

Research Article

An Acetamide Derivative as a Camptothecin Sensitizer for Human Non-Small-Cell Lung Cancer Cells through Increased Oxidative Stress and JNK Activation

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In recent years, combination chemotherapy is a primary strategy for treating lung cancer; however, the issues of antagonism and side effects still limit its applications. The development of chemosensitizer aims to sensitize chemoresistant cancer cells to anticancer drugs and therefore improve the efficacy of chemotherapy. In this study, we examined whether N-[2-(morpholin-4-yl)phenyl]-2-{8-oxatricyclo[7.4.0.0.2,7]trideca-1(9),2(7),3,5,10,12-hexaen-4-yloxy}acetamide (NPOA), an acetamide derivative, sensitizes human non-small-cell lung cancer (NSCLC) H1299 cells towards camptothecin- (CPT-) induced apoptosis effects. Our results demonstrate that the combination of CPT and NPOA enhances anti-lung-cancer effect. The cytometer-based Annexin V/propidium iodide (PI) staining showed that CPT and NPOA cotreatment causes an increased population of apoptotic cells compared to CPT treatment alone. Moreover, Western blotting assay showed an enhancement of Bax expression and caspase cascade leading to cell death of H1299 cells. Besides, CPT and NPOA cotreatment-mediated disruption of mitochondrial membrane potential (MMP) in H1299 cells may function through increasing the activation of the stressed-associated c-Jun N-terminal kinase (JNK). These results showed that NPOA treatment sensitizes H1299 cells towards CPT-induced accumulation of cell cycle S phase and mitochondrial-mediated apoptosis through regulating endogenous ROS and JNK activation. Accordingly, NPOA could be a candidate chemosensitizer of CPT derivative agents such as irinotecan or topotecan in the future.

1. Introduction

Lung cancer is one of the common causes of death worldwide in both men and women [1]. Non-small-cell lung cancer (NSCLC) is one of the members of lung cancer types, and NSCLC accounts for about 80% of all lung cancers.

Previous studies have shown that NSCLC often grows and spreads quickly [2]. Despite chemotherapy being one of the main therapeutic options for the treatment of NSCLC patients [3], the acquired chemoresistance often causes poor prognosis and recurrence in advanced NSCLC patients [4]. Therefore, improved efficacy and reduced cross-resistance of

chemotherapeutic agents for NSCLC patients are urgent and are still under development [5].

Combination chemotherapies, a combination of two or more anticancer drugs, are aimed to enhance the efficacy of monochemotherapy [6]. However, the side effects of combination chemotherapies, such as neutropenia, febrile neutropenia, and sepsis, still cannot be avoidable completely during treatment, causing reduced efficacy of NSCLC treatments [7]. CPT, a quinoline alkaloid, is isolated from the Chinese ornamental tree *Camptotheca acuminata*. CPT and its derivatives are utilized to inhibit the proliferation of cancer cells, including lung cancer [8]. Two clinically used CPT derivatives irinotecan (CPT-11) and topotecan are widely used to inhibit the growth of lung cancer in combination with cisplatin, paclitaxel [9], and bevacizumab [10]. However, combination chemotherapy may cause antagonism and result in an attenuated efficacy of anticancer drugs [11, 12].

Chemotherapy sensitizers or chemosensitizers are used for enhancing the efficacy of anticancer drugs [13]. For example, PZ-39 (N-(4-chlorophenyl)-2-[(6-[[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]amino]-1,3-benzothiazol-2-yl)sulfanyl]acetamide), an acetamide-containing compound, has been reported to significantly enhance the anti-breast-cancer activity effect of mitoxantrone, an anthracenedione [14]. Furthermore, amiloride, an acetamide-containing compound, enhances erlotinib-induced apoptosis of human pancreatic cancer cells [15]. Accordingly, the development of chemotherapy sensitizer could reduce antagonism and sensitize cancer cells to chemotherapeutic agents.

Acetamide-containing compounds are reported to exert various bioactivities, including anti-inflammatory, antibacterial, and antifungal activity [16–18]. For instance, 40006, an acetamide derivative, has been shown to inhibit inflammation of murine macrophage J774A.1 cells through reducing endogenous ROS [19]. Furthermore, acetamide derivatives have also been reported to exert anticancer activity [19, 20]. N-Butyl-2-(2-fluorophenyl) acetamide (SCK6), an acetamide compound, has been reported to inhibit proliferation and to induce apoptosis of human lung squamous carcinoma CH27 cell via G₁ cell cycle arrest [21]. Besides, combinations with acetamide derivatives and other chemotherapeutic agents have been reported to treat various cancer cells. For instance, the combination of acetamide derivatives N-(2-hydroxyphenyl) acetamide (NA-2) and Temozolomide (TMZ) synergistically induces apoptosis of human glioblastoma U87 cells [22], suggesting that acetamide derivatives could be a chemosensitizer for enhancing other chemotherapy agent-induced anticancer effects.

To overcome the limitations mentioned above of CPT-based treatment against NSCLC, we preliminarily examined a panel of acetamide derivatives and screened out a candidate N-[2-(morpholin-4-yl)phenyl]-2-{8-oxatricyclo[7.4.0.0,2,7]trideca-1(9),2(7),3,5,10,12-hexaen-4-yloxy}acetamide (NPOA). In this study, the synergistic effect and the mechanism of NPOA-mediated synergistic anti-NSCLC effect by CPT-based treatment were investigated. Furthermore, NPOA-mediated regulation of endogenous reactive oxygen species (ROS), the disruption of mitochondrial membrane

potential (MMP), and the role of MAPK JNK in CPT-induced apoptosis of NSCLC cells were also discussed.

