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Non-thermal plasma induces apoptosis accompanied by protective autophagy via activating JNK/Sestrin2 pathway

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Abstract

Although non-thermal plasma (NTP) has been proved to be an effective way to kill cancer cells, the mechanism of NTP-induced cancer cell death is still not clear. In this study, we found that NTP exposure caused reactive oxygen species generation and apoptosis in A549 and MDA-MB-231 cells. Meanwhile, NTP treatment also induced autophagy, as evidenced by the formation of acidic vesicular organelles and conversion of LC3-I to LC3-II. Suppression of autophagy by chloroquine significantly increased NTP-induced cell death, indicating that NTP-induced autophagy acted in a protective role from apoptosis. Furthermore, NTP treatment significantly increased Sestrin2 (Sesn2) expression and activated the JNK signaling pathway. Knocking down Sesn2 with special siRNA enhanced NTP-induced cell death, while pretreatment with JNK inhibitor abolished the increase of Sesn2 and LC3 formation, and promoted cell apoptosis induced by NTP. These indicated that the JNK/Sesn2 pathway was involved in autophagy and apoptosis induced by NTP. These findings provided evidence that supplement with autophagy inhibition might be a useful strategy to improve the tumor cell killing effect of NTP.

Supplementary material for this article is available online

Keywords: non-thermal plasma, apoptosis, protective autophagy, c-JUN N-terminal kinase, Sestrin2

(Some figures may appear in colour only in the online journal)

1. Introduction

Non-thermal plasma (NTP) has emerged as a new approach to kill cancer cells in recent years [1, 2]. Numerous studies have demonstrated that NTP induced generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), caused DNA damages, cell cycle arrest and apoptosis *in vitro* and abrogated tumor growth *in vivo* [3, 4]. NTP treatment decrease the expression of MMP-9 and suppress cell migration and invasion [5]. Interestingly, NTP treatment cause the higher level of intracellular ROS and selectively killed cancer cells [4]. Recent studies have found that NTP treatment induce damage-associated molecular patterns (DAMPs) release and immunogenic cell death [6]. It has been reported that NTP treatment mainly causes oxidative stress and modulates various signaling pathways, such as JNK/MAPK, TNK-ASK and Sestrin2/iNOS pathways in cancer cells, thus leading to cell death [7–10]. However, the mechanisms underlying of NTP-induced cancer cell death are still not well understood.

Autophagy, type II programmed cell death, is an evolutionarily conservative process that degrades and circulates some dysfunctional or excessive proteins and organelles under various stimuli [11]. Autophagy exhibited its dual roles in regulating cell survival and cell death [12]. Several studies have revealed that autophagic cell death is activated by chemotherapy or oxidative stress in cancer cells [13, 14]. Hara *et al* reported that autophagy played a cytoprotective process against unfavorable conditions or diseases [15, 16]. NTP-induced ROS has been regarded as a main cause of cell death besides apoptosis in various cancer cells, but ROS induced autophagy has not yet been reported. It is pertinent to explore the occurrence of autophagy and its possible roles in NTP-induced cell death.

As a member of Sestrin family, Sestrin2 (Sesn2 or Hi95) can be induced to defend against various stresses, such as oxidative stress, hypoxia and DNA damage [17, 18]. Sesn2 also has dual roles in regulation of cell viability. Upregulation of Sesn2 was observed to protect cells against DNA damages or H_2O_2-induced cell death [19]. Recently, Sesn2 was found to be a novel mediator in autophagy induction [20]. Previous studies reported that Sesn2 played a key role in regulating autophagic death [21]. Moreover, expression of Sesn2 was found to have significantly increased upon glucose deprivation, while overexpression of Sesn2 reduced oxidative damages and apoptosis [22, 23]. Many studies reported that JNK and Sesn2 were involved in regulating cell growth and death [24, 25]; however, the function of JNK/Sesn2 pathway in NTP-induced autophagy and apoptosis is still unknown.

In this study, we found that NTP treatment triggered protective autophagy and apoptotic cell death as well, and ROS was identified to play a critical role in the occurrence of autophagy and apoptosis. Moreover, we found that NTP treatment induced autophagy in a Sesn2-dependent manner via activation of the JNK pathway since suppression of JNK/Sesn2 pathway inhibited autophagy and enhanced the cell killing effect of NTP. Our results indicated that JNK/Sesn2 pathway played a major role in the network to regulate the crosstalk between apoptosis and autophagy. These findings suggested that autophagy and Sesn2 could be new targets to enhance the anticancer effect of NTP.

2. Materials and methods

2.1. Cell culture and reagents

A549 (Human lung cancer cell line) and MDA-MB-231 (Human breast cancer cell line) were purchased from ATCC (American Type Culture Collection) and cultured in DMEM (Dulbecco's modified Eagle's medium; Hyclone, Logan, USA) supplemented with 10% FBS (fetal bovine serum) and 1% penicillin-streptomycin (Gibco, Carlsbad, CA, USA). Both cells were incubated at 37 °C in humidified 5% CO_2 atmosphere.

Cyto-ID® autophagy detection kit was obtained from Enzo Life Sciences (Farmingdale, NY, USA). Autophagy antibody sampler kit and the primary antibodies for PARP (poly (ADP-ribose) polymerase), Caspase 3, JNK, phospho-JNK, LC3, Sesn2 and β-actin were purchased from CST (Cell Signaling Technology, Beverly, MA, USA). CQ (chloroquine) and AO (Acridine Orange) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Non-thermal dielectric barrier discharge (DBD) plasma treatment

Non-thermal DBD plasma was used in our previous studies, as shown in figure 1(a) [26, 27]. Briefly, the device mainly consists of three parts: plasma reaction chamber, high voltage power supply and gas source. The plasma reactor chamber contains four electrodes, one gas inlet and one gas outlet (figure 1(b)). The high voltage electrode consists of copper column (32 mm in diameter) with a quartz glass (1 mm thickness) at the bottom as an insulating layer, and copper columns (37 mm in diameter) are used as the negative electrode. NTP is produced by 12 kV voltage (peak to peak) at a frequency of 24 kHz. The average discharge power density is about 0.9 W cm^{-2}. In this study, helium (He, purity: 99.999%,) was used as the work gas. Before the treatment, we injected 3 min working gas (helium) which flow rates is 120 L h^{-1} to expel air as much as possible from plasma chamber (figure 1(c)).

