dependent activation of p38 in innate immunity,[11] lifespan limitation,[12] apoptosis,[13] and autophagy.[14] Findings underscored a critical role played by p38 in tight control of the autophagy process at the maturation step, and abrogation of p38 by SB203580 (a specific inhibitor of p38) is sufficient to interfere with the normal autophagic maturation step.[15] JNK activation has been strongly implicated in inflammatory responses, neurodegeneration, and apoptosis. Recent study indicates that, in human cervix carcinoma HeLa cells, JNK participates in CH11 (Fas agonistic antibody)-induced autophagy, which can be blocked by

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JNK inhibitor SP600125.[16)]

In this study, we demonstrated that p38 inhibitor SB203580 and ROS scavengers (catalase and SOD) repress activation of p53, which might function *via* ROS-p38 pathway based on our previous research. However, JNK was not influenced by scavenging of ROS. The results suggest that activation of p53 induced by silibinin might depend on ROS-p38 and JNK MAPK pathways.

MATERIALS AND METHODS

Reagents Silibinin was obtained from Beijing Institute of Biological Products (Beijing, China); its purity was determined about 99% by HPLC measurement. Silibinin was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution and diluted by Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, U.S.A.) before the experiments. DMSO concentration in all cell cultures was kept $<0.1\%$, which had no detectable effect on cell growth or death. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescein diacetate (DCF-DA), monodansyl cadaverine (MDC), 3-methyladenine (3-MA), pifithrin-α (PFT-α), SB203580, SP600125, catalase, SOD, rapamycin, PD98059, and wortmannin were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Polyclonal antibodies against caspase-3 (sc-7148), p38α/β (sc-7149), p-p38 (sc-101759), p53 (sc-6243), p-p53 (sc-135630), JNK (sc-571), p-JNK (sc-135642), extracellular regulated kinase 1 (ERK1) (sc-93), ERK2 (sc-154), p-ERK1/2 (sc-101761), Akt1/2/3 (sc-8312), p-Akt1/2/3 (sc-109903), Actin (sc-7210), BECN1 (sc-11427), MAP LC3β (sc-28266), and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cell Culture HT1080, human fibrosarcoma cells, were obtained from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) and cultured in DMEM medium supplemented with 10% heat inactivated (56 °C, 30 min) fetal bovine serum (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China), 2 mM L-glutamine (Gibco, Grand Island, NY, U.S.A.), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37 °C in 5% CO_2. The cells in the exponential phase of growth were used in the experiments.

Autophagic Vacuoles Labeled by MDC Staining A fluorescent compound, MDC, has been used as tracer for autophagic vacuoles. After incubation with silibinin for the indicated time periods, HT1080 cells were stained with 50 μM MDC at 37 °C for 40 min, then the morphology was observed by fluorescence microscopy (Olympus, Tokyo, Japan).

Growth Inhibition Assay The growth inhibitory effect of silibinin on HT1080 cells was measured by MTT assay.[17)] The cells were dispensed in 96-well flat bottom microtiter plates (NUNC, Roskilde, Denmark) at a density of 1.5×10^4 cells per well. After 24-h incubation, they were treated with the test agents for the indicated time periods. A 20-μl aliquot of MTT solution (5.0 mg/ml) was added to each well followed by 4-h incubation and the optical density was measured by ELISA reader (Tecan Spectra, Wetzlar, Germany). The percentage of cell growth inhibition was calculated as follows:

$$\text{inhibitory ratio (\%)}=(A_{490,\text{ control}}-A_{490,\text{ sample}})/(A_{490,\text{ control}}-A_{490,\text{ blank}})\times100$$

Western Blotting Analysis Both adherent and floating HT1080 cells were harvested, washed twice with ice-cold phosphate buffered saline (PBS), then lysed in lysis buffer (50 mM Hepes (pH 7.4), 1% Triton-X 100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM edetic acid, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin) on ice for 1 h. After centrifugation of the cell suspension at $13000\times\boldsymbol{g}$ for 15 min, protein content of the supernatant was determined by Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, U.S.A.). The protein lysates were separated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, U.S.A.). Proteins were detected using polyclonal antibody and visualized using anti-rabbit, anti-mouse, or anti-goat immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) and 3,3-diaminobenzidine tetrahydrochloride (DAB) as the substrate of HRP.