2. Materials and Methods

2.1. Preparation of Compounds. N-[2-(morpholin-4-yl)phenyl]-2-{8-oxatricyclo[7.4.0.0,2,7]trideca-1(9),2(7),3,5,10,12-hexaen-4-yloxy}acetamide (NPOA) was purchased from the chemical supplier Enamine™ (Kiev, Ukraine). CPT was purchased from Sigma-Aldrich (St. Louis, MO, USA). Both CPT and NPOA were dissolved in DMSO (below 0.01%) immediately before assays.

2.2. Reagents and Antibodies. DMEM with high glucose, F-12 Nutrient Mixture, trypan blue, dimethyl sulphoxide (DMSO), fetal bovine serum (FBS), antibiotics penicillin G, and streptomycin were purchased from Gibco BRL (Gaithersburg, MD, USA). Ribonuclease A (RNase A) was purchased from Sigma-Aldrich. Antibodies against phospho-JNK (Thr¹⁸³/Tyr¹⁸⁵) (#9101), total-JNK (#9102), cleaved caspase 9 (#7237), and cleaved caspase 3 (#9961) were purchased from Cell Signaling Technology (San Jose, CA, USA). Antibody against Bax (GTX61026) was obtained from Genetex (Alton Parkway, Irvine, CA, USA). Antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Santa Cruz Biotechnology Co. (Santa Cruz, CA, USA). Both anti-mouse and anti-rabbit IgG secondary antibodies conjugated to peroxidase were purchased from Leadgene Biomedical (#20102 and #20202, resp., Tainan, Taiwan). Annexin V-Fluorescein isothiocyanate (FITC) staining kit was purchased from Strong Biotech Co. (Taipei, Taiwan).

2.3. Cell Culture. Non-small-cell lung cancer cell lines A549 and H1299 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in the medium DMEM: F-12 (ratio 3 : 2) and supplemented with 2 mM glutamine, 8% FBS, and the antibiotics 100 µg/mL streptomycin and 100 units/mL penicillin at 37°C in a humidified condition of 5% CO₂. Cells were checked using a PCR-based assay described previously for confirming no contamination of mycoplasma [23].

2.4. Proliferative Inhibition Assay. The proliferation rate was determined by a trypan blue exclusion-based assay. Briefly, 3 × 10⁴ NSCLC H1299 cells were seeded and treated with vehicle or the indicated concentrations of CPT and NPOA alone or cotreatment for 24 h and 48 h, respectively. After incubation, cells were stained with 0.2% trypan blue and counted by Countess™, an automatic cell counter (Invitrogen, Carlsbad, CA, USA).

2.5. Analysis of Drug Synergism. The drug synergism was analyzed according to the previous work [24]. Briefly, H1299 cells were treated with serial dilutions (from 0.125 to 1 µM) of CPT combining with serial dilutions of NPOA (from 10 to 40 µM). The values of combination index (CI) were analyzed using the software CalcuSyn (Biosoft, Cambridge, UK), a computer program based on the method of Chou and Talalay

[25, 26]. The 95% confidence intervals for the dose-response values were used for determining the data. Synergism ($CI < 1$), additivity ($CI = 1$), or antagonism ($CI > 1$) presents the effect of drug combinations, respectively.

2.6. Apoptosis Assessment. Annexin V/PI dual staining was performed for detecting the externalization of phosphatidylserine (PS) from the cellular plasma membrane, a hallmark of apoptosis. Briefly, 1×10^5 cells were seeded onto a 6-well plate and treated with CPT and NPOA alone or cotreatment for 24 h and 48 h, respectively. Subsequently, cells were harvested and stained with Annexin V/PI, and cells were analyzed by a flow cytometer Muse™ (Cell Analyzer, Merck Millipore, Billerica, MA, USA).

2.7. Oxidative Stress. The changes of intracellular redox state were determined by superoxide (O_2^-) sensitive fluorescent dye dihydroethidium (DHE) (Merck, Darmstadt, Germany). In brief, 1×10^5 cells were seeded onto a 6-well plate and treated with CPT and NPOA alone or cotreatment for 6 h, respectively. Subsequently, cells were stained with $1 \mu M$ DHE for 30 min in darkness. After incubation, the cells were analyzed by a flow cytometer Muse™ (Cell Analyzer, Merck Millipore).

2.8. Assessment of Mitochondrial Membrane Potential ($\Delta\Psi_m$). The changes of mitochondrial membrane potential ($\Delta\Psi_m$) during cell apoptosis was determined by a cationic dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) staining assay according to a previous study of Hsu et al.'s work with minor modifications [27]. The green fluorescence intensity of JC-1 is inversely proportional to the level of MMP ($\Delta\Psi_m$). Briefly, H1299 cells (1×10^5 cells/well) were seeded in a 6-well plate and treated with $0.5 \mu M$ CPT alone and $10 \mu M$ NPOA alone or their combination for 24 h, respectively. After incubation, cells were harvested and stained with $1 \mu M$ of the JC-1 solution in serum-free medium and incubated at $37^\circ C$ in the dark for 30 minutes. Afterward, cells were washed and detected by a flow cytometer Muse Cell Analyzer, Merck Millipore.

