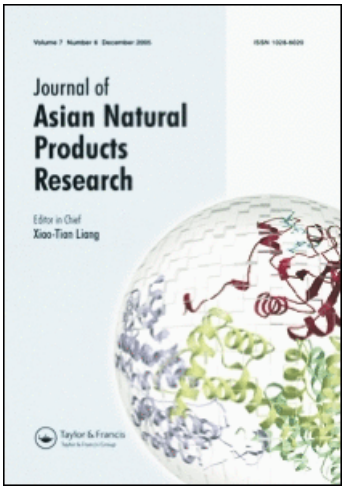

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[a] China-Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, Shenyang, China [b] Department of Clinical and Biomedical Sciences, Showa Pharmaceutical University, Tokyo, Japan

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[a]*China–Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, Shenyang 110016, China;* [b]*Department of Clinical and Biomedical Sciences, Showa Pharmaceutical University, Tokyo 194-8543, Japan*

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Our previous results demonstrated that silibinin induced autophagic and apoptotic cell death dependent on reactive oxygen species (ROS especially H_2O_2 and $O_2^{\cdot-}$) in HT1080 cells. In this study, we further show that p38–NF-κB pathway is involved in silibinin-induced ROS-mediated autophagy. Cells were pretreated with serum-free media for 24 h before being treated with silibinin. Generation of ROS and autophagy was detected in 15 min and 1 h, respectively. Development of autophagy was supported by an upregulated expression of Beclin-1 and conversion of light chain (LC3-I–LC3-II). Expression of p38/p-p38 and transposition of NF-κB from cytoplasm to nuclei were also increased. Inhibitors of p38 and NF-κB and scavengers of H_2O_2 and $O_2^{\cdot-}$ reduced both generation of ROS and simultaneous occurrence of silibinin-induced autophagy. Besides, expression of p38/p-p38 and transposition of NF-κB from cytoplasm to nuclei were decreased by these two ROS scavengers. ROS and p38–NF-κB pathway were possibly cooperated in a positive feedback mechanism. Inhibition of p38, NF-κB, H_2O_2, or $O_2^{\cdot-}$ rescued cells from silibinin-induced death in a long-term (12 h) manner. According to the previous study that silibinin-induced autophagy was a positive regulator of apoptotic cell death, it was possible that ROS and p38–NF-κB mediated silibinin-induced autophagy and eventually led to cell death.

Keywords: silibinin; autophagy; p38; NF-κB; H_2O_2; $O_2^{\cdot-}$

1. Introduction

Silibinin (Figure 1, **1**), which is well known for its hepato-protective properties [1], exerted anticancer activities to several kinds of tumor cells [2,3]. Our previous study demonstrated that **1** induced reactive oxygen species (ROS)-mediated autophagic and apoptotic death in human fibrosarcoma HT1080 cells [3]. Although type II programed cell death (PCD), autophagy, was considered to be a defence mechanism for cell to ensure survival, especially in starvation [4], several researches proved that autophagy led to total devastation of the cell in a way distinguished from type I PCD, apoptosis [3,5]. ROS-mediated p38–NF-κB pathway was found to participate in cell-protective autophagic process [4]; however, in this study, p38–NF-κB pathway contributed to autophagic cell death mediated by **1**-induced ROS generation.

p38 belongs to the family of mitogen-activated protein kinases (MAPKs). As a tumor suppressor, p38 plays an essential role in cell differentiation, growth inhibition, and apoptosis [6]. Although p38 was found to be a negative regulator of

*Corresponding author. Email: ikejimat@vip.sina.com

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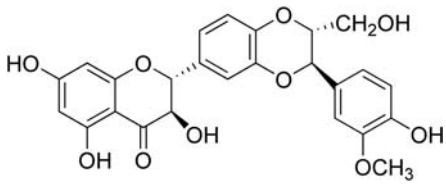

Figure 1. Structure of silibinin.

both basal autophagy and starvation-induced autophagy [7], findings underscored the critical role played by p38 in the 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 [8].

