

Targeted inhibition of p38MAPK-enhanced autophagy in SW620 cells resistant to photodynamic therapy-induced apoptosis

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Received: 28 May 2014 / Accepted: 18 March 2015 / Published online: 9 August 2015
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Abstract Photodynamic therapy (PDT) is a promising and noninvasive treatment that can induce apoptosis, autophagy, or both depending on the cell phenotype. In this work, chlorin e6 (Ce6) was used to photosensitize human colorectal cancer SW620 cells. In cells, apparent autophagy and apoptosis with dependence on intracellular reactive oxygen species (ROS) generation were detected. p38MAPK activation followed by ROS generation might be a core component in Ce6 mediate PDT (Ce6-PDT)-induced autophagy and apoptosis signaling pathway. By using p38MAPK siRNA, the results showed a marked enhancement on cell apoptosis in Ce6-PDT with increased annexin (+) apoptotic cells, nuclear condensation, caspase-3, and PARP cleavage. Besides, impairment of p38MAPK also promoted the autophagic response to photodamage as indicated by conversion of LC3 and monodansyl cadaverine (MDC) labeling patterns. It appears that Ce6-PDT induced ROS production involving activation of p38MAPK, probably to prevent SW620 cells from photodamage. Moreover, autophagy inhibitor 3-methyladenine/bafilomycin A1 greatly aggravated Ce6-PDT-induced apoptosis in SW620 cells with knockdown of p38MAPK. Taken together, this study suggests that

autophagy could represent a promising field in cancer treatment and p38MAPK may be a potential therapeutic target to enhance the efficacy on clinical evaluation for the treatment of colorectal cancer.

Keywords PDT · p38MAPK · ROS · Autophagy · Apoptosis

Introduction

Colorectal cancer is the second most fatal cancer in the world, considering the fact that over one million new cases were diagnosed and about half of them died every year [1]. Surgery remains the most effective curative treatment for colorectal cancer, but the risk of recurrence is high. Although 75–80 % of newly diagnosed cases are localized or regional tumors, around 50 % of patients suffer recurrence and side effects after surgery [2, 3].

Photodynamic therapy (PDT) is a clinically established treatment modality for a range of cancers and wet age-related macular degeneration [4–6]. It utilizes the combined action of photosensitizer, light, and molecular oxygen to generate reactive oxygen species (ROS), particularly singlet oxygen, to eradicate cancer cells.

Chlorin e6 (Ce6) is a second-generation photosensitizer, which is a derivative of natural chlorophyll α by partial saturation of one of the four pyrrole rings [7, 8]. It has been shown to have better selectivity and prolonged retention time in tumor cells and absorption maximum at the longer infrared wavelength of 654 nm and clear faster from the organism [9, 10].

It is clear that the target of PDT is not restricted to a single cellular component or a single signaling pathway [11], but the molecular mechanisms underlying PDT-induced antitumor activities still remain unclear. Activation of mitogen-

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activated protein kinases (MAPKs) is known to play a major role in ROS-induced signaling pathways response to PDT [12]. The MAPKs, which mainly consist of the extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinases (JNK), and p38MAPK, have been shown to regulate a wide variety of cellular events, including cell survival and death [13]. In human cells, p38MAPK exists in four isoforms, i.e., p38 α , p38 β , p38 γ , and p38 δ . Each isoform differs in tissue distribution, regulation, and specific biological effects. Miki et al. found that the p38MAPK expression level in colorectal cancer tissues was significantly higher than that in peripheral normal tissues [14]. p38MAPK is required for cell proliferation and survival, and its inhibition leads to cell cycle arrest, apoptosis, or autophagic cell death [15]. p38MAPK may play distinct or even opposite role under different PDT conditions, such as the applied sensitizers, cell models, light exposure parameters, and so on [16]. A complex network of intracellular kinase cascades controls autophagy and survival.

Autophagy is a cellular degradation process in which portions of the cell's cytoplasm and organelles are sequestered in a double-membrane bound vesicle named autophagosome. Fusion of autophagosomes with lysosomes results in the formation of autolysosomes, where the proteins and organelles are degraded. In contrast to the majority of human cancers, colorectal cancer exhibits high levels of autophagy [17–19] which is associated with the intestinal proliferative/undifferentiated and progenitor cell populations. However, the role of autophagy in colorectal cancer is paradoxical. Studies indicate that activation of autophagy can suppress the tumor function, but it also contributes to colorectal tumorigenesis. Induction of autophagy leads to proliferative arrest or apoptosis in human colorectal cancer cells [20, 21]. The evidence suggests autophagy has a dual role in colorectal cancer, and by regulating autophagy activity, it may bring some positive influence in colorectal cancer treatment.