2.3. Cell viability assay

Cell viability after NTP treatment was measured with the CCK-8 kit (Cell Counting Kit-8; Beyotime, Shanghai, China). Cells were treated with CCK-8 working solution for 1 h at 37 °C, then the supernatant (100 μl) was added to 96-well plates, and measured OD450 using the Varioskan Flash microplate reader (Thermo Fisher Scientific, Rockford, IL, USA).

2.4. Intracellular ROS measurement

ROS production was measured with the fluorescent probe DCFH-DA (Beyotime, Shanghai, China) following the

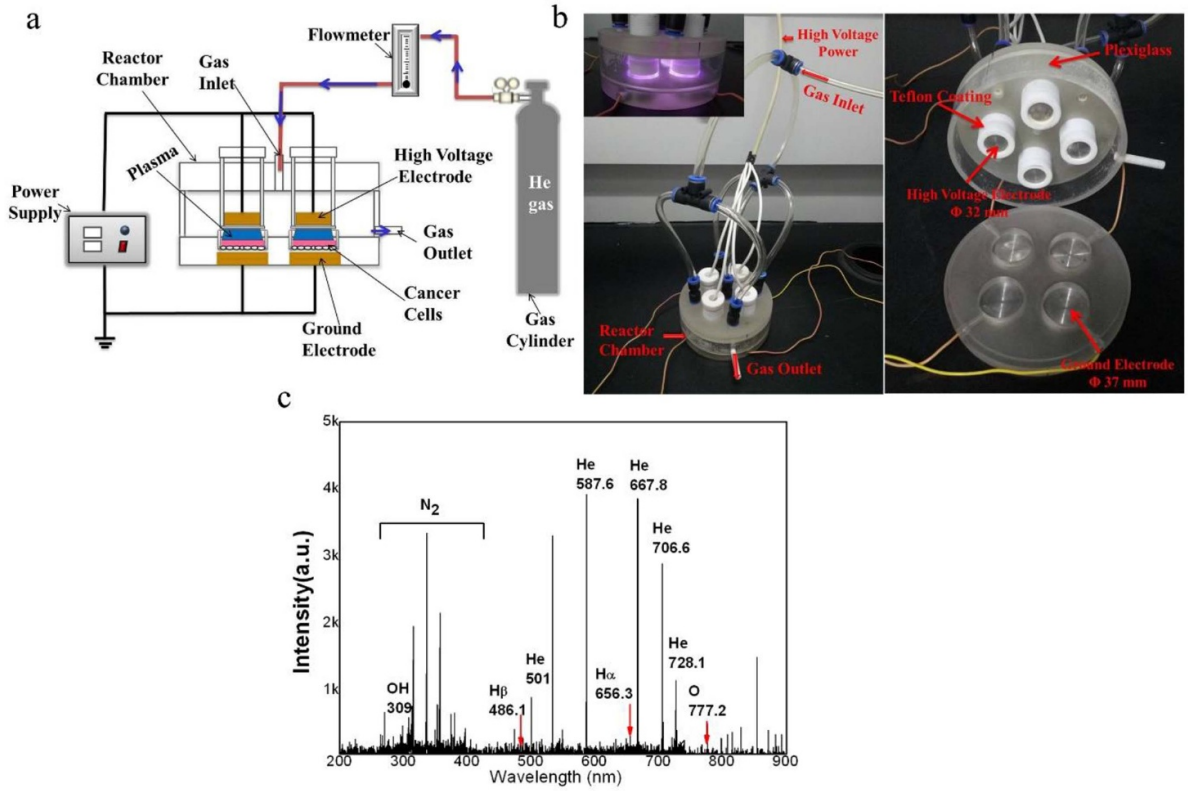

Figure 1. Schematic of the non-thermal plasma device. (a) Schematic of the plasma system. (b) Photographs of reactor chamber. (c) Typical optical spectrum of helium plasma.

manufacturer's instructions. The cells were stained with DCFH-DA (10 μM) for 30 min at 37 °C at 1–2 h post treatment, and then captured fluorescent images with fluorescence microscope (Leica DMI 40008, Germany).

2.5. Apoptosis detection

NTP-induced apoptotic cell death was measured with Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, Bedford, MA, USA) according to the instruction manual. Cells were harvested and incubated with Annexin V-FITC (5 μl) and PI (5 μl) for 15 min in the dark at room temperature, and finally measured using flow cytometry (Accuri C6, BD Biosciences, Bedford, MA, USA) within 1 h.

2.6. Autophagy detection

Cells were harvested and stained with Cyto-ID® autophagy detection kit according to the manufacturer's instructions, and then observed with fluorescence microscope (Leica DMI 40008, Germany).

2.7. Quantification of acidic vesicular organelles (AVOs)

At 24 h after NTP treatment, the cells were washed three times with PBS and stained with AO (Acridine Orange, 1 μg ml^{-1}) for 15 min at 37 °C as described previously [28]. Subsequently, the cells were washed three times with PBS and harvested for flow cytometry analysis.

2.8. Western blot

Cells were harvested and lysed with RIPA lysis buffer (Beyotime, Shanghai, China). The lysate was centrifuged at 12 000× g for 10 min at 4 °C and the supernatants were collected. Concentration of protein was detected with BCA Protein Assay Reagent Kit (Beyotime, Shanghai, China). Equivalent amounts of protein (50 μg) were separated with 8%–12% SDS-PAGE and transferred to PVDF (polyvinylidene difluoride) membrane (Millipore Corporation, Bedford, MA, USA). The membranes were then blocked with 5% nonfat dry milk and incubated with special primary antibodies overnight at 4 °C, and labeled with fluorescent second antibodies for 1 h at room temperature. Finally, the membranes were measured with Odyssey-CLx (Li-Cor, USA).

2.9. RNA interference

The special small interfering RNAs (siRNA) specific for Sesn2 and the negative control were purchased from Santa Cruz Biotechnology. The cells were transfected with siRNA by using lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. At 24 h post-transfection, the cells were treated with NTP for subsequent experiments.

2.10. Statistical analysis

All experiments were repeated at least three times and data were expressed as mean ± standard deviation (S.D.).