2.9. Western Blotting Assay. Western blotting assay was described previously with minor modifications [28]. In brief, $20 \mu g$ protein was resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked with 5% nonfat milk in PBS-T buffer (PBS containing 0.1% Tween-20) and incubated with the primary and its corresponding secondary antibodies against specific proteins. The signals of specific protein were detected by a chemiluminescence-based WesternBright™ ECL detection solution (Advansta, Menlo Park, CA, USA).

2.10. Statistical Analysis. Differences between CPT and NPOA cotreatment and CPT-treated alone were analyzed in at least triplicated experiments. The difference was analyzed by one-way analysis of variance (ANOVA). $*p < 0.05$ was considered statistically significant.

3. Results

3.1. CPT and NPOA Cotreatment Synergistically Enhances the Antiproliferation of H1299 Cells. To determine whether NPOA synergistically enhances CPT-induced antiproliferation of NSCLC cells, the multidrug effect analysis of Chou-Talalay method was used for analyzing the synergism of CPT and NPOA combination. The calculated 50% lethal concentration (LC_{50}) of CPT for reducing cell viability is $0.5 \mu M$ in H1299 cells. Besides, we found the treatment of NPOA was noncytotoxic in H1299 cells, whereas it synergistically enhanced CPT-induced cytotoxicity of H1299 cells (Supplementary Table 1 in Supplementary Material available online at <http://dx.doi.org/10.1155/2016/9128102>). Therefore, we next confirmed the synergism of CPT and NPOA combination on antiproliferation of NSCLC cell lines H1299 and A549 by trypan blue exclusion assay. As shown in Figures 1(a) and 1(b), the treatment of two compounds alone slightly inhibited the viability of two NSCLC cells for 24 h and 48 h. On the contrary, CPT and NPOA cotreatment markedly inhibited the viability of two NSCLC cells compared to both CPT and NPOA treatments alone, especially at 48 h treatment ($**p < 0.001$). Moreover, we performed colony formation assay to confirm the markedly inhibited cell proliferation of two NSCLC cells after CPT and NPOA cotreatment (Figures 1(c) and 1(d)).

3.2. NPOA Sensitizes NSCLC Cells towards CPT-Induced Mitochondrial-Mediated Apoptosis. To determine whether combining CPT and NPOA inhibited cell growth by inducing apoptosis, the flow cytometer-based detection assay was determined by Annexin V/PI dual staining. In this assay, the percentages of Annexin V-positive/PI-negative were presented as early apoptosis, and the percentages of Annexin V-positive/PI-positive were presented as late apoptosis. The H1299 cells were incubated with indicated concentration of $0.5 \mu M$ CPT alone, $10 \mu M$ NPOA alone, or their combination for 24 h and 48 h, respectively. As shown in Figures 2(a) and 2(b), CPT treatment alone slightly induced apoptosis of H1299 cells at 24 h and 48 h, whereas the treatment of NPOA alone did not. However, CPT and NPOA cotreatment significantly increased the percentage of apoptotic cells in H1299 cells after 48 h treatment. In addition, the NPOA markedly enhanced CPT-induced apoptosis through regulating Bax and cleaved caspase 9 and caspase 3 (Figure 2(c)). These results showed that the NPOA synergistically enhanced CPT-induced apoptosis of H1299 cells.

3.3. CPT and NPOA Cotreatment Induces the Disruption of Membrane Potential in H1299 Cells. To determine whether CPT and NPOA cotreatment-induced apoptosis of NSCLC cells was through the modulation of mitochondria-mediated apoptosis pathway, JC-1, a cyanine dye, was used to detect the depolarization of mitochondrial membrane potential (MMP), a hallmark of mitochondria-mediated apoptosis [29]. The H1299 cells were cultured with indicated concentration of $0.5 \mu M$ CPT alone and $10 \mu M$ NPOA alone or their combination for 24 h. As depicted in Figures 3(a) and 3(b),