The aim of this study is to investigate the role of p38MAPK in the process of Ce6-PDT-induced autophagy and apoptosis in human colorectal cancer SW620 cells (represent a final stage of colorectal cancer development) and to further explore targeting inhibition of p38MAPK-enhanced autophagy in SW620 cells resistance to photodynamic therapy-induced apoptosis.

Materials and methods

Chemicals and reagents

Chlorin e6 (Ce6) was purchased from Sigma Chemical Company (St. Louis, MO, USA), and the purity was greater than 95 %. Ce6 was dissolved in sterilized PBS (0.1 M, pH 7.4) at a stock concentration of 2.5 mg/ml at -20°C .

N-Acetylcysteine (NAC), dimethyl sulfoxide (DMSO), monodansylcadaverine (MDC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide tetrazolium (MTT), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), *N*-acetylcysteine (NAC), 3-methyladenine (3-MA), bafilomycin A1 (BafA1), antibodies against microtubule-associated protein 1 light chain 3 (LC3), and β -actin were purchased from Sigma Chemical Company (St. Louis, MO, USA). 2', 7'-Dichlorofluorescein diacetate (DCFH-DA) was supplied by Molecular Probes Inc. (Invitrogen, CA, USA). Antibodies against caspase-3, phospho-JNK (Thr 183/Tyr 185), phospho-ERK 1/2 (Thr 202/Tyr 204), phosphor-p38MAPK (Thr 180/Tyr 182), and p38MAPK (Ab182) were from Cell Signaling Technology (Beverly, USA). Guava Nexin Reagent was obtained from Millipore Corporation (Billerica, MA, USA).

Cell culture

The human colorectal cancer SW620 cells were obtained from the cell bank of the Chinese Academy of Science, Shanghai, China. The cell line was maintained in L-15 medium supplemented with 10 % fetal bovine serum (FBS), 1 % penicillin–streptomycin (100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin), and 1 % glutamine in 100 cm^2 tissue culture flasks under a humidified 5 % CO_2 and 95 % air atmosphere at 37°C .

Photodynamic treatment procedure

SW620 cells (4×10^5 cells/ml, 300 μl) were cultured in 24 well plates to 80 % of confluence and subjected to photodynamic treatment. Cells were incubated with 0.5 $\mu\text{g}/\text{ml}$ Ce6 in serum-free L-15 medium for 4 h, allowing sufficient time for cells to uptake the sensitizer rapidly to achieve a maximum level. Then, the medium was replaced with serum-free L-15 medium without Ce6 after two washes with PBS, and cells were irradiated with light per well for 1 min. The semiconductor laser (excitation wavelength, 650 nm) was used as a source for evoking photodynamic effect. Irradiance was measured by the radiometer system (Institute of Photonics & Photon-technology, Department of Physics, Northwest University). The total light dose of 3 J/cm^2 was applied in this study. During and post-PDT treatment, no significant temperature variation was observed in the cell culture medium. After irradiation, the cells were normal cultured in a 5 % CO_2 incubator at 37°C before further investigation.

Intracellular ROS generation examination

Intracellular ROS production was studied by measuring the fluorescence intensity of dichlorofluorescein (DCF) as described by other studies [22]. 2', 7'-DCF-diacetate, a nonfluorescent cell-permeant compound, is cleaved by endogenous esterase within the cell, and the de-esterified product can be

converted into the fluorescent compound DCF upon oxidation by intracellular ROS. To estimate the intracellular ROS, we added DCFH-DA before PDT treatment for 30 min at 37 °C; after irradiation, cells (100 μ l, 4×10^4 cells) were collected and analyzed for ROS production by flow cytometry (Guava easyCyte 8HT, Millipore, USA) with the excitation wavelength at 488 nm, and histograms were analyzed by using FCS Express V3 software.

To determine the role of ROS in Ce6-PDT-induced cell apoptosis and autophagy, the ROS general scavenger NAC (5 mM) was added to culture medium prior to PDT treatment by 1 h and then treated as described above.

DAPI staining for apoptotic morphology observation

DAPI is a fluorescence probe, which binds to natural double-stranded DNAs and shows the change of the nuclei morphology. DAPI staining was performed to detect changes of nuclei morphology of SW620 cells after PDT treatment. At 24 h after irradiation, cells were stained with DAPI (4 μ g/ml) at room temperature for 30 min. Then, the stained cells (4×10^5 cells/ml) were washed three times with PBS and observed using a fluorescence microscopy with standard excitation filters (Nikon E-600, Japan).