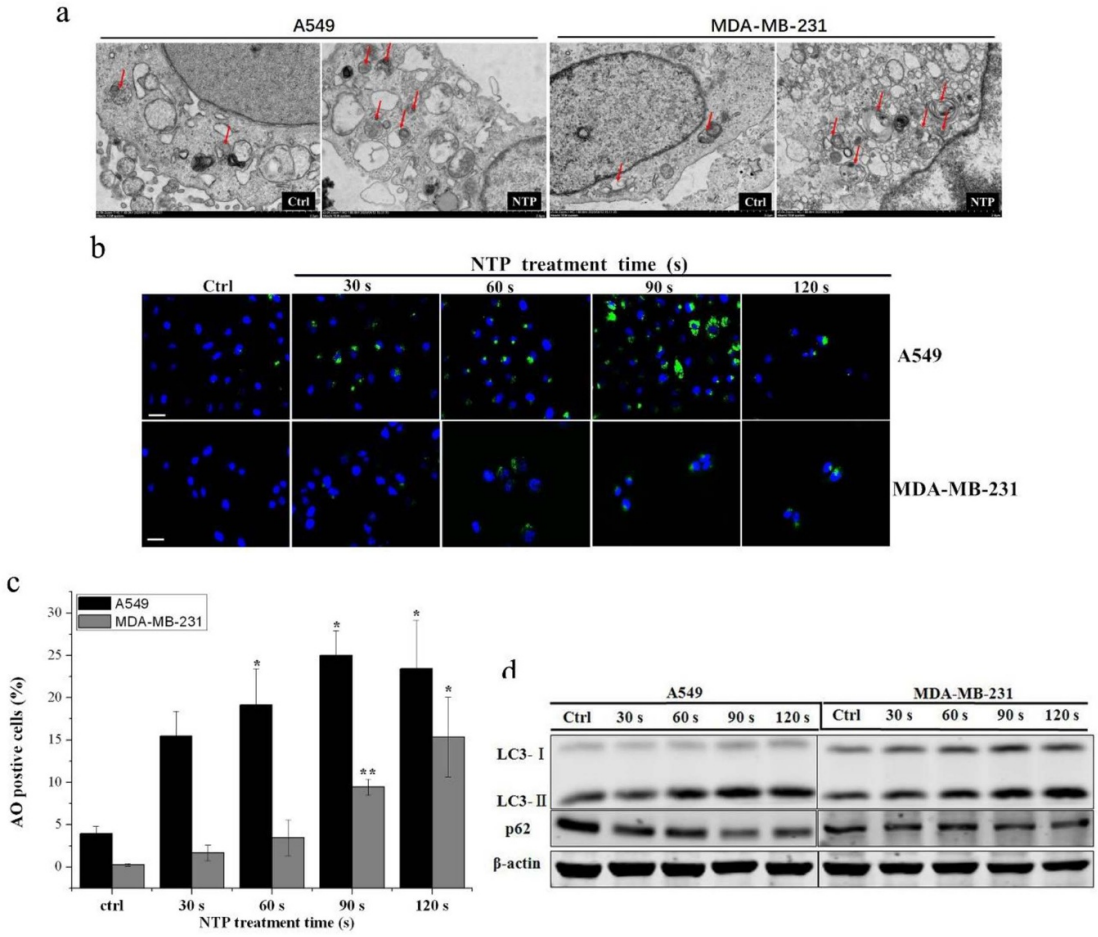

Figure 2. NTP treatment induced autophagy in A549 and MDA-MB-231 cells. (a) Representative TEM images of autophagy induced by NTP in A549 and MDA-MB-231 cells. (b) Representative images of autophagy positive cells after NTP treatment. (c) Acridine orange (AO) positive cells after NTP exposure. (d) Expression of autophagy-related proteins. Data are expressed as means ± S.D. for three independent experiments with triplicate each (significant vs. Ctrl, $^{*}p < 0.05$; $^{**}p < 0.01$).

Differences between results of two groups were evaluated with the Student's *t*-test. Results with $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. NTP treatment triggers autophagy

The formation of autophagosomes is an important sign of autophagy. In this study, the number of autophagosomes induced by NTP was captured with a transmission electron microscopy (TEM). The images were shown in figure 2(a) and the autophagosomes were indicated with red arrows. As autophagy is accompanied by increased AVOs, we used AO staining to quantify the accumulation of AVOs. As shown in figures 2(b) and (c), NTP treatment induced significant accumulation of AVOs in A549 and MDA-MB-231 cells. It is well known that LC3-II accumulation indicated autophagy occur [29]. p62 is also an important indicator of autophagic flux, which forms a complex with LC3-II located on the membrane of autophagy cells, and finally degradation in autolysosomes [30]. Furthermore, expression of LC3-II and p62 induced by NTP treatment was measured with western blotting. Results in figure 2(d) showed that the expression level of LC3-II increased with the NTP exposure dose in both A549 and MDA-MB-231 cells, but the expression of p62 exhibited an opposite trend. These results demonstrate that NTP treatment induces autophagy in a dose-dependent manner in both A549 and MDA-MB-231 cells.

3.2. NTP induces apoptotic cell death

It is well known that NTP treatment induce apoptotic cell death in many cancer cells [31]. In this study, the cytotoxicity of NTP treatment was also determined with the CCK-8 assay at 24 h after NTP exposure. As shown in figure 3, NTP treatment decreased the cell viability in a dose-dependent manner in A549 and MDA-MB-231 cells, and NTP treatment significantly increased apoptotic cell death in A549 and MDA-MB-231 (figures 4(a) and (b)). Similar trends were found in the activation of apoptosis related protein PARP and Caspase 3 (figure 4(c)). These data indicate that NTP exposure induces apoptotic cell death in both A549 and MDA-MB-231 cells.