Flowcytometric Analysis Using MDC and DCF-DA After incubation with test agents for the indicated time periods, HT1080 cells were harvested, rinsed with PBS, then stained with 50 μM MDC at 37 °C for 40 min. After incubation, the cells were washed once with PBS. The samples were analyzed by FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

The treated HT1080 cells were incubated with 10 μM DCF-DA at 37 °C for 30 min. Intracellular ROS mediated oxidation of DCF-DA to the fluorescent compound 2′,7′-dichlorofluorescein (DCF). Then the cells were harvested and suspended in PBS. Samples were analyzed by FACScan flow cytometer.

Statistical Analysis All data represented at least three independent experiments and are expressed as mean±S.E.M. The data were analyzed by ANOVA using Statistics Package for Social Science (SPSS) software (version 13.0; SPSS, Chicago, IL, U.S.A.) and least significant differences (LSD)-*post hoc* test was employed to assess the statistical significance of difference between control and treated groups. A p-value <0.05 was considered statistically significant.

RESULTS

Silibinin Induced Autophagic and Apoptotic Death *via* p53 in HT1080 Cells In this study, the cells were cultured in medium with 10% fetal bovine serum, then cultured in serum-free-medium for 24 h to mimic starvation further. This 24-h-serum withdrawal did not lead to any detectable changes to HT1080 cells (data not shown). Silibinin induced autophagy in the cells, confirmed by observation of notable autophagocytic vacuoles, which were stained with MDC, a selective fluorescent marker for autophagic vacuoles.[18)] The specific autophagic inhibitor, 3-MA,[19)] decreased autophagocytic vacuoles (Fig. 2A, a). MTT assay showed that silibinin significantly inhibited cell growth, and this inhibition was reversed by 3-MA, but augmented by rapamycin (Fig. 2A, b), stimulating autophagy by inhibiting mammalian target of rapamycin (mTOR) in nutrient-insufficient autophagy.[20)] The results suggest that autophagy induced by silibinin possibly facilitates apoptosis and cell death.