NF-κB is formed through the dimerization of five subunits: p65, c-Rel, RelB, NF-κB1, and NF-κB2 [9]. The p65 subunit provides the gene regulatory function. In response to stimuli, degradation of inhibitory κB proteins allows the translocation of NF-κB from the cytoplasm to the nucleus and subsequently binding to target genes in charge of various kinds of cellular responses [10]. NF-κB was thought to suppress PCD, according to the first evidence provided by RelA knockout mice that died of massive liver apoptosis [11]. However, the followed researches indicated NF-κB functions as a double-edged sword. For instance, NF-κB was essential for promoting apoptosis in thymocytes [12]. Meanwhile, recent studies expounded intricate relationship between NF-κB and autophagy [13] and indicated a two-way regulation of NF-κB to autophagy. NF-κB was reported to suppress autophagy in oridonin-treated murine fibrosarcoma L929 cells [4], while its enhancement of autophagic death was validated in oridonin- [5] and **1**-treated HT1080 cells [3].

On the basis of our previous researches (**1**-induced ROS-dependent autophagic cell death, followed by apoptotic death), further investigation was carried out to elucidate the relationship between ROS and autophagy. In this study, it was first reported that **1**-induced generation of ROS was in coordination with p38–NF-κB pathway, resulting in autophagy and death of HT1080 cells in serum-free media (SFM).

2. Results and discussion

2.1 Generation of ROS and autophagy was detected in 1-treated HT1080 cells in a short-term manner

Our previous studies demonstrated that **1**-treated HT1080 cells in SFM showed more significant development of ROS, autophagic death, and apoptotic death than the cells in media with 10% fetal bovine serum (FBS), and the experimental results of cell cultured in SFM were parallel to those in media with FBS [3]. In this study, the cells were cultured in media with 10% FBS and then transferred to SFM for 24 h to mimic starvation. This 24-h serum withdrawal did not lead to any detectable changes to HT1080 cells (data not shown). In our previous study, autophagy was induced in 12 h by 200 μM silibinin in media with FBS and 20 μM silibinin in SFM [3]. In this study, after pretreatment with SFM for 24 h, augmentation of cell sensitivity to **1** was obtained, according to the detection of increased ROS generation in 15 min (Figure 2A, a) and monodansyl cadaverine (MDC)-positive ratio (autophagy) in 1 h (Figure 2A, b) after **1** treatment; however, these augmentations declined in longer culture periods (Figure 2A, a, b). Development of autophagy was supported by observation of autophagocytic vacuoles stained with MDC in **1**-treated cells (Figure 2B).

Beclin-1 (BECN1) and microtubule-associated protein-light chain 3β (MAP LC3β) play significant roles in autophagy. BECN1 is one of the first mammalian proteins discovered to mediate autophagy [14]. There are two forms of LC3β: cytosolic LC3-I and membrane-bound LC3-II. LC3-I is formed by the removal