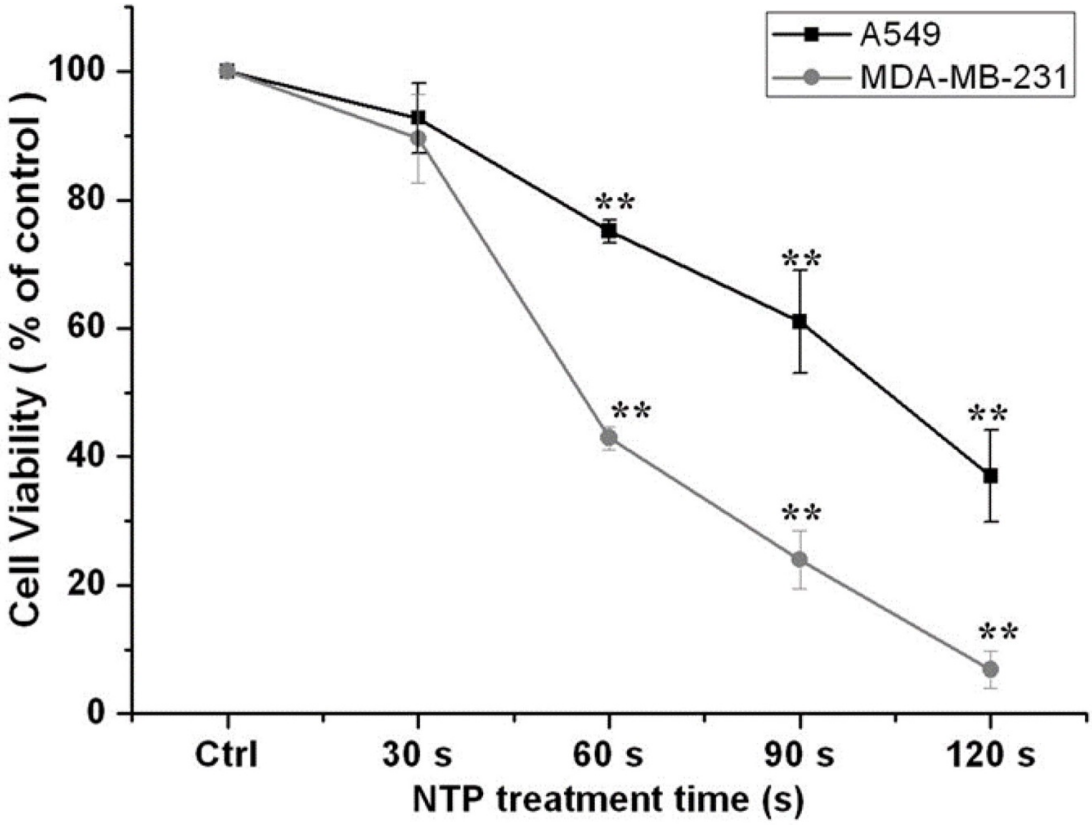

Figure 3. Cell viability after NTP treatment (significant vs. Ctrl, $^{*}p < 0.05$; $^{**}p < 0.01$).

3.3. Autophagy protects cells from NTP-induced death

Autophagy has dual roles in cell survival and cell death [12]. To confirm the roles of autophagy in NTP-induced cell death, the autophagy inhibitor CQ (chloroquine, 20 μM) was used to inhibit autophagy in both A549 and MDA-MB-231 cells. In our study, the cells were treated for 24 h with CQ and treated with NTP. The results found that the combined treatment of CQ and NTP more efficiently reduced the cell viability and enhanced apoptosis compared with either NTP or CQ treatment alone (figure 5(a)). These results strongly suggest that autophagy protects A549 and MDA-MB-231 cells from NTP-induced apoptotic cell death.

3.4. ROS-mediated autophagy and apoptosis induced by NTP

ROS is an important regulator involved in apoptosis and autophagy [32, 33]. However, whether NTP-induced apoptosis and autophagy were related with ROS production. Our results also found that NTP exposure increased intracellular ROS production, and it was strongly inhibited by using ROS scavenger, N-acetyl-L-cysteine (NAC, 10 mM) (figures 6(a) and (b)). Furthermore, pretreatment with NAC remarkably decreased the level of apoptosis (figures 6(c) and (d)) and autophagy (figure 6(e)) induced by NTP. These results indicate that NTP-induced apoptosis and autophagy are mediated by cellular ROS generation. In addition, the production of H_2O_2 in cell culture medium after NTP treatment (30, 60, 90, 120 s) was measured and analyzed the contribution of H_2O_2. The results showed NTP treatment increased the production of H_2O_2 in cell culture medium. Pretreatment with catalase can significantly inhibit NTP-induced cell death (90 s). In addition, the same concentration H_2O_2 (NTP treatment, 90 s) induced cell death is less than NTP directly treatment in A549 and MDA-MB-231 cells (figure S1 (available online at stacks.iop.org/JPD/53/465201/mmedia)).

3.5. Sesn2 is involved in autophagy and apoptosis induced by NTP

As a crucial mediator for ROS, Sesn2 is induced in response to oxidative damages [19]. To investigate whether NTP exposure changed Sesn2 expression, we detected the protein level of Sesn2 after NTP treatment. Our results found that NTP treatment significantly upregulated the expression of Sesn2 in A549 and MDA-MB-231 cells (figure 7(a)).

Sesn2 has been reported to be involved in apoptosis and autophagy induction [34]. To further elucidate the functional role of Sesn2 in the regulation of autophagy induced by NTP, the special siRNA was used to knock down the expression of Sesn2. The transfection efficiency was confirmed with western blotting, and results showed that transfection with the siRNAs led to downregulation of Sesn2 (figure 7(b)). The results showed that suppression of Sesn2 expression dramatically reduced cell viability and enhanced apoptosis after NTP treatment (figures 7(b) and (c)). Moreover, Sesn2 knockdown