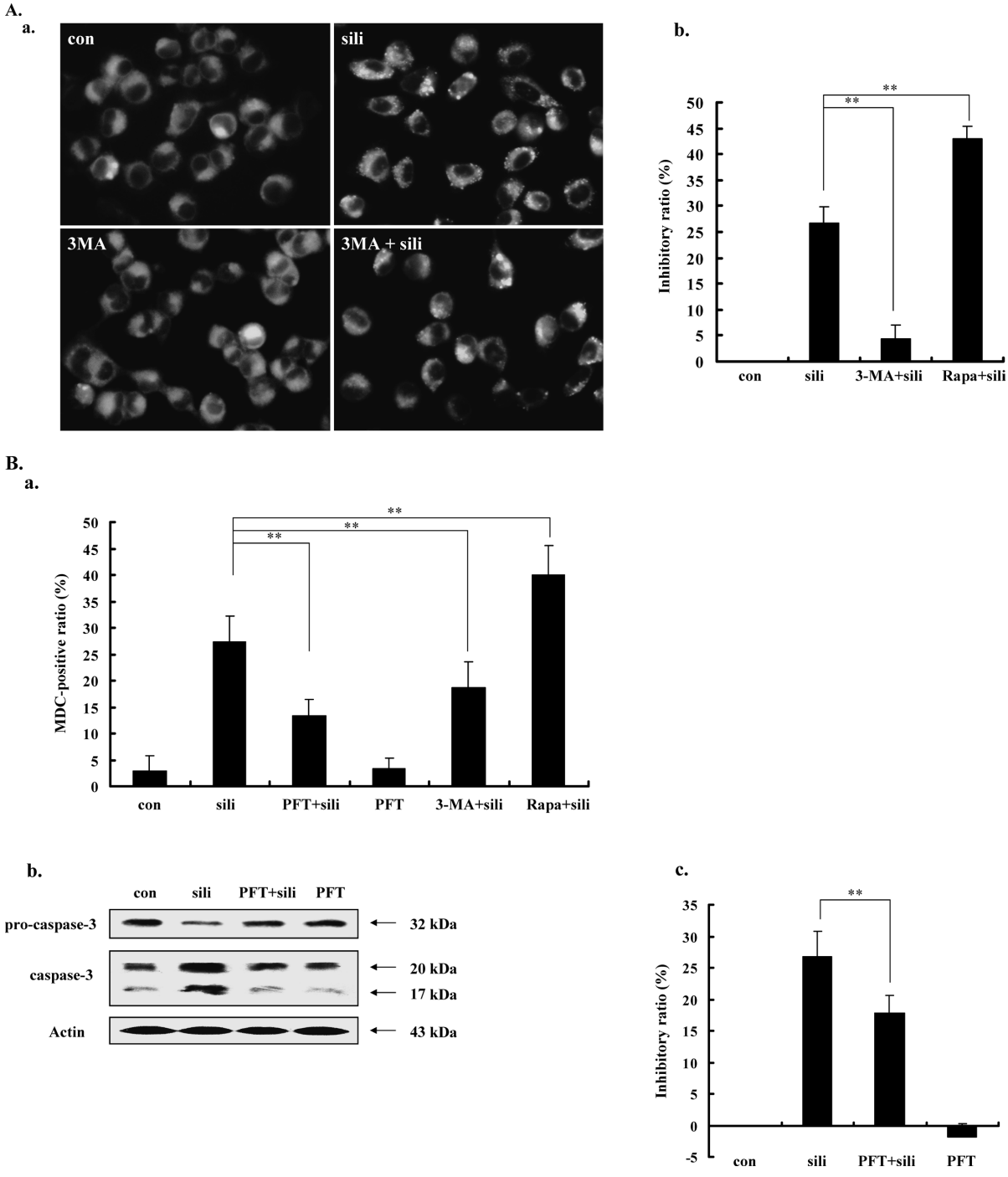

Fig. 2. p53 Plays a Crucial Role in Silibinin-Induced Autophagy and Apoptosis in HT1080 Cells

In this study, the cells were cultured in medium with 10% fetal bovine serum, then cultured in serum-free medium for 24 h to mimic starvation further. This 24-h-serum withdrawal did not lead to any detectable changes to HT1080 cells. The cells were treated with indicated inhibitors for 1 h, and cultured with 40 μM silibinin for 4 h. con: control. (A) Autophagic (a) and inhibitory ratio (b) was detected in 3MA (800 μM)-pretreated and silibinin-treated cells by MDC staining and MTT assay, respectively. (B) (a) Flow cytometric analysis was applied to determine the effect of PFT-α (PFT, 20 μM) on silibinin-induced autophagy in HT1080 cells. Rapa: rapamycin, 20 μM. (b) Western blotting analysis was applied to investigate the effect of PFT-α on the cleavage of pro-caspase-3 to activated caspase-3. (c) MTT assay showed the effect of PFT-α on cytotoxicity of silibinin to HT1080 cells. The data are presented as mean±S.E.M. of results for three independent experiments. ** $p<0.01$.

Pifithrin-α (PFT-α) is a specific inhibitor of p53.[21] It reversibly inhibits p53-transcriptional activity, and subsequently inhibits p53-induced apoptosis and cell cycle arrest.[22] Flow cytometric analysis showed that PFT-α reduced silibinin-induced autophagy staining with MDC, and this autophagy was inhibited by 3-MA, but enhanced by rapamycin (Fig. 2B, a). Caspase-3 is a member of the family of asparate-specific cysteinyl proteases, and has been identified as a key mediator of apoptosis of mammalian cells by cleaving a variety of key cellular proteins, such as inhibitor of caspase-activated DNase (ICAD), poly(ADP-ribose) polymerase (PARP), and others.[23] Western blotting analysis showed that PFT-α reversed cleavage of unactivated pro-caspase-3 to activated caspase-3, indicating that p53 was involved in silibinin-induced apoptosis (Fig. 2B, b). Growth inhibition assay indicated that PFT-α attenuated silibinin-induced cell death (Fig.