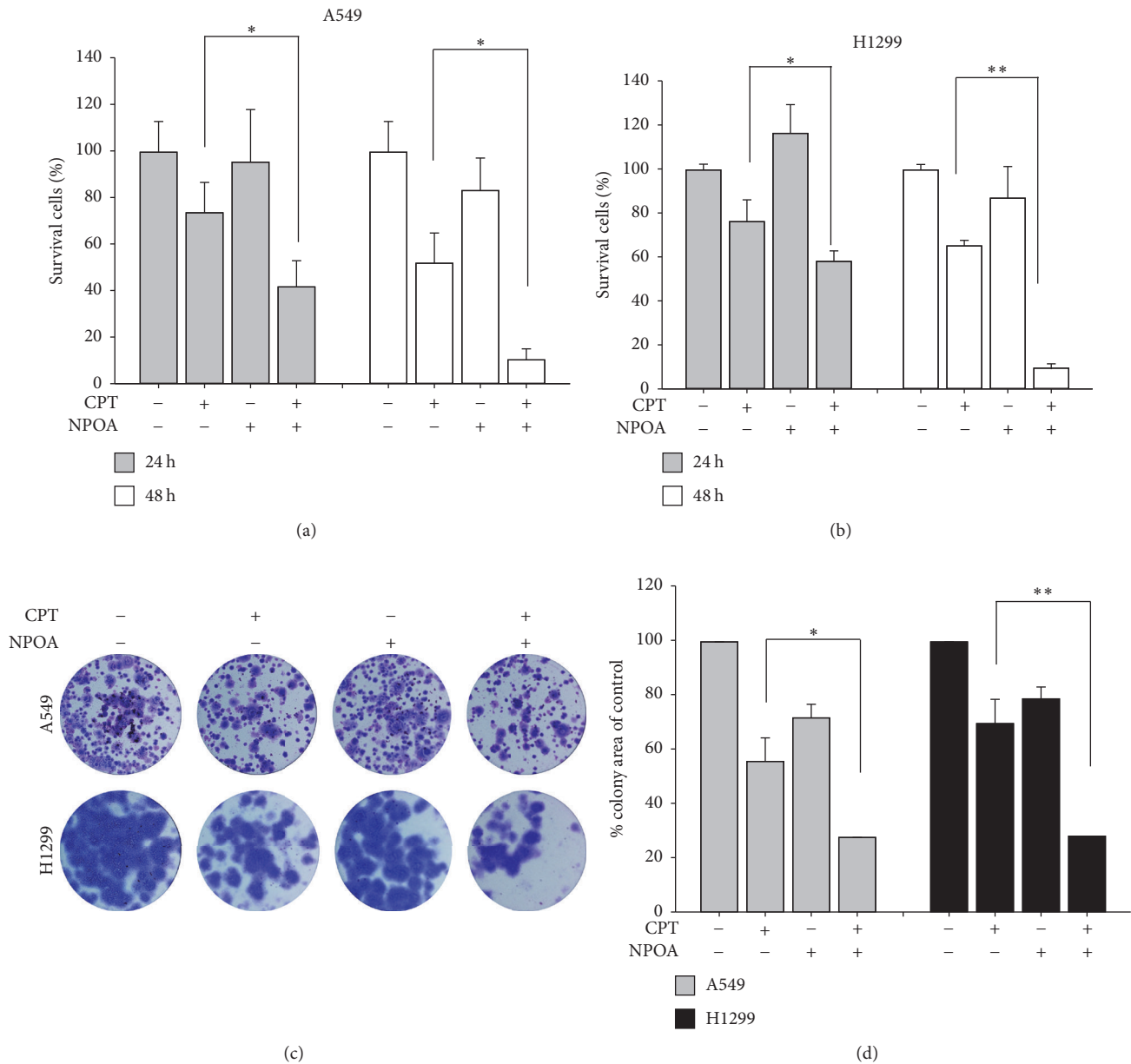
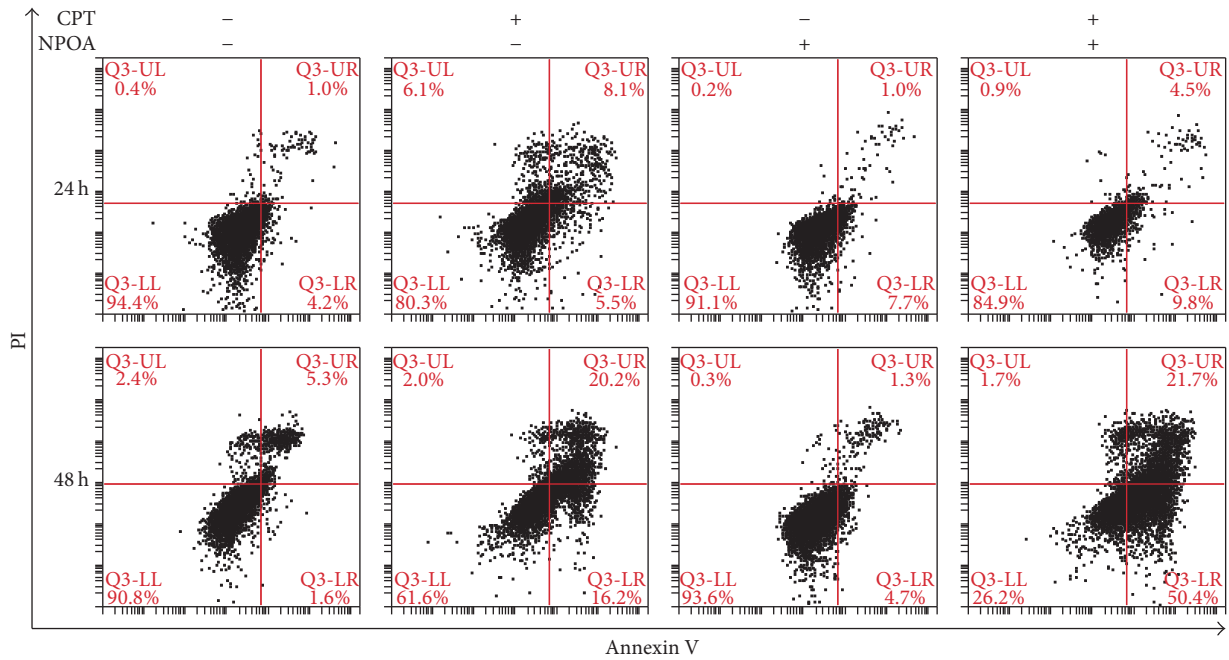


FIGURE 1: CPT and NPOA cotreatment inhibits cell proliferation of two NSCLC cells. The two NSCLC cell lines, A549 and H1299, were incubated with $0.5 \mu\text{M}$ CPT and $10 \mu\text{M}$ NPOA alone or in combination, respectively. (a and b) The rates of two NSCLC cell survival lines were determined using trypan blue exclusion assay. The quantification analysis of survival cells. (c) CPT and NPOA cotreatment inhibited the colony formation of two NSCLC cells for 11 days. Afterward, the cells were fixed in 4% paraformaldehyde and Giemsa stained. (d) The quantitative analysis of the colony area. Data are presented as means \pm SD (* $p < 0.05$; ** $p < 0.001$).