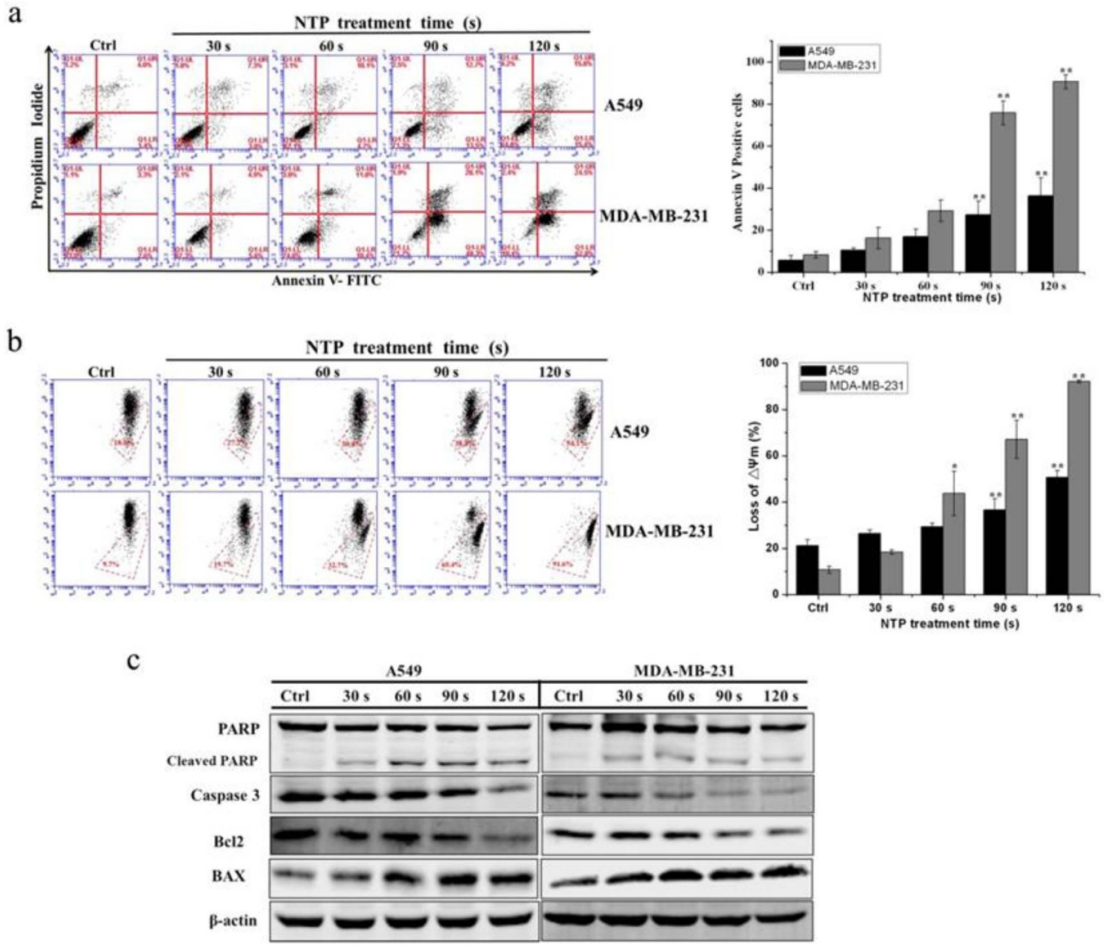

Figure 4. NTP treatment induced apoptosis in A549 and MDA-MB-231 cells. (a) Apoptosis induction after NTP exposure. (b) MMP changes after NTP exposure. (c) Expression of apoptosis-related proteins after NTP exposure. (Significant vs. Ctrl, $^{*}p < 0.05$; $^{**}p < 0.01$).

significantly decreased NTP-mediated LC3-II accumulation (figure 7(e)). Our results indicate that NTP treatment activates Sesn2-dependent protective autophagy, thereby mitigating cells from oxidative stress induced by NTP.

3.6. JNK pathway regulates NTP-induced Sesn2 expression and autophagy

It is well known that the JNK pathway plays an important role in regulating both apoptosis and autophagy [35, 36]. Conway *et al* found that NTP treatment significantly increased intracellular ROS level and activated JNK pathway, then induced Caspase-independent cell death in U373MG cells [37]. Our previous study found that NTP exposure upregulated the expression of HO-1 against oxidative damages via activation of JNK/Nrf2 signaling pathway in A549 cells [25]. We hypothesized that JNK pathway plays double roles in cell death induced by NTP. In this study, we explored whether JNK pathway was involved in mediating NTP-induced autophagy. The results showed that NTP treatment up-regulated the phosphorylation of JNK (figure 8(a)) and pretreatment with the JNK specific inhibitor (SP600125) at 2 h before NTP exposure enhanced the cell killing effect of NTP (figure 8(b)), and increased the ratio of apoptotic cells (figure 8(c)). Furthermore, inhibition of JNK with SP600125 also attenuated Sesn2 induction and LC3-II formation after NTP treatment in A549 cells (figure 8(d)). Similar results were also observed in MDA-MB-231 cells. These findings indicate that the JNK pathway is involved in apoptosis and autophagy after NTP treatment through regulating the expression of Sesn2.

4. Discussion

NTP has attracted significant attention in cancer therapy research for its effective and selective killing effect in various cancer cells [38]. Numerous studies have indicated that NTP treatment caused apoptotic cell death *in vitro* and inhibited tumor growth *in vivo* [39, 40]. ROS generation contributed to NTP treatment-caused DNA damages, apoptosis and cell cycle arrest [3, 41]. However, the molecular mechanism of NTP-induced cell death is still not well understood.

Autophagy has dual roles in regulating cell death and survival in response to various stimuli [12]. Previous studies found that NTP treatment increased lysosome biogenesis and autophagy in mesothelioma cells [42]. However, the mechanism of NTP-induced autophagy is still unknown. In this study,