2B, c). These results suggest that p53 is involved in silibinin-induced autophagy and apoptosis in HT1080 cells.

Silibinin Activated p53 *via* ROS-p38 Pathway Western blotting analysis showed that p38/phosphorylated-p38 (p-p38, the activated form of p38) and p53/p-p53 (the activated form of p53) were both upregulated in a time-dependent manner (Fig. 3A). ROS was previously demonstrated a potential upstream regulator of p38.[4] Catalase (scavengers of H_2O_2) and SOD (scavengers of $O_2^{\cdot -}$) reduced levels of both p38 and p-p38. p53 as well as its activated form were also decreased in ROS scavengers-treated group (Fig. 3B). Kim *et al.*[24] reported that p38 partly regulated activation of p53-related signaling. To investigate the functions of p38 and p53, PFT-α and SB203580 (p38 specific-inhibitor[25]) were applied in silibinin-treated cells. Expression of p53/p-p53 was inhibited by SB203580, while p38/p-p38 were not affected by PFT-α (Fig. 3C), indicating that silibinin might activate p53 through p38 by inducing ROS generation.

JNK Mediated Silibinin-Induced Activation of p53, but Uncorrelated with ROS To investigate the role of JNK in silibinin-induced autophagic cell death, a specific inhibitor of JNK, SP600125,[26] was applied in our following experiments. Detected by flow cytometric analysis and MTT assay, respectively, SP600125 attenuated silibinin-induced autophagy (Fig. 4A, a) and death (Fig. 4A, b) in HT1080 cells. Silibinin elevated expressions of both JNK and its activated form p-JNK time-dependently (Fig. 4B). JNK did not affect generation of ROS in silibinin-treated cells (Fig. 4C, a), and catalase or SOD had no effect on expression of JNK/p-JNK (Fig. 4C, b), suggesting that JNK expression might be not correlated with ROS in this process. Previous evidence indicates that p53 was partially regulated by JNK in pseudolaric acid B-induced HeLa cell death.[27] SP600125 markedly reversed upregulation of p53/p-p53, while PFT-α had no visible effect on JNK (Fig. 4D).

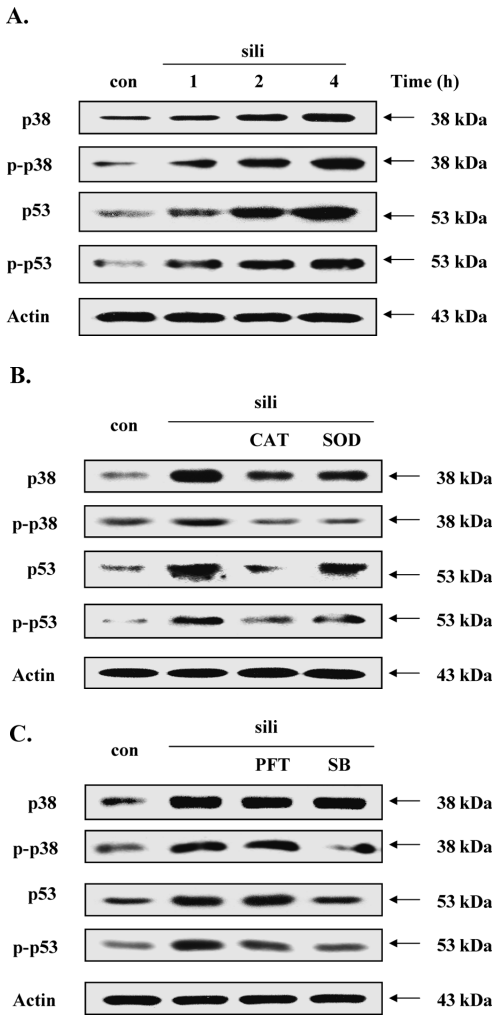

Fig. 3. ROS and p38 Are Potential Upstream Regulators of p53

Cells were cultured in serum-free-medium (SFM) for 24 h, then added by indicated inhibitors for 1 h, and cultured with 40 μM silibinin. con: control. (A) Expression of p38/p-p38 and p53/p-p53 was detected in silibinin-treated cells by Western blotting analysis. (B) Effects of catalase and SOD were determined in silibinin-induced cells by detecting levels of p38/p-p38 and p53/p-p53. CAT: catalase, 500 U/ml; SOD: 100 U/ml. (C) Relation between p38 and p53 was investigated by Western blotting analysis. SB: SB203580, 15 μM; PFT: PFT-α, 20 μM.