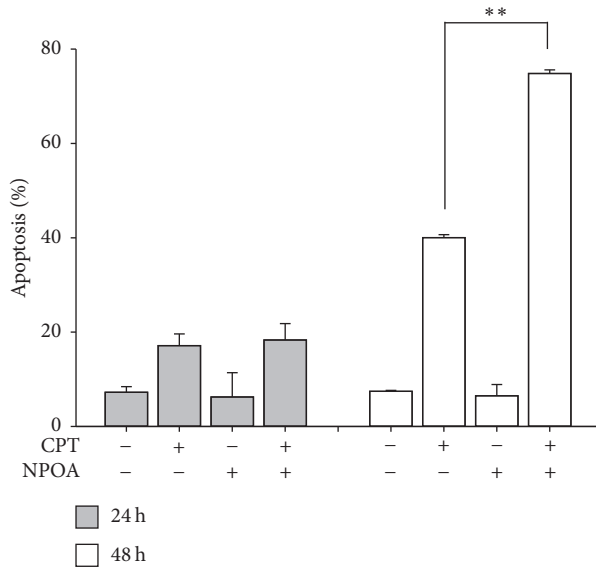
NPOA enhanced CPT-induced depolarization of MMP in H1299 cells. Moreover, the JC-1 fluorescence-based imaging assays also confirmed the synergism of NPOA on CPT-induced mitochondrial membrane depolarization of H1299 cells (Figure 3(c)). These results showed that a dramatic loss of MMP ($\Delta\Psi_m$) was induced by CPT and NPOA cotreatment in H1299 cells.

3.4. NPOA Enhances CPT-Induced Endogenous ROS Production of H1299 Cells. A high level of reactive oxygen species

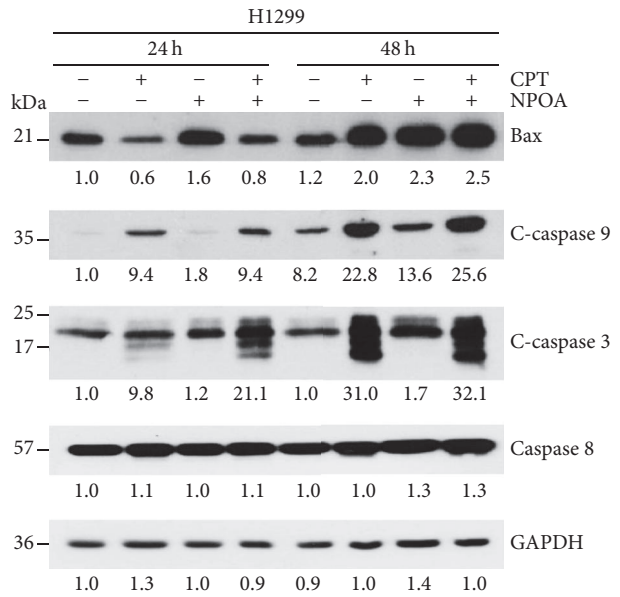
(ROS) is considered to induce apoptosis of cancer cells via mitochondrial pathway [30]. Next, we examined the synergistic effect of NPOA on CPT-induced anti-H1299 cells through upregulating endogenous ROS. The dihydroethidium (DHE) staining can detect endogenous ROS level by combining flow cytometric analyses. We found that the NPOA treatment markedly increased CPT-induced ROS production in H1299 cells compared to the CPT or NPOA treatment alone (Figures 4(a) and 4(b)). These results suggest that NPOA enhanced CPT-induced ROS in H1299 cells may play a pivotal role. On



(a)



(b)



(c)

FIGURE 2: CPT and NPOA cotreatment induced apoptosis of H1299 cells. The cells were treated with 0.5 μ M CPT alone, 10 μ M NPOA alone, or their combination for 24 h and 48 h, respectively. (a) The cells were double-stained with Annexin V and PI and analyzed by flow cytometer-based detection assay. (b) The quantitative analysis of (a). Data are presented as means \pm SD (** $p < 0.001$). (c) The results of Western blot assay showed the changes of mitochondrial apoptotic Bax protein, cleaved caspase 9 and cleaved caspase 3, and full-length caspase 8. Abbreviations: C-caspase 9 indicates cleaved caspase 9 and C-caspase 3 indicates cleaved caspase 3. GAPDH as an internal control for equal loading.