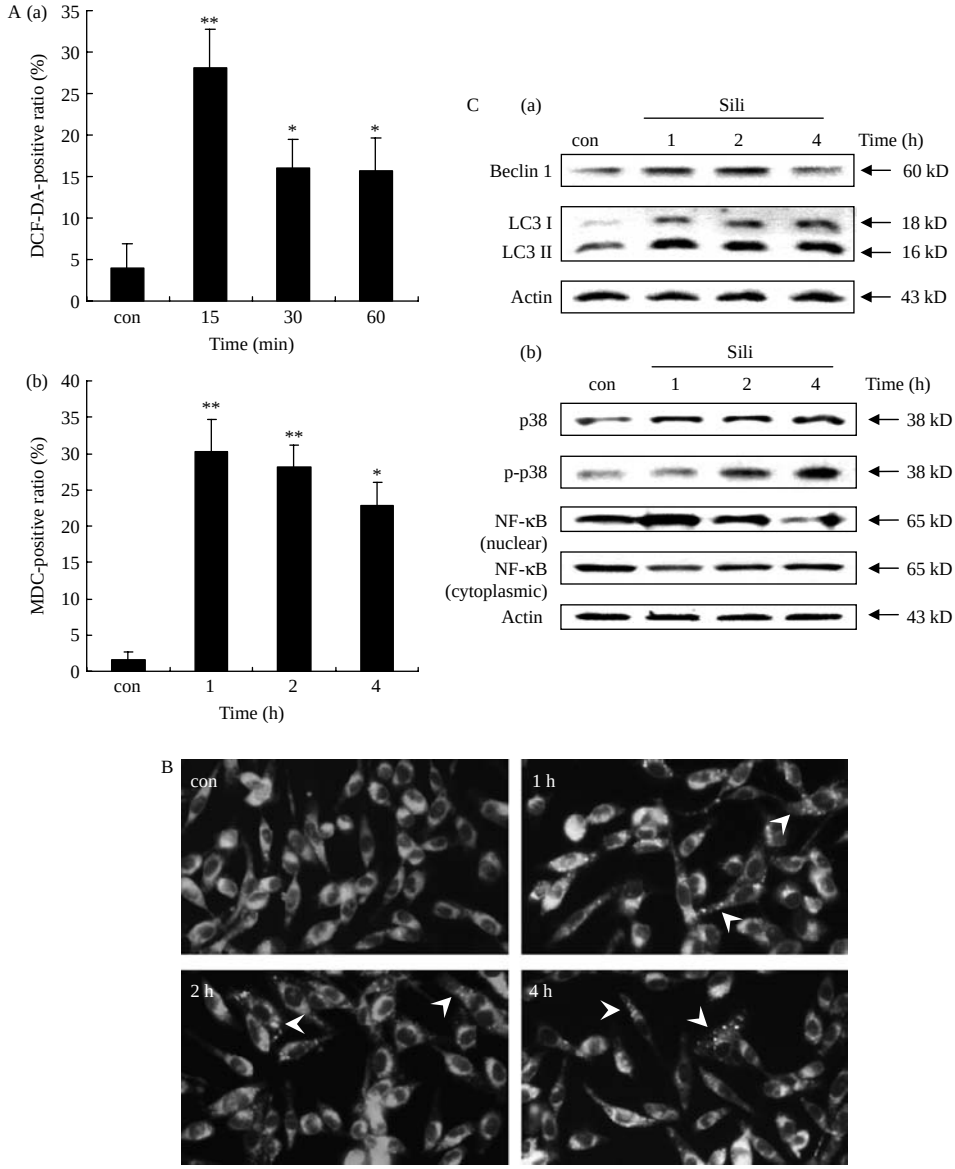

Figure 2. Generation of ROS and autophagy were observed shortly after **1** treatment in SFM-pretreated HT1080 cells. The cells were cultured in SFM for 24 h and then added by 40 μM **1** for indicated time periods. con: control. A: quantitative analysis detected a positive ratio of DCF-DA (a) and MDC (b) staining by flow cytometric analysis. The data are presented as the mean ± SEM of the results for three independent experiments. $*P < 0.05$; $**P < 0.01$ vs. control group. con: control. B: autophagic vacuoles were observed in **1**-treated cells (× 400 magnification); the cells were labeled with MDC. C: a: autophagy-related proteins were upregulated in **1**-treated cells. b: upregulation of p38/p-p38 and transposition of NF-κB from the cytoplasm to the nucleus were detected in **1**-treated cells.

of the C-terminal 22 amino acids from the newly synthesized LC3, followed by the conversion of LC3-I–LC3-II. The amount of LC3-II is concerned with the degree of autophagosome formation [15]. Data showed increased levels of BECN1 and

LC3-II/LC3-I ratio after silibinin treatment for 1 and 2 h (Figure 2C, a), accompanied with augmented expression of p38/phosphorylated-p38 (p-p38) and transposition of NF-κB from cytoplasm to nuclei (Figure 2C, b), suggesting that **1** induced autophagy in cells, and both p38 and NF-κB might have participated in autophagy process. These expression levels declined in longer time (Figure 2C).