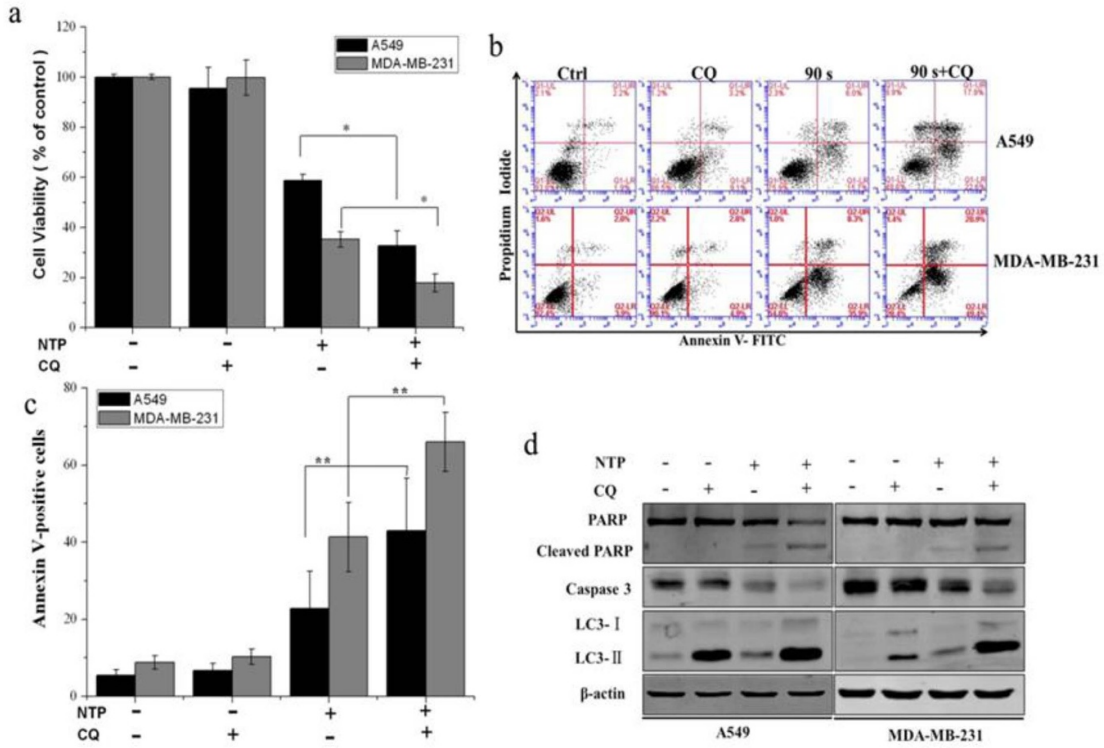

Figure 5. Inhibition of autophagy enhanced the cytotoxicity of NTP in A549 and MDA-MB-231 cells. (a) Cell viability, (b) and (c) apoptosis and (d) autophagy were measured after NTP exposure (90 s) with or without CQ (20 μM) pretreatment. $^{*}p < 0.05$; $^{**}p < 0.01$.

we found that NTP treatment decreased cell viability and induced apoptosis in a dose-dependent manner in both A549 and MDA-MB-231 cells. We further explored the occurrence of autophagy and its function in NTP-induced cell death. Our results found that NTP treatment increased LC3-II expression and degraded p62. In addition, NTP treatment also increased AO positive cells. To determine the function of autophagy, the autophagy inhibitor (CQ, 20 μM) was used to block autophagy induction. It was observed that pretreatment with CQ significantly enhanced the cell killing effects of NTP. These results provide strong evidence that NTP treatment induces protective autophagy and apoptosis.

ROS is an important initiator of apoptosis and autophagy [33]. Previous studies reported that NTP treatment induced ROS accumulation and caused cell death [41, 43, 44]. Moreover, other studies found that NTP treatment or NTP-activated medium protected cells from oxidative stress [25, 45]. Therefore, the role of ROS in NTP-induced autophagy and apoptosis was studied. Our results showed that NTP treatment markedly increased the levels of cellular ROS and led to cell death, which were prevented by pretreatment with NAC, a scavenger of ROS, in A549 and MDA-MB-231 cells. The same effects were found in inhibiting the activation of apoptosis related proteins (PARP and Caspase 3) induced by NTP treatment. In addition, NAC pretreatment also inhibited NTP-induced autophagy through reduction of LC3-II expression. Taken together, these results indicate that ROS production plays a key role in NTP-induced apoptosis and autophagy in A549 and MDA-MB-231 cells.

Sesn2 is an antioxidant protein, which protects cells against various stresses. Jegal *et al* found that eupatilin treatment activated Sesn2-dependent autophagy and resisted oxidative stress induced by AA+ iron [46]. Liang *et al* revealed that Isorhapontigenin (ISO) treatment effectively induced autophagic cell death via upregulating Sesn2 expression in bladder epithelial cells [21]. Seo *et al* reported that glucose deprivation significantly increased the expression of Sesn2 against glucose deprivation-induced ROS generation and apoptosis in hepatocyte-derived cells [23]. Recent research found that NTP activated iNOS and increased NO production via Sesn2 upregulation, and then promoted NTP-induced cell apoptosis in melanoma cell lines [10]. Interestingly, our results showed that NTP treatment also increased the expression of Sesn2 and knockdown of Sesn2 with special siRNA significantly enhanced NTP-induced cell death and reduced LC3-II formation in both A549 and MDA-MB-231 cells. These results indicate that NTP treatment induces apoptosis and protective autophagy by regulating the expression of Sesn2.

In addition, the JNK pathway might participate in the induction of Sesn2. Yi *et al* found that AngII treatment significantly induced Sesn2 expression via activation of the JNK/c-Jun signaling pathway in human umbilical vein endothelial cells [47]. Zhang *et al* reported that the upregulation of Sesn2 expression caused by excisanin A or serum deprivation was mediated by the JNK pathway in human carcinoma cell lines CNE1 and CNE2 [48]. Previous studies also found that 4-hydroxy-trans-2-nonenal treatment triggered autophagy through activation of the JNK signaling pathway and pro-