MDC staining assay for autophagy detection

MDC is a marker for lysosomal activity and fused autolysosomes [23]. In this study, MDC staining was used to confirm the abundance of autophagic vacuoles in PDT-treated cells. Briefly, at different times post-PDT, cells (4×10^5 cells/ml) were washed with PBS and stained with MDC at a final concentration of 20 mM for 20 min at 37 °C. After labeling, cells were washed with PBS, and images were obtained with a fluorescence microscope (Nikon E600). Excitation wavelength was 360–380 nm, and emission wavelength was 520–560 nm.

Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (SDS-PAGE) and immunoblotting were performed according to standard procedures. Briefly, after PDT treatment, cells were harvested and collected by centrifugation, then lysed with RIPA buffer on ice. The protein content of the lysate was measured using the BCA protein assay reagent. Similar amount of protein was analyzed on SDS-PAGE and transferred onto polyvinylidene fluoride membranes (0.22 μ m, Millipore). Membranes were then incubated at room temperature for 1 h in blocking buffer (5 % low-fat milk powder in tris-buffered saline-Tween 20 (0.05 %) (TBST). The membranes were then incubated overnight at 4 °C with primary antibodies. The bound primary antibodies were then tagged with IRDye 680-conjugated IgG (Li-cor, Biosciences,

Lincoln, NE) at room temperature for 1 h. The infrared fluorescence was detected with the Odyssey infrared imaging system (Li-Cor Bioscience, Lincoln, NE), and autographs were quantified using Bio-Rad Quantity One software (Bio-Rad). β -Actin was used to ensure equal loading.

siRNA transfection

SW620 cells were transfected with p38MAPK siRNA, and nontargeting (NT) siRNA was obtained from Sangon Biotech Co. (Shanghai, China). Transfection was performed using TurboFect Transfection Reagent (Thermo, 00110997) according to the manufacturer's protocol. The total proteins from cells were extracted at 72 h after transfection and subjected to analyses with Western blotting.

Annexin V-PE/7-AAD staining assay

Apoptotic cells were measured with the Guava Nexin Assay (Millipore, USA) according to the manufacturer's protocol. SW620 cells (4×10^5 cells/ml) with p38MAPK siRNA/NT siRNA were collected and divided randomly into groups with BafA1/3-MA pretreatment or not. For BafA1/3-MA groups, the cells were incubated 1 h with 40 μ M BafA1/1 mM 3-MA before photodynamic treatment. Instead of BafA1/3-MA, an equivalent quantity of serum-free L-15 medium was used for the other groups. After irradiation, each group was digested and 100 μ l Annexin V-PE and 7-ADD binding buffer was added to each sample with 100 μ l cells and then incubated for 20 min at room temperature in the dark. The Annexin V-PE (+) was determined by flow cytometry, and analysis was performed using FCS Express V3 software.

Cell viability detection

SW620 cells (100 μ l, 4×10^4 cells) were pretreated with or without 5 mM NAC in 96-well flat-bottomed tissue culture plates that were irradiated with different light dose (3–12 J/cm²) after incubated at 0.5 μ g/ml Ce6. Cell viability was determined at 24 and 48 h post-PDT by adding 10 μ l MTT solution (5 mg/ml in PBS) to each well, and the mixture was incubated for 4 h at 37 °C in a CO₂ incubator. After incubation, the medium was replaced by DMSO. The formazan crystals were dissolved in DMSO, and the absorbance at 570 nm was recorded using a microplate reader (Bio-Tek ELX800, USA) against the reference value at 630 nm. The results were shown as percentage of control.

Statistical analysis

All values were expressed as means \pm SD of three independent experiments. Differences among different groups were assessed

with one-way ANOVA followed by Scheffé's post hoc test. Statistical significance was established at a value of $p < 0.05$.

Results

Ce6-PDT-induced autophagy and apoptosis were correlated with the accumulation of ROS in SW620 cells

ROS, a group of highly reactive molecules, especially singlet oxygen, have been shown to play a key role in PDT-induced cell death. As shown in Fig. 1a, the percentage of cells with DCF fluorescence increased significantly after PDT, suggesting that an amount of intracellular ROS was formed. By pretreating with ROS general scavenger NAC [24] before PDT, the data indicated that NAC greatly eliminated accumulation of intracellular ROS. NAC inhibited Ce6-PDT-induced autophagy as demonstrated by the reduction of the formation of MDC punctate staining (Fig. 1b) and the conversion of LC3 (Fig. 1d). We also found that large condensed chromatin and nuclear damage caused by Ce6-PDT were decreased (Fig. 1c) with NAC pretreatment, and the cleavage of caspase-3 and PARP was also reduced (Fig. 1d). The results suggest that ROS participated in regulating autophagy and apoptosis induction by Ce6-PDT treatment. In addition, NAC significantly prevented cytotoxicity of SW620 cells caused by Ce6-PDT (Fig. 1e).