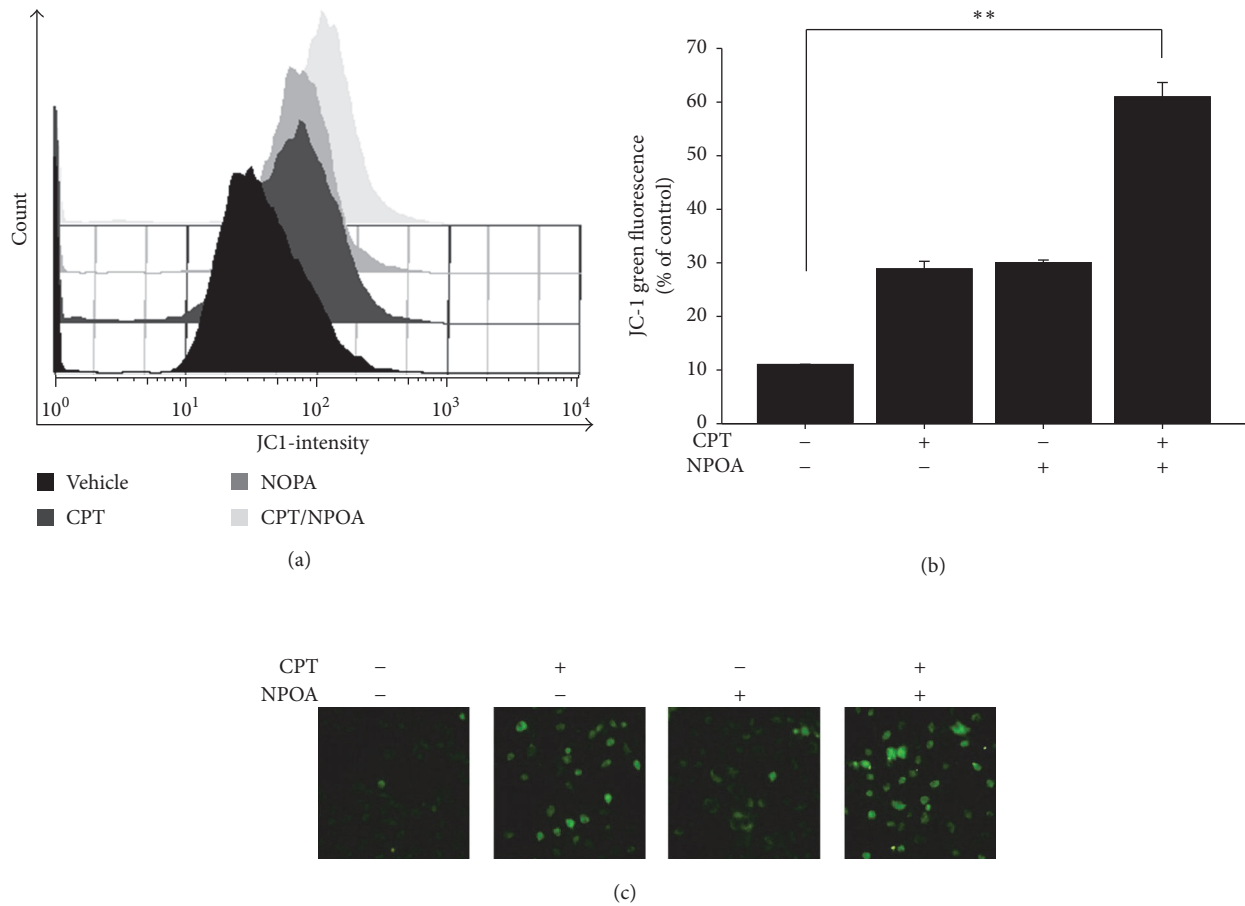


FIGURE 3: NPOA enhanced CPT-induced apoptosis through mitochondrial pathway. H1299 cells were treated with CPT alone and NPOA alone or their combination for 24 h, respectively. Afterward, H1299 cells were analyzed using flow cytometer- and fluorescence microscopy-based JC-1 assays, respectively. (a) The changes of mitochondrial membrane potential were determined by flow cytometer-based JC-1 staining assay. (b) The quantification analysis of (a). Data are presented as means \pm SD (** $p < 0.001$). (c) The green fluorescence of JC-1 indicates the decrease of mitochondrial membrane potential, a hallmark of apoptosis at the early stage. Magnification 200x.

the contrary, the blockage of endogenous ROS by N-acetyl-L-cysteine (NAC), a potent ROS scavenger, moderately reduced endogenous ROS of H1299 cells following CPT and NPOA cotreatment (Figures 4(c) and 4(d)). The result suggests that the CPT and NPOA cotreatment induced apoptosis of H1299 cells through regulating endogenous ROS.

3.5. ROS Scavenger Attenuates CPT and NPOA Cotreatment-Induced Apoptosis of H1299 Cells. To determine whether the blockage of CPT and NPOA cotreatment-induced ROS production of H1299 cells after treatment with NAC reduces apoptosis, the H1299 cells were precultured with 2 mM NAC for 3 h, followed by CPT and NPOA cotreatment for 6 h. NAC significantly inhibited CPT and NPOA cotreatment-induced apoptosis and depolarization of MMP of H1299 cells (Figure 5). These results suggest that NPOA sensitizes CPT induction towards apoptosis of H1299 cells through modulating endogenous ROS. The results showed that a dramatical loss of MMP ($\Delta\Psi_m$) was induced by CPT and NPOA cotreatment in H1299 cells.

3.6. The Upregulation of JNK Phosphorylation and Caspase Activation in H1299 Cells following CPT and NPOA Cotreatment. To further explore the mechanism as to how NPOA increases CPT-induced apoptosis of H1299 cells, we investigated whether NPOA increased CPT-induced apoptosis-associated protein in H1299 cells. The requirement of JNK activation for mitochondrial-mediated apoptosis is well documented [31]. Next, we examined whether CPT and NPOA cotreatment induced the JNK phosphorylation of H1299 cells. As described in Figure 6(a), the Western blot analysis showed that CPT and NPOA cotreatment synergistically enhanced the JNK phosphorylation of H1299 cells compared to CPT treatment alone. On the contrary, we used JNK inhibitor, SP600125 (SP), to inhibit JNK activity and to evaluate the effect of JNK inhibition on apoptosis of H1299 cells. The inhibition of JNK activity attenuated CPT and NPOA cotreatment-induced mitochondrial membrane depolarization of H1299 cells (Figure 6(b)). The H1299 cells were precultured with JNK inhibitor (10 μ M) for 3 h, followed by cotreatment with CPT and NPOA for 48 h combining