p53 Partially Mediated Autophagy and Death by Inhibiting the Functions of MEK/ERK and PI3K/Akt MAPK kinase (MEK)/extracellular regulated kinase 1/2 (ERK1/2), and phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB/Akt) are implicated in cell survival pathways and play critical roles in cell growth and proliferation. PD98059 is a potent inhibitor of MEK/ERK1/2,[28] and wortmannin can inhibit PI3K/Akt pathway.[29] Although PD98059 showed no effect on silibinin-induced autophagy (Fig. 5A, a), it augmented inhibitory ratio in silibinin-treated group (Fig. 5A, b). Nonetheless, wortmannin augmented autophagy and cell death induced by silibinin (Figs. 5A, c, d). Western blotting analysis showed that SP600125 and PFT-α reversed silibinin-induced inhibition of activation of ERK1/2 and expression of Akt/p-Akt (Fig. 5B). These results suggested that PD98059 and wortmannin themselves had little effect on cell death, indicating that silibinin might activate JNK-p53 pathway and subsequently and indirectly inhibit functions of MEK/ERK1/2 and PI3K/Akt survival pathways. Beclin1 (BECN1) is one of the first mammalian proteins discovered to mediate autophagy,[30] and microtubule-associated protein-light chain 3β (MAP LC3β) is the first mammalian protein identified autophagosome membrane-associated.[31] Expression of Beclin1 and conversion of LC3 I to LC3 II (which is considered as marker of autophagosome formation) were both elevated by silibinin. PFT-α and SP600125 downregulated Beclin1, while wortmannin increased expression of Beclin1, and PD98059 showed no notable change (Fig. 5C). Likewise, conversion of LC3 I to LC3 II was reversed by PFT-α and SP600125, and was enhanced by wortmannin markedly; PD98059 did not affect the conversion (Fig. 5C). Therefore it was supposed that, in the process of silibinin-induced autophagic and apoptotic cell death, p53 was activated by ROS-p38 and JNK pathways, and led to cell death partly by inhibition of MEK/ERK1/2 and PI3K/Akt survival pathways.

DISCUSSION

Autophagy has been reported regulated by ROS including $O_2^{\cdot -}$ and H_2O_2 induced by starvation of glucose, L-glutamine, pyruvate, amino acids, or serum, and $O_2^{\cdot -}$ is the major form participting in ROS-regulated autophagy.[32] Furthermore, ROS is described as a kind of signaling molecule in starvation-induced autophagy in which starvation stimulates especially H_2O_2.[33] Our previous study illustrated that in HT1080