Activation of p38MAPK in Ce6-PDT treated SW620 cells

To assess whether Ce6-PDT could activate MAPKs, the states of phosphorylation of p38MAPK, JNK, and ERK were investigated by Western blot. As shown in Fig. 2a, the activation of p38MAPK was shown immediately after PDT and reached maximum after 1 h incubation, then slightly decreased. The time for phospho-JNK (p-JNK) to increase was similar to phospho-p38MAPK (p-p38MAPK), but the expression level was relatively lower. Compared with control, no significant increase in phosphorylation of ERK1/2 (p-ERK1/2K) was observed. We also observed that ROS scavenger NAC reduced the activation of p38MAPK apparently (Fig. 2b). The data demonstrated Ce6-PDT-induced p38MAPK activation depending on ROS production in SW620 cells.

Ce6-PDT-induced apoptosis was potentiated by autophagy inhibition in SW620 cells with knockdown of p38MAPK

To confirm the role of p38MAPK in SW620 cells, we applied the p38MAPK siRNA to dissect its effect on cellular apoptotic and autophagic response to Ce6-PDT treatment. In the control experiment, siRNA specifically reduced the expression of their corresponding target

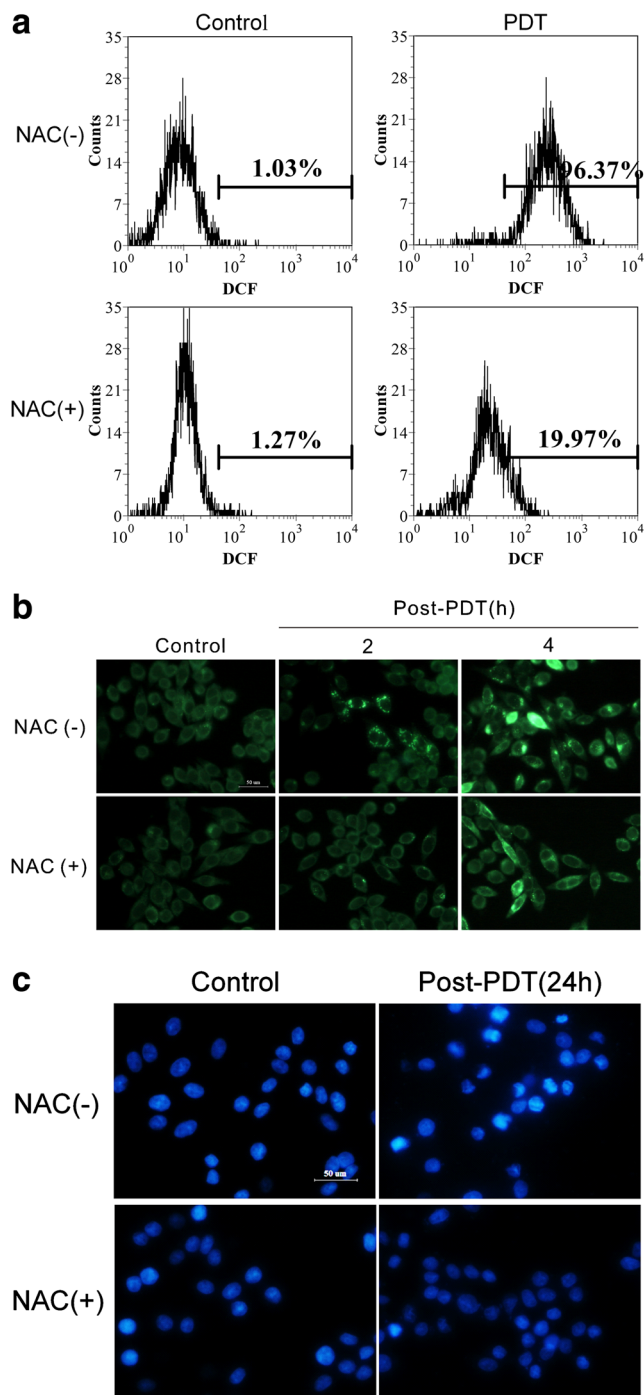


Fig. 1 Ce6-PDT-induced apoptosis, autophagy, and growth inhibition were correlated with an increase of cellular ROS. Intracellular ROS (**a**), autophagic cells (**b**), and apoptotic nuclei (**c**) were measured with or without ROS scavenger NAC. Western blot analysis of caspase-3, LC3II/I and cleaved PARP after Ce6-PDT treatment (**d**). The ratios of LC3II/I, cleaved caspase-3/ β -actin, and cleaved PARP/ β -actin were statistically analyzed. $**p < 0.01$ versus PDT(-), $^{\#}p < 0.05$ between groups. Cell viability was determined by MTT assay (**e**). $*p < 0.05$ versus PDT

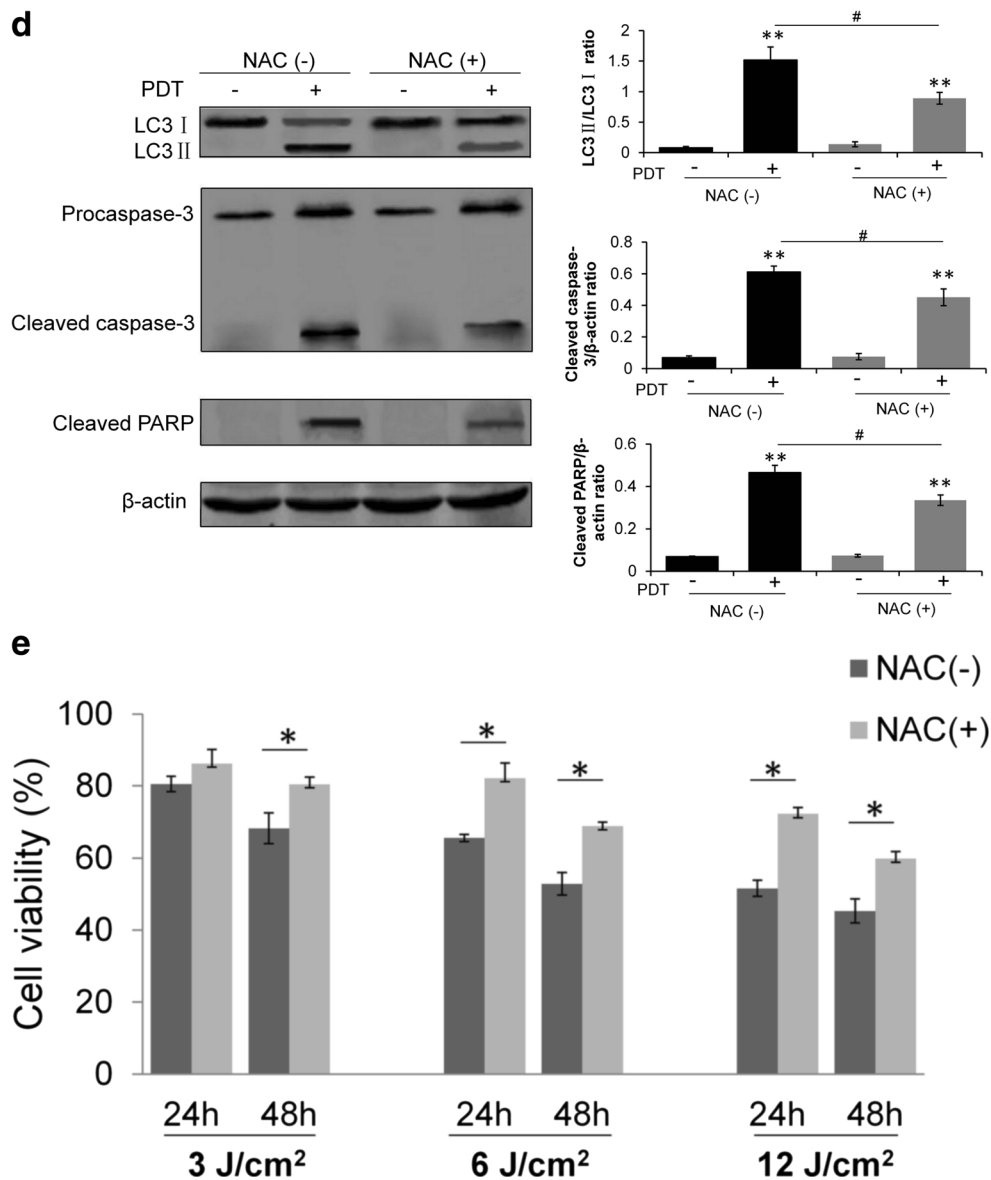


Fig. 1 (continued)