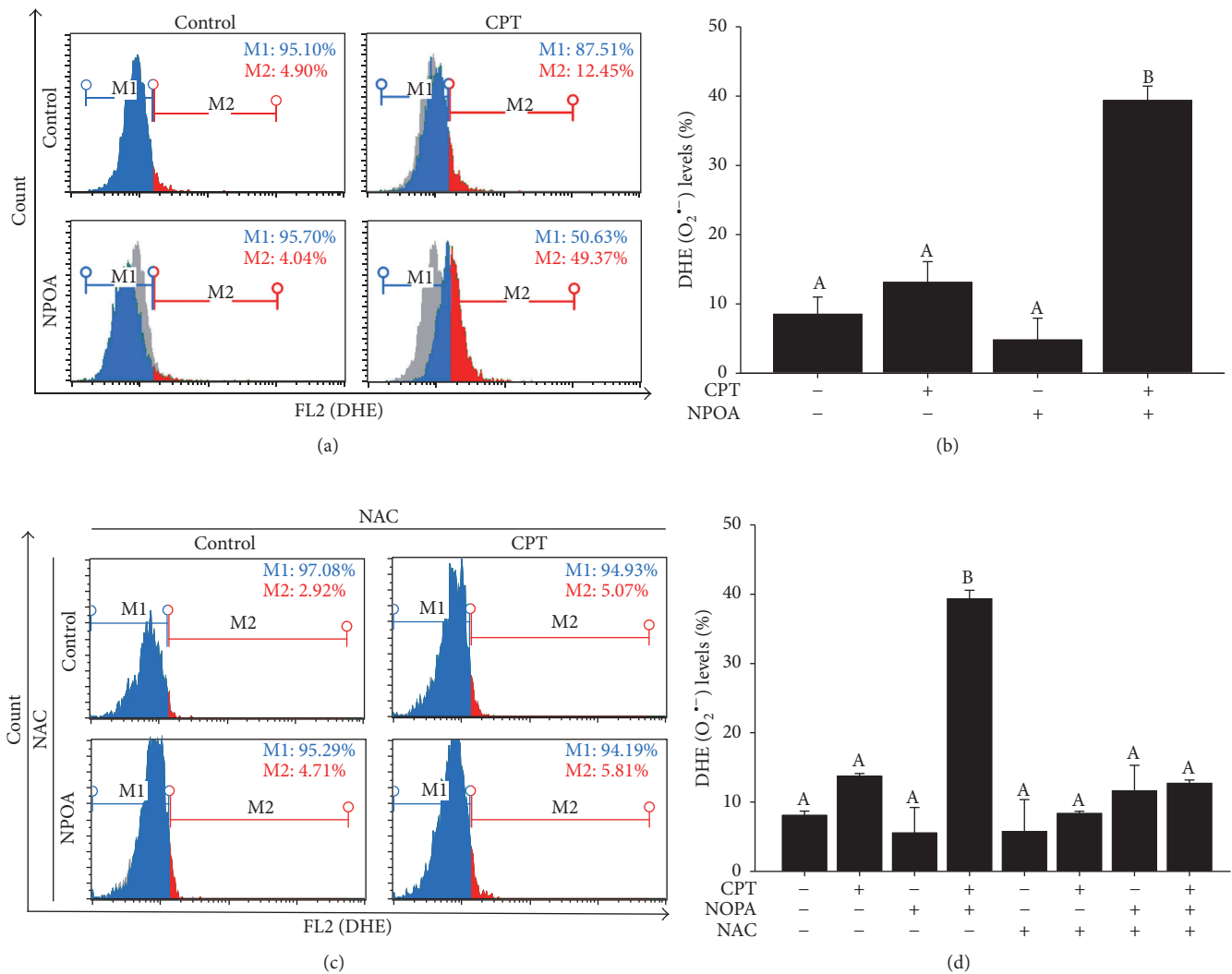


FIGURE 4: NPOA increased CPT-induced ROS production in H1299 cells. The cells indicate the concentration of CPT and NPOA alone or in combination for 6 h. (a) The levels of ROS production were determined by flow cytometer-based dihydroethidium (DHE) staining assay. (b) The quantification analysis of endogenous ROS. Data are presented as means \pm SD. (c) H1299 cells were pretreated with 2 mM NAC for 3 h before CPT alone or CPT and NPOA cotreatment. (d) The quantification analysis (c). Data are presented as means \pm SD (^A $p > 0.05$, ^B $p < 0.001$).

the flow cytometry-based analysis. The blockade of JNK attenuated CPT and NPOA cotreatment-induced apoptosis of H1299 cells (Figures 6(c) and 6(d)). Therefore, we suggest that NPOA enhanced CPT-induced mitochondrial-mediated apoptosis of H1299 cells through upregulating JNK activation. These results showed that the NPOA increased CPT-induced apoptosis of H1299 cells through upregulating JNK activation.

4. Discussion

Currently, the treatments for lung cancer include surgical resection, radiation therapy and chemotherapy [32]. The chemotherapeutic treatments, such as platinum-based or CPT-based chemotherapy, have been shown to prolong the survival rate of advanced NSCLC patients. However, the recurrence rate of NSCLC patients after chemotherapy

treatment is still up to 50% [33]. Furthermore, the acquired chemoresistant cancer cells are highly correlated with high recurrence and poor prognosis in these patients [34]. Combination chemotherapies, a combination of two or more chemotherapeutic drugs, are widely used to treat cancer, including lung cancer [35]. Unfortunately, the combination chemotherapies do not always enhance the efficacy of anti-cancer drugs. The combination of docetaxel and cisplatin has been reported to cause antagonism in treating NSCLC EBC-1 (squamous cell carcinoma) and RERF-LCMS cells (adenocarcinoma) [36]. Recently, the antagonism was also observed in 15 human NSCLC cell lines by the treatment of combining gefitinib and cisplatin [37].