2.2 p38 and NF-κB were proved to be involved in 1-induced autophagy which was abrogated by p38 inhibitor SB203580 and NF-κB inhibitor PDTC

SB203580 was applied as a specific inhibitor of p38 [16]. The metal chelator pyrolidine dithiocarbamate (PDTC) was used as an anti-oxidant inhibitor of NF-κB [17]; however, on the precise mechanism of action of PDTC, none of the studies demonstrated that it inhibited NF-κB by decreasing oxygen radicals in cells. Besides, there were results suggesting that, rather than acting as an anti-oxidant, PDTC had a pro-oxidant effect on the cells which led to the oxidation of NF-κB and thereby inhibited DNA binding directly [18]. Experimental data indicated that both SB203580 and PDTC significantly reduced **1**-induced generation of ROS (Figure 3A, a), suggesting that in this status, p38 and NF-κB were potential regulators of ROS generation.

Autophagic antagonist 3-methyladenine (3-MA) and agonist rapamycin were applied to assist this experiment. The ability of 3-MA to suppress the formation of autophagosomes suggested that it might be regarded as a specific inhibitor of autophagy [19]. Rapamycin (the positive

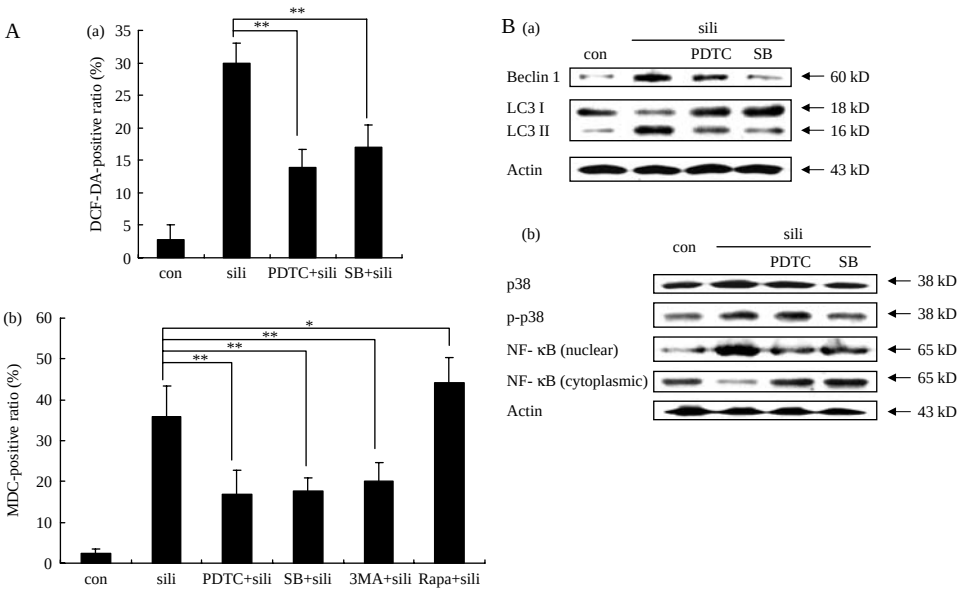

Figure 3. Inhibition of p38 and NF-κB decreased **1**-induced autophagy in HT1080 cells. The cells were cultured in SFM for 24 h, then added by indicated inhibitors for 1 h, and then cultured with 40 μM **1** for 1 h. con: control; PDTC: 2 μM; SB: SB203580, 15 μM; 3MA: 800 μM; Rapa: rapamycin, 20 μM. A: ROS generation (a) and autophagy (b) in cells were detected by a flow cytometry staining with DCF-DA and MDC, respectively. The data are presented as the mean ± SEM of the results for three independent experiments. $*P < 0.05$; $**P < 0.01$. B: a: Western blotting indicated that both PDTC and SB203580 decreased the **1**-induced upregulation of autophagy-related proteins. b: transposition of NF-κB from cytoplasm to nucleus was inhibited by SB203580, while PDTC could not decrease the upregulation of p38 and p-p38 induced by **1**.