(Fig. 3a). Subsequent analysis shows not only apoptosis but also autophagy induced by Ce6-PDT that was enhanced by p38MAPK siRNA. In autophagy detection, the formation of acidic vesicular organelles by MDC staining was significantly increased in cells with p38MAPK siRNA than cells with/without NT siRNA (Fig. 3b). As shown in Fig. 3c, there is more conversion of LC3 induced by Ce6-PDT in p38MAPK knockdown cells ($p < 0.01$). In apoptosis evaluation, more caspase-3 activation ($p < 0.01$) and annexin V (+) apoptotic cells (41.5 % in PDT+ p38MAPK siRNA vs 16.15 % in PDT+ NT siRNA, $p < 0.01$) were detected (Fig. 3c, d). Moreover, the percentage of annexin V (+) cells was further increased to 47.85 and 51.45 % ($p < 0.05$) when

pretreatment with 3-MA and BafA1, respectively (Fig. 3d). These results suggest that autophagy may play a pro-survival role in the process of Ce6-PDT in SW620 cells with knockdown of p38MAPK.

Discussion

PDT has been undergoing clinical evaluation for the treatment of breast cancer, colorectal cancer, esophageal squamous cell carcinoma, and other malignancies [25]. As a promising therapeutic approach, PDT is able to induce not only apoptotic cell death, but simultaneously also a kind of autophagy, depending on the cell

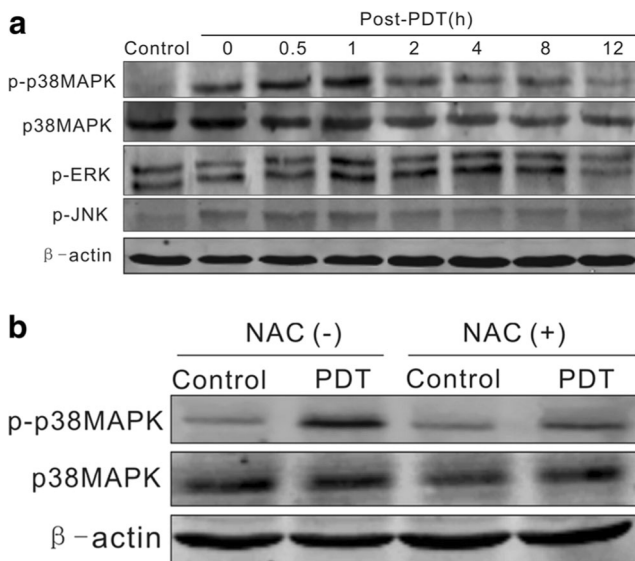


Fig. 2 Activation of p38MAPK induced by Ce6-PDT. **a** At different time post Ce6-PDT, SW620 cells were collected and subjected to Western blot analysis. **b** The phosphorylated and total p38MAPK levels were detected by NAC pretreatment before PDT

phenotype and dose of photosensitizer [26]. Our previous studies regarding the potential of Ce6 as a photosensitizer have suggested that Ce6 is effective to inhibit cancer growth by induction of apoptosis [27]. We speculated that the apoptosis is a general antitumor mechanism of Ce6-PDT on human cancers. This study tries to demonstrate the therapeutic potential of Ce6-PDT on human colorectal cancer cells SW620. Our finding reveals that both apoptotic and autophagic responses are dependent on p38MAPK signal pathway, and inhibition of p38MAPK contributed greatly to Ce6-PDT-induced SW620 cell apoptosis.