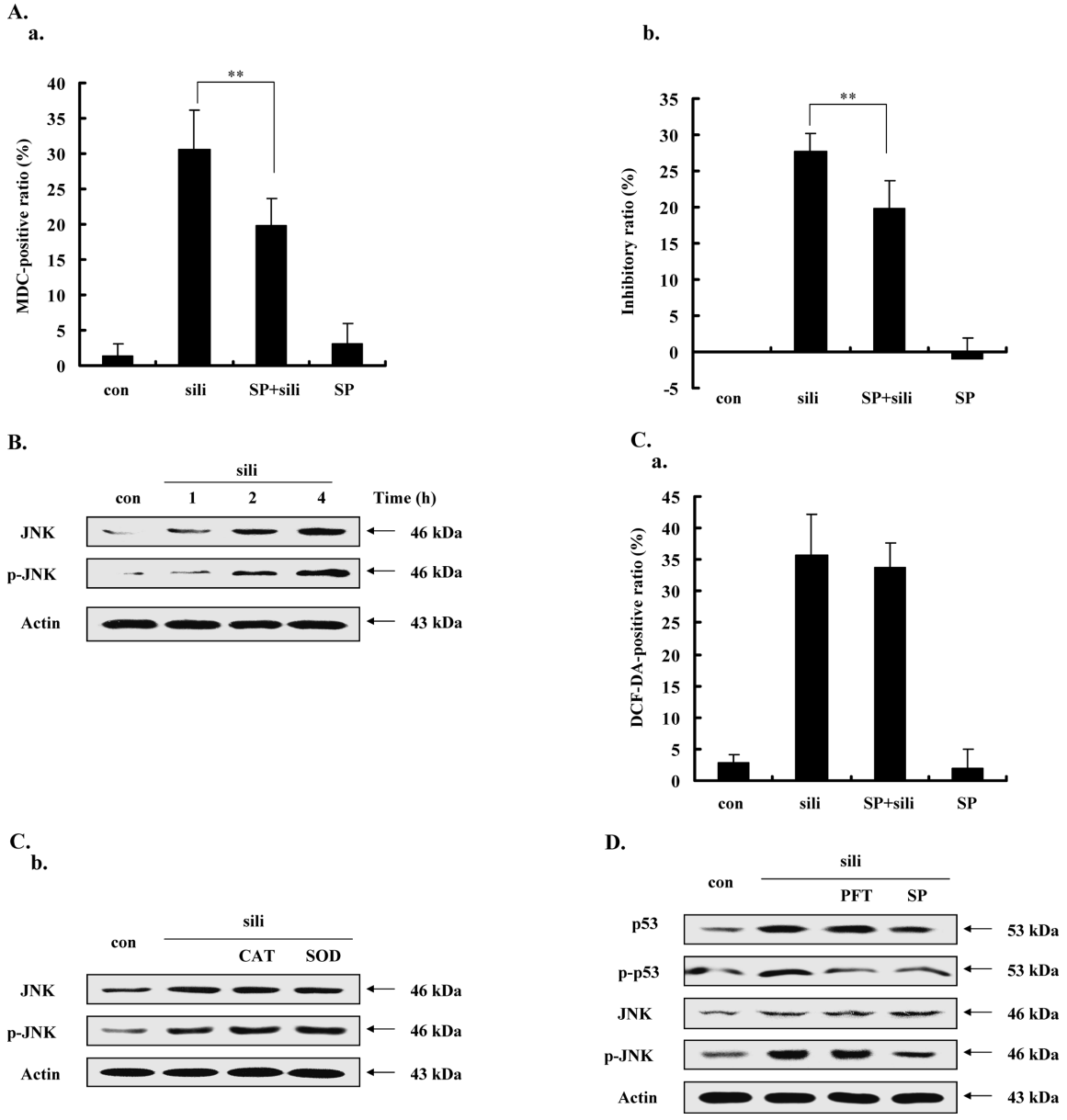

Fig. 4. JNK Participated in Silibinin-Induced Autophagic Death by Activating p53, but Not Correlated with ROS

Cells were cultured in SFM for 24 h, then added by indicated inhibitors for 1 h, and cultured with 40 μM silibinin. con: control. (A) Autophagic (a) and inhibitory ratio (b) in SP600125 (SP, 5 μM)-pretreated and silibinin-treated cells were detected by MDC staining and MTT assay, respectively. (B) Expression of JNK/p-JNK was determined by Western blotting analysis. (C) (a) Flow cytometric analysis by DCF-DA staining showing effect of SP600125 on generation of ROS. (b) Effects of catalase (CAT, 500 U/ml) and SOD (100 U/ml), on expression of JNK/p-JNK was detected by Western blotting analysis. The data are presented as mean$\pm$S.E.M. of the results for three independent experiments. $^{**}p<0.01$. (D) Western blotting analysis was applied to determine relation between JNK and p53. PFT: PFT-α, 20 μM.