group) is used to stimulate autophagy in some cells by inhibiting mTOR which regulated nutrient-insufficient autophagy [20]. Results of MDC-positive ratio showed that both SB203580 and PDTC decreased the extent of **1**-induced autophagy to a level similar to 3-MA-pretreated group (the negative group) (Figure 3A, b), suggesting that p38 and NF-κB were possibly the two regulators of **1**-induced autophagy. Combining the regulative effect of ROS to autophagy discussed in our previous study [3] and all of these results, p38 and NF-κB were possibly responsible for the regulation of **1**-triggered ROS generation which induced autophagy in HT1080 cells.

To justify this conclusion and further investigate the relationship between p38 and NF-κB, Western blotting analysis was subsequently carried out (Figure 3B). **1**-induced augmentation of the expression of BECN1 and the conversion of LC3-I–LC3-II were effectively reversed by SB203580 and PDTC, confirming the crucial function of p38 and NF-κB to autophagy (Figure 3B, a). In addition, transposition of NF-κB from the cytoplasm to the nuclei was inhibited in the cells pretreated with SB203580, while there was no detectable fluctuation of p38/p-p38 expression in PDTC-pretreated group (Figure 3B, b), implicating that NF-κB might be a downstream effector that was regulated by p38 in this process. p38–NF-κB–ROS was supposed to be a possible pathway that participated in **1**-induced autophagy in HT1080 cells.

2.3 A mechanism of positive feedback between ROS and p38–NF-κB pathway was implicated in HT1080 cells

Our previous researches showed superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) as two main forms of **1**-induced ROS which subsequently resulted in autophagic death in HT1080 cells [3]. Superoxide dismutase (SOD) has been reported to be a classical scavenger of $O_2^{\cdot-}$ [21] and catalase is well known as a specific H_2O_2 scavenger [22]. Both of the two scavengers effectively decreased the generation of ROS induced by **1** (Figure 4A, a) and, on the other hand, significantly reduced MDC-positive ratio in cells (Figure 4A, b). Meanwhile, SOD and catalase decreased **1**-induced augmentation of BECN1 expression and conversion of LC3-I–LC3-II, suggesting that autophagic degree was downregulated when ROS was scavenged. Besides, the expression of p38/p-p38 and the transposition of NF-κB were also declined in SOD group and catalase group, indicating that p38 and NF-κB were activated by ROS production, which was inhibited by ROS scavengers (Figure 4B). According to the regulative effect of p38 and NF-κB to ROS (Figure 3A, a), ROS and p38–NF-κB pathway might interact in a way of co-augmentation, in other words, positive feedback, in **1**-induced autophagic process.

2.4 1-Induced autophagy was a lethal factor and inhibitors described above were found to protect HT1080 cells from death

Previous studies found that in **1**-treated HT1080 cells, autophagy occurred before apoptosis; meanwhile, 3-MA rescued cells from **1**-induced death, indicating that autophagy positively regulated apoptosis to cell death [3]. **1** showed no detectable inhibition to cell proliferation in the short-term (1 h) manner (data not shown). According to these, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in a long-term (12 h) manner was applied. Data showed that SB203580 and PDTC reversed inhibition effect of **1** to cells (Figure 5A); additionally, the two ROS scavengers, SOD and catalase, also effectively rescued the cells from **1**-induced cell death (Figure 5B). On the basis of the previous results of the