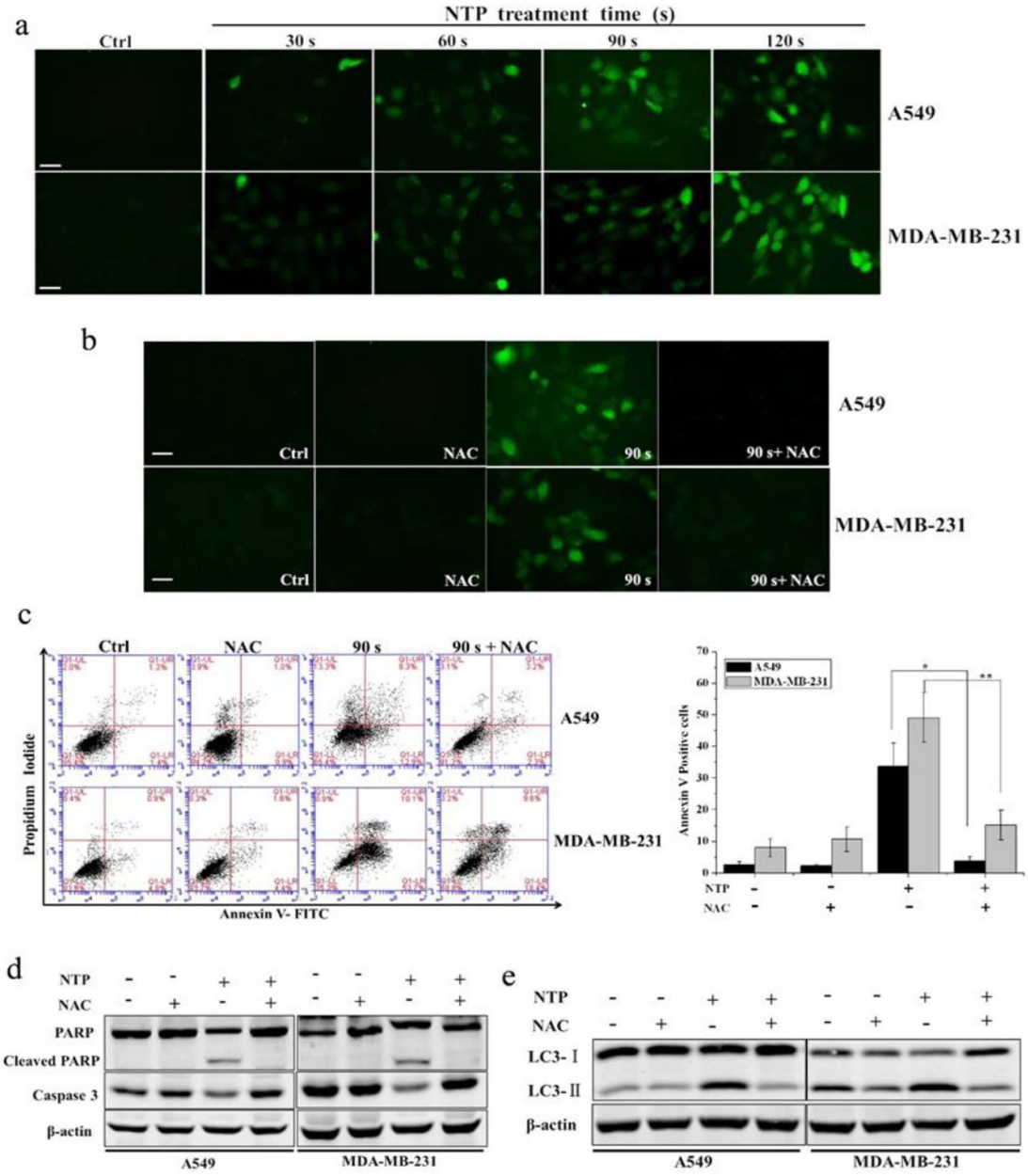

Figure 6. ROS mediating NTP-induced apoptosis and autophagy. (a) Representative images of ROS fluorescence captured at 1–2 h after NTP treatment. (b) Effect of NAC (10 mM) on NTP-induced ROS generation, (c), (d) Apoptosis and (e) autophagy. $^{*}p < 0.05$; $^{**}p < 0.01$.

moted cell survival during oxidative stress [24]. Previous researches reported that NTP treatment increased intracellular ROS generation, activated JNK pathway and induced cell apoptosis [31, 37]. In addition, our pervious study revealed that NTP exposure improved the phosphorylation level of JNK and heme oxygenase-1 expression against apoptosis in A549 cells [25]. Therefore, we speculated that NTP treatment induced autophagy and apoptosis by activating ROS/JNK pathway. In this study, we found that NTP exposure also induced JNK phosphorylation and combined treatment with JNK inhibitor (SP600125) markedly increased cell death, and decreased Sesn2 expression and LC3-II formation. These findings suggest that NTP exposure induces autophagy and apoptotic cell death mediated by the ROS/JNK/Sesn2 pathway in both A549 and MDA-MB-231 cells.

5. Conclusions

In summary, our results indicated that NTP exposure stimulated ROS accumulation, induced apoptosis and protective autophagy in both A549 and MDA-MB-231 cells. NTP treatment induced Sesn2 expression in a dose-dependent manner which was mediated by JNK signaling pathway. These findings indicated that NTP exposure could lead to two seemingly opposite consequences: inducing apoptotic death and

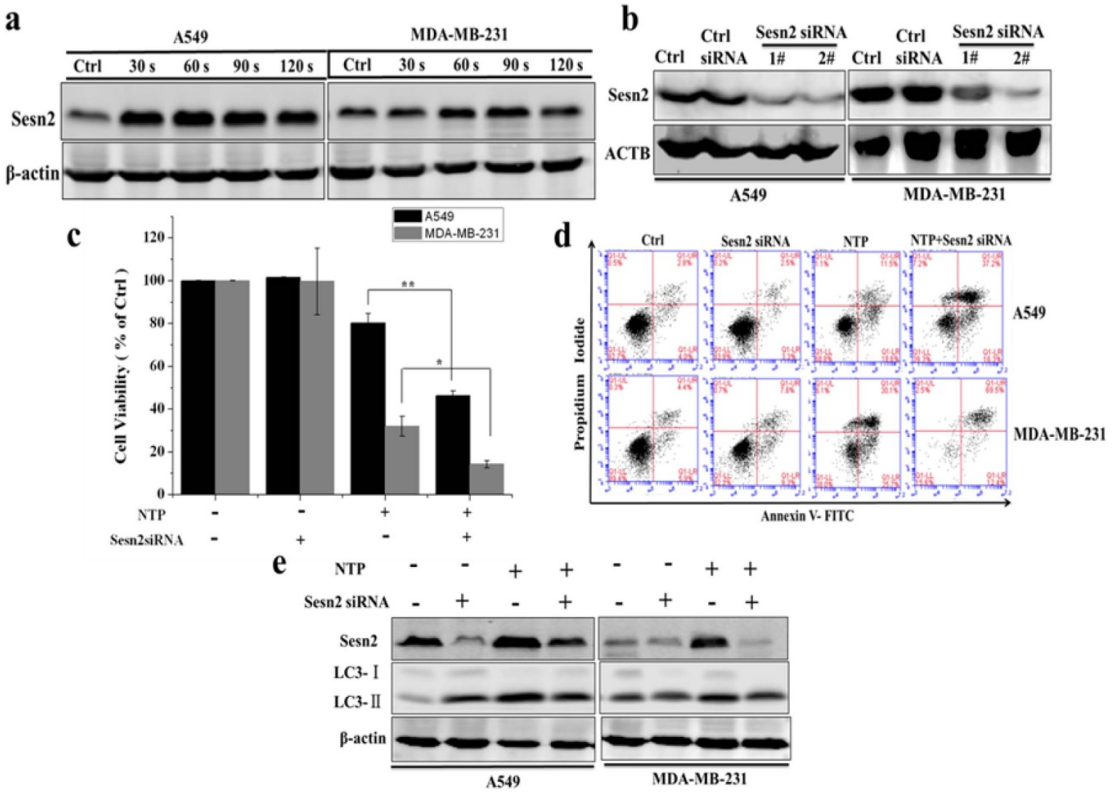

Figure 7. Sesn2 was involved in NTP-induced autophagy in A549 and MDA-MB-231 cells. (a) Expression of Sesn2 induced by NTP treatment. (b) Efficiency of Sesn2 silencing at 24 h after transfection and NTP treatment (90 s), (c) cell viability, (d) apoptosis and (e) autophagy were analyzed. Data are expressed as mean ± S.D. for three independent experiments with triplicate each. $^{*}p < 0.05$; $^{**}p < 0.01$.