cells in serum-free medium, silibinin induced $O_2^{\cdot -}$ and H_2O_2 as two major forms of ROS, which were subsequently involved in autophagic cell death.[3)]

p38 blocked cancer cell autophagy in a previous study[34)]; however, other research has demonstrated positive effects of p38 on autophagic cancer cell death.[35)] Vitale *et al.*[36)] demonstrated that depletion of p38 by short interfering RNA (siRNA) abolished phosphorylation of p53; furthermore, vitamin C was suggested to inhibit p53-induced senescence by preventing ROS generation, which in turn led to activation of p38 MAPK.[37)] In human melanoma A375 cells, ROS-p38-p53 pathway was reported involved in autophagy and apoptosis mediated by mitochondria.[38)]

Inhibition of the stress kinase JNK abrogated autophagy in HeLa cells[16)] and glioma cells.[39)] Since JNK in association with p53 plays an important role in p53 stability, activation of p53 by stress and damage stimuli often correlates with induction of JNK. JNK was shown to increase levels of the exogenously transfected form of p53 in p53 null cells as well as of endogenous p53 in breast cancer MCF7 cells.[40)] Recent evidence indicates critical roles of p53 and JNK in regulation of autophagy and the interplay between them in antitumor compound 2-methoxyestradiol (2-ME)-treated Ewing sarcoma cells[41)]; moreover, p53 phosphorylation, which is mediated by JNK, contributed to HW1 (agonistic single chain variable fragment antibody against human death receptor 5 (DR5))-induced autophagic cell death in colon carcinoma HCT116 cells.[42)]

p53 expression is regulated through activation of p38 and JNK pathways during inflammatory responses.[43)] In prostate