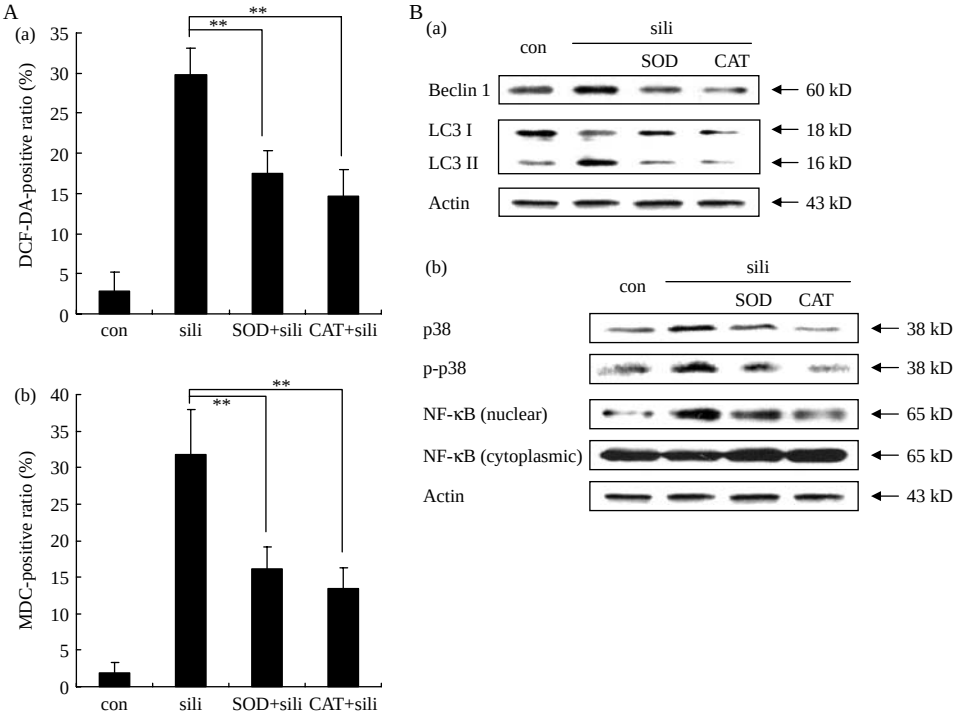

Figure 4. ROS scavengers inhibited **1**-induced autophagy in HT1080 cells through p38–NF-κB pathway. The cells were cultured in SFM for 24 h, then added by SOD or catalase for 1 h, and then cultured with 40 μM **1** for 1 h. con: control; SOD: 100 U/ml; CAT: catalase, 500 U/ml. A: Flow cytometric analysis indicated that SOD and catalase decreased the positive ratio of DCF-DA (a) and MDC (b) in **1**-treated cells. The data are presented as the mean ± SEM of the results for three independent experiments. $*P < 0.05$; $**P < 0.01$. B: SOD and catalase reversed **1**-induced upregulation of autophagy-related proteins and p38/p-p38, and transposition of NF-κB from cytoplasm to nucleus was also inhibited.

regulative effect of autophagy to apoptosis in **1**-treated cells and the regulative effect of p38–NF-κB and ROS to autophagy, it was indicated that SB203580, PDTC, SOD, and catalase might protect cells by inhibiting autophagy. The induction of autophagy through ROS and p38–NF-κB pathway was a possible mechanism involved in cancer-inhibitory function of **1**.

3. Experimental

3.1 Chemicals

Compound **1** was obtained from Beijing Institute of Biological Products (Beijing, China), and its purity was determined to be about 99% by HPLC measurement. Compound **1** was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution and diluted by DMEM (Gibco, Grand Island, NY, USA) before the experiments. DMSO concentration in all cell culture was kept below 0.1%, which had no detectable effect on cell growth or death. MTT, 2′,7′-dichlorofluorescein diacetate (DCF-DA), MDC, ammonium PDTC, SB203580, catalase, SOD, 3-MA, and rapamycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Polyclonal antibodies against BECN1 (sc-11427), MAP LC3β (sc-28266), p38α/β (sc-7149), p-p38 (sc-101759), NF-κB p65 (sc-372), actin (sc-7210), and horseradish peroxidase (HRP)-conjugated secondary