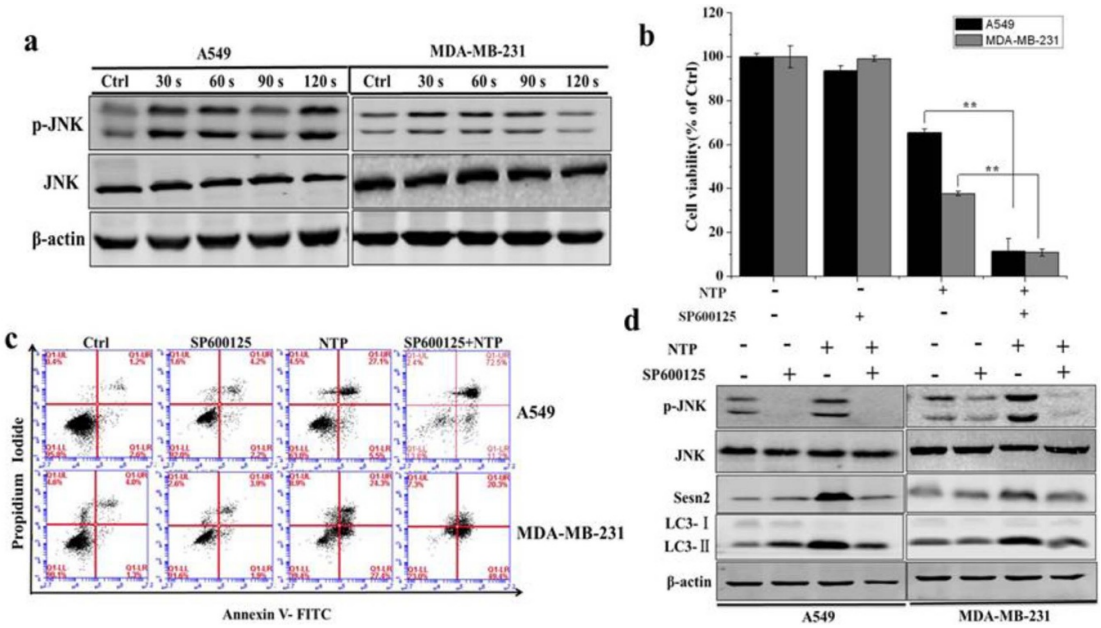

Figure 8. JNK pathway mediated the Sesn2-dependent autophagy after NTP exposure. (a) Phosphorylation of SAPK/JNK pathways after NTP treatment. After co-treatment with JNK inhibitor and NTP (90 s), (b) cell viability, (c) apoptosis and (d) the protein levels of p-JNK, Sesn2, LC3 were analyzed. $^{*}p < 0.05$; $^{**}p < 0.01$.

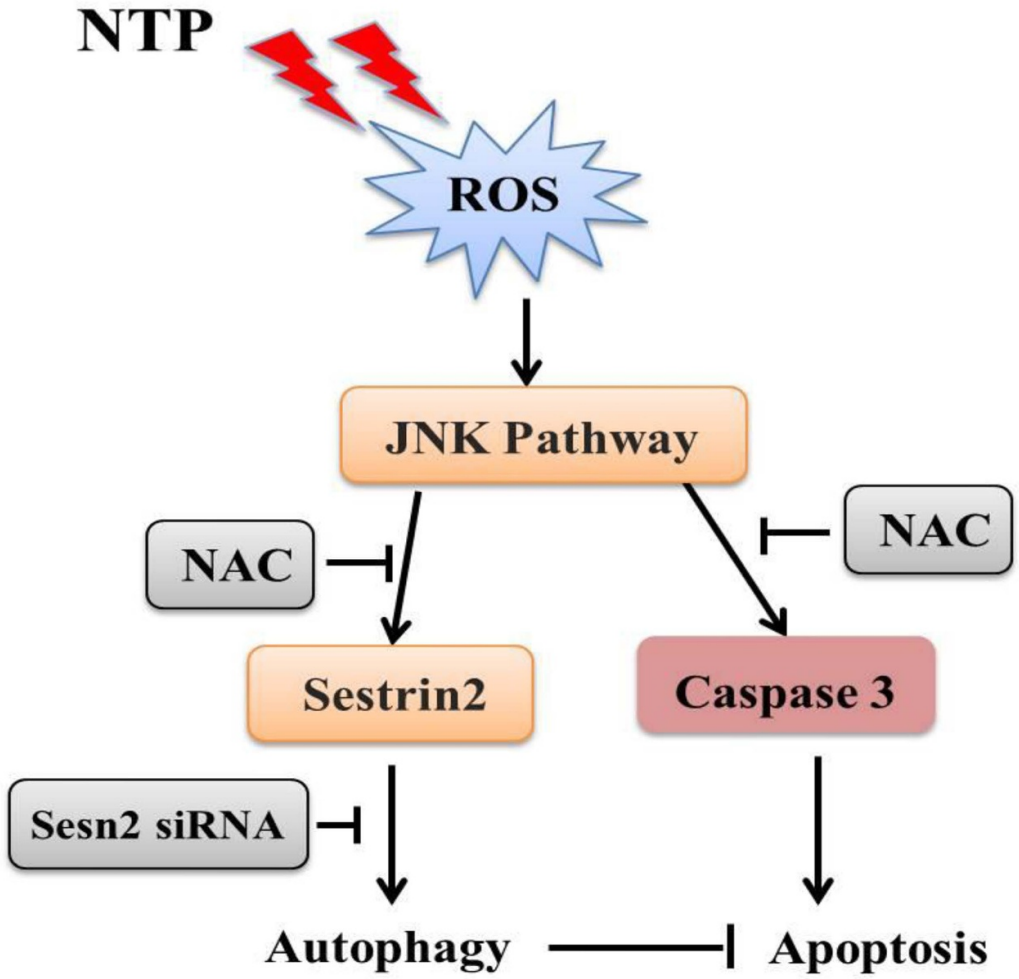

Figure 9. Possible signaling pathways of autophagy and apoptosis induced by NTP. NTP treatment induces ROS generation, then activates JNK pathway to increase the expression of Sesn2, and initiates protective autophagy to protect cells from NTP. Blocking the JNK/Sesn2 pathway can inhibit the autophagy induction and enhance the tumor cell killing effects of NTP.

triggering a protective autophagy in A549 and MDA-MB-231 cells (figure 9).

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