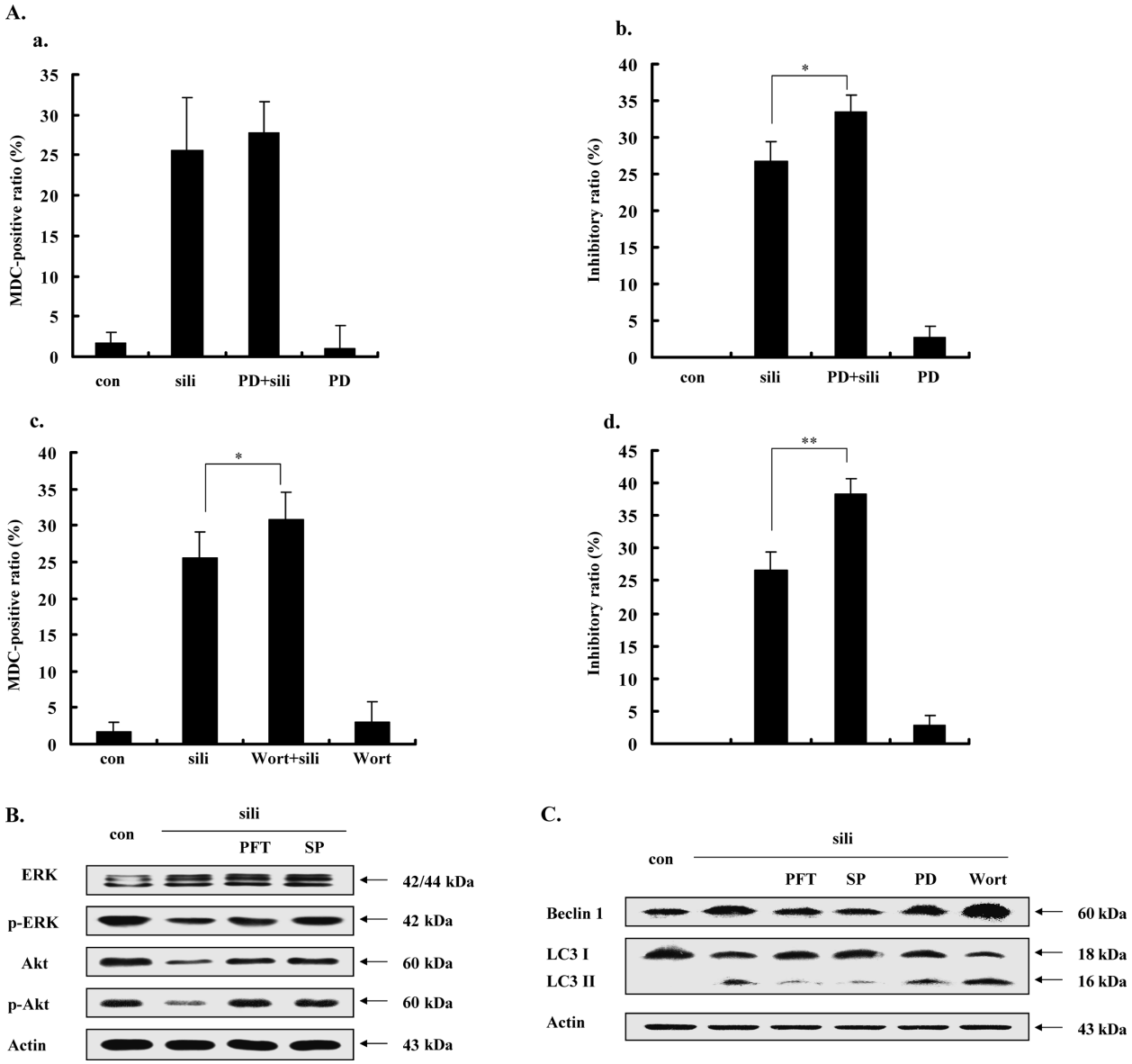

Fig. 5. MEK/ERK and PI3K/Akt, Which Protect Cells against Silibinin, Were Suppressed by p53

Cells were cultured in SFM for 24 h, then added by indicated inhibitors for 1 h, and cultured with 40 μM silibinin for 4 h. con: control; PD: PD98059, 5 μM; Wort: wortmannin, 500 nM; PFT: PFT-α, 20 μM; SP: SP600125, 5 μM. (A) Effects of PD98059 on silibinin-induced autophagy (a) and growth inhibition (b) as detected by MDC staining and MTT assay, respectively. Effects of wortmannin on autophagic (c) and inhibitory ratio (d) in silibinin-treated cells were detected by MDC staining and MTT assay, respectively. The data are presented as mean±S.E.M. of the results for three independent experiments. $*p<0.05$; $**p<0.01$. (B) Effects of PFT-α and SP600125 on expression of ERK/p-ERK and Akt/p-Akt were detected by Western blotting analysis. (C) Effects of indicated inhibitors on expression of Beclin1 and conversion of LC3 I to LC3 II were detected by Western blotting analysis.

cancer cells, inhibition of NF-κB or its close regulator p38 resulted in suppression of p53 induction and apoptosis; meanwhile, inhibition of JNK strongly reduced p53 induction, and almost completely suppressed 2-ME-induced apoptosis.[44] However, apoptosis was indicated to be induced through activation of p53 *via* JNK and p38 signaling in H-Ras MCF10A cells,[45] Lewis lung carcinoma (LLC) cells, hepatoma HepG2 cells, and Molt-4 leukemia cells.[46]

We previously demonstrated that p53 regulated autophagy in addition to its tumor-suppressing activity, and p53 activation was involved in oridonin-induced apoptotic and autophagic cell death in HT1080 cells.[47] LY294002, an inhibitor of class I PI3K, also induced autophagy by activating p53 and caspase-3 in gastric cancer SGC7901 cells.[48] Conversely, absence or inhibition of p53 was reported to cause autophagy in G_1 phase of the colon carcinoma HCT116 cell cycle.[49] Inhibition of p53 led to autophagy in enucleated cells, and cytoplasmic, not nuclear, p53 was able to repress autophagy in $p53^{-/-}$ cells of human, mouse, and nematode.[50] Consolidating these findings, p53 plays a dual role in the control of autophagy: on one side, nuclear p53 can induce autophagy through transcriptional effects; on the other, cytoplasmic p53 may act as a repressor of autophagy.[51] The mechanisms of autophagy inhibition by cytoplasmic p53 are drastically distinct from both its transcriptional function in nucleus and its pro-apoptotic function at mitochondria.[52] Although p53-mediated suppression of autophagy might promote tumorigenesis, however, its biological role remains to be elucidated.

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