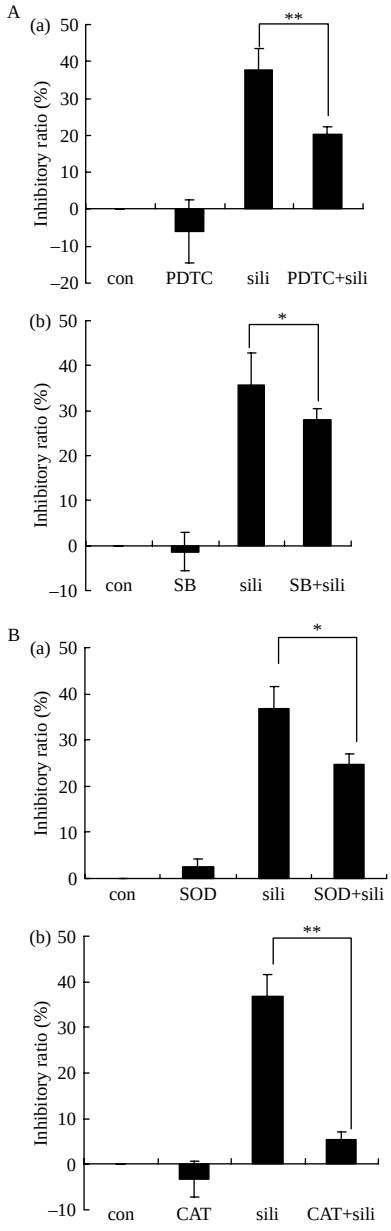

Figure 5. Inhibition of p38 and NF-κB, and the generation of ROS rescued cells from **1**-induced death in a long-term manner. The cells were cultured in SFM for 24 h, then added by indicated inhibitors for 1 h, and then cultured with 40 μM **1** for 12 h. con: control; PDTC: 2 μM; SB: SB203580, 15 μM; SOD: 100 U/ml; CAT: catalase, 500 U/ml. The data are presented as the mean ± SEM of the results for three independent experiments. $*P < 0.05$; $**P < 0.01$.

antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

3.2 Cell culture

The cells were cultured in DMEM supplemented with 10% heat-inactivated (56°C, 30 min) FBS (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China), 2 mM L-glutamine (Gibco), 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.

3.3 Autophagic vacuoles labeled by MDC staining

A fluorescent compound, MDC, was used as a tracer for autophagic vacuoles. After incubation with **1** for the indicated time periods, HT1080 cells were stained with 50 μM MDC at 37°C for 40 min, and then the morphology was observed under a fluorescence microscope (Olympus, Tokyo, Japan).

3.4 Flow cytometric analysis using MDC and DCF-DA

After incubation with tested agents for the indicated time periods, HT1080 cells were harvested, rinsed with PBS, and 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 a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

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

3.5 Western blot analysis

Both adherent and floating HT1080 cells were harvested, washed twice with ice-cold PBS, and then lyzed 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 PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) on ice for 1 h. After centrifugation of the cell suspension at 13,000*g* for 15 min, the protein content of supernatant was determined by Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). The protein lysates were separated in 12% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). Proteins were detected using polyclonal antibody and visualized using anti-rabbit, anti-mouse, or anti-goat IgG conjugated with HRP and 3,3-diaminobenzidine tetrahydrochloride as the substrate of HRP.

3.5.1 Preparation of nuclear and cytoplasmic extraction of proteins

Nuclear and cytoplasmic proteins were extracted according to previous methods [23].

3.6 Growth inhibition assay

The growth inhibitory effect of **1** on HT1080 cells was measured by MTT assay. The cells were dispensed in 96-well flat-bottomed 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 tested 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 using an ELISA reader (Tecan Spectra, Wetzlar, Germany). The percentage of cell growth inhibition was calculated as follows: inhibitory ratio $(\%) = (A_{490,\text{control}} - A_{490,\text{sample}})/(A_{490,\text{control}} - A_{490,\text{blank}}) \times 100$.

3.7 Statistical analysis

All data represented at least three independent experiments and were expressed as mean ± SEM. The data were analyzed by ANOVA using Statistical Package for Social Science (SPSS) software (version 13.0; SPSS, Chicago, IL, USA), and LSD-post hoc test was employed to assess the statistical significance of difference between control and treated groups. *p*-values of less than 0.05 were considered statistically significant.

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