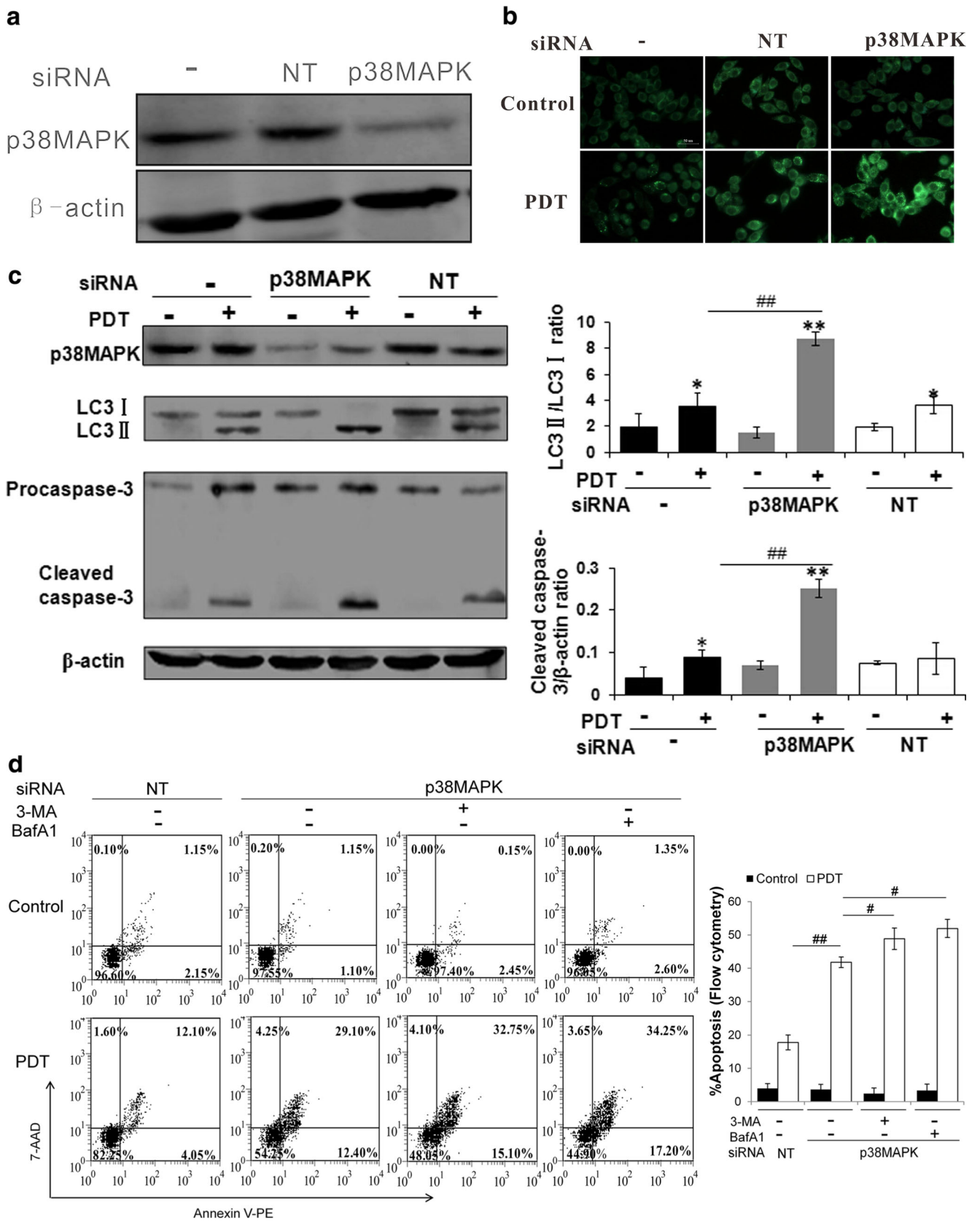
In the presence of light, activated photosensitizer can generate reactive oxygen species that are toxic to tumors. As shown in Fig. 1a, an increase in intracellular ROS levels was observed after Ce6-PDT treatment. ROS, a group of highly reactive molecules, especially singlet oxygen, have been shown to play a key role in PDT-induced cell death [26]. The formed unstable singlet molecular oxygen and ROS will not be migrated within more than a micron from the site of formation. As a result, photodamage can be quite specific. Because of their high reactivity with all macromolecular constituents, such as lipids, DNA, and proteins, ROS represent a source of cytotoxicity and are therefore reduced by cellular detoxifying and antioxidant enzymes or agents. In our study, exogenous ROS scavenger NAC obviously decreased autophagy, cell apoptosis, and increased cell viability (Fig. 1), suggesting ROS dominated by O_2^1 was the key component that participated in the Ce6-PDT photochemical process. Multiple intracellular components and signaling pathways have been implicated in PDT-induced cell death. MAPKs are triggered by a variety

Fig. 3 Ce6-PDT-induced apoptosis and potentiation by autophagy inhibition with knockdown of p38MAPK. **a** p38MAPK expression with or without siRNA p38MAPK by Western blot. **b** MDC staining. **c** Western blotting analysis of caspase-3 activation and LC3 conversion. The ratios of LC3II/I and cleaved caspase-3/ β -actin were statistically analyzed. * $p < 0.05$ and ** $p < 0.01$ versus PDT (-), ### $p < 0.01$ between groups. **d** Annexin V-PE/7-ADD staining of apoptosis populations. # $p < 0.05$; ## $p < 0.01$ between PDT groups

of oxidant stresses, which mediate the intracellular signaling cascades that ultimately regulate cell proliferation, differentiation, and apoptosis [28]. In our study, we observed Ce6-PDT could activate MAPK pathway, especially p38MAPK by the rapid induction of intracellular ROS stress under PDT treatment. More likely, p38MAPK activation should be a core component in Ce6-PDT-induced apoptosis and autophagy signaling pathway.