In a comparison of combination chemotherapy, the chemosensitizer itself exerts low- or noncytotoxic effects but enhances the efficacy when cotreated with anticancer drugs. For instance, PZ-39, an acetamide-containing compound,

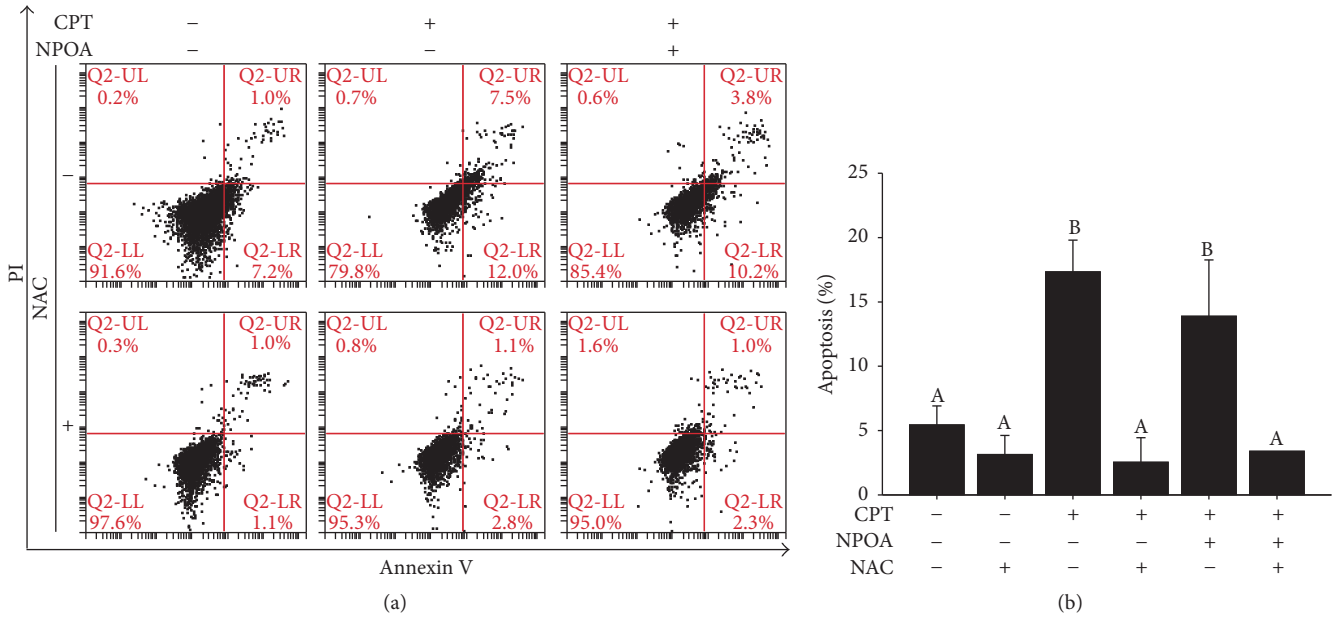


FIGURE 5: The ROS scavenger attenuated CPT and NPOA cotreatment-induced apoptosis of NSCLC cells. The H1299 cells were pretreated with 2 mM NAC for 3 h and then subject to cotreatment of CPT and NPOA for 24 h, as described in the Materials and Methods section. (a) The cells were double-stained with Annexin V and PI and analyzed by flow cytometer-based detection assay. (b) The quantitative analysis of (a). Data are presented as means \pm SD (^A $p > 0.05$, ^B $p < 0.001$). (c) The results of photography showed the JC-1 green fluorescence image and indicated that NAC rescued the loss of mitochondrial membrane potential of H1299 cells after CPT and NPOA cotreatment. Magnification 200x.

was shown to sensitize human breast cancer cell line MCF-7 towards mitoxantrone-induced cell killing [14]. Likewise, amiloride, an acetamide-containing compound, was reported to sensitize four tested human pancreatic cancer cell lines towards erlotinib-induced apoptosis through inhibiting the PI3K/AKT pathway [15]. Accordingly, chemotherapy sensitizer may reduce the risk of side effects and antagonism raised by combination chemotherapies and therefore benefit the cancer treatment.

In this study, we first examined whether an acetamide-containing compound NPOA enhanced the sublethal dose of CPT-induced anti-NSCLC using two NSCLC cells. Our results from trypan blue exclusion assay and Annexin V/PI analysis showed that the NPOA sensitized two NSCLC cells

towards CPT-induced antiproliferation and apoptosis (Figures 1 and 2). Interestingly, NPOA treatment alone did not exert significant cytotoxicity in two NSCLC cells, whereas a significant increase of Bax in H1299 cells following NPOA treatment was observed. Bax (Bcl-2-associated X protein), a proapoptotic protein of Bcl-2 family, is essential for regulation of apoptosis. For instance, Lalier et al. reported that expression of Bax sensitizes cells to mitochondrial-mediated apoptosis in cancer cells, including lung cancer [38]. We therefore investigated whether the CPT and NPOA cotreatment increased proapoptotic proteins of H1299 cells, including Bax, cleaved caspase 9 and cleaved caspase 3, and mitochondrial depolarization (Figure 3), suggesting that Bax may play a role in contributing to the synergism of CPT and