p38MAPK has been reported to be highly activated in many human tumors, e.g., colorectal, esophageal, and breast cancer [29]. There are different reports concerning the role of p38MAPK activation in apoptosis, cell growth, and survival. The protection mechanism of HeLa cells against hypoxia-PDT induced apoptosis was also found to be a rapid activation of p38MAPK [30]. In contrast, the enhanced phosphorylation of p38MAPK in response to Pc4-PDT suggests a possible role in promoting apoptosis in colon tumor xenografts [31]. In LY-R cells, one step in PDT-induced apoptosis is promoted by p-p38MAPK [32]. Therefore, it is of great importance to know whether p38MAPK promoted proliferation or death in SW620 cells by Ce6-PDT treatment. In our study, we demonstrated that p38MAPK deficiency caused increased cytotoxic and apoptosis (Fig. 3). By p38MAPK siRNA, it was clearly seen that more enhanced cell apoptosis was observed after PDT, implying that p38MAPK might have the function of anti-apoptosis in SW620 cells after Ce6-PDT treatment.

Research for new therapeutic targets has been on the rise over the past few years, and autophagy processes appear to be one of the most promising approach. Recently, autophagy has been found to be highly activated in colon cancer cells [33]. Autophagy is a homeostatic cellular recycling mechanism and has recently attracted the interest in the field of cancer therapy because it is also designated as type II programmed cell death [34]. Autophagy induction after mitochondrial damage has recently been found to be accompanied by the production of ROS. ROS in the induction of cell death are closely related with MAPK pathways. The role of the p38MAPK signal in the negative control of autophagy has been described in hepatocytes responding to hypo-osmotic stress, amino acids, or insulin addition [35]. In this study, when SW620 cells were treated with p38MAPK siRNA, it promoted autophagic response to Ce6-PDT (Fig. 3). These findings suggest that p38MAPK could mediate ROS-induced apoptosis as well as autophagy in Ce6-PDT-treated SW620 cells.



Understanding the signaling pathways involved in the regulation of autophagy as well as autophagy process itself leads to new directions in the development of anticancer therapies. In our subsequent studies, autophagy inhibitor 3-MA/BafA1 further aggravated Ce6-PDT-induced p38MAPK siRNA cell apoptosis (Fig. 3). The results implied that autophagy was a cytoprotective process in SW620 cells with knockdown p38MAPK after Ce6-PDT treatment. This may be based on the ability of autophagy to recycle photo-damaged mitochondria before cytochrome C can be released [36]. It is evident that the apoptosis and autophagy pathways share several key signaling components and several opportunities for crosstalk exist. Several studies have demonstrated that DNA damage induced by ROS, DNA damage agents, or ionizing radiation led to the following sequential events, including PARP activation, ATP depletion, AMPK activation, mTOR suppression, and induction of autophagy [37]. In our study, we confirmed that Ce6-PDT enhanced the level of PARP cleavage, trigger cellular ROS generation. Further investigations are required to get full understanding of the contributions of autophagy for determining the outcome of combined autophagy inhibition and apoptosis induction.

Furthermore, compared with standard antitumor therapies such as surgery and chemo- and radiotherapy, PDT may be targeted more precisely, less invasive, and safer [38]. However, the inability of visible light to penetrate tissues deeply enough leads to a discounted therapeutic efficacy and might be the cause of the tumor relapse [39]. In view of that tumor unlikely to be cured by the application of a single therapeutic method, the potential combination of PDT and some other curative protocols have become a promising option in cancer treatment [40]. Previous studies showed that PDT can be combined with chemotherapeutic drugs like avastin, paclitaxel, and cisplatin, showing apparent effects in inhibiting tumor cell proliferation [41]. Our results showed that inhibitions of p38MAPK could potentiate PDT efficacy where autophagy offers protection from photokilling, suggesting the targeted inhibition of p38MAPK and autophagy may be a useful strategy for colorectal cancer treatment by combination with PDT.

Acknowledgments This work was supported by the National Natural Science Foundation of China (no. 81472846) and the Fundamental Research Funds for the Central Universities (GK201502009).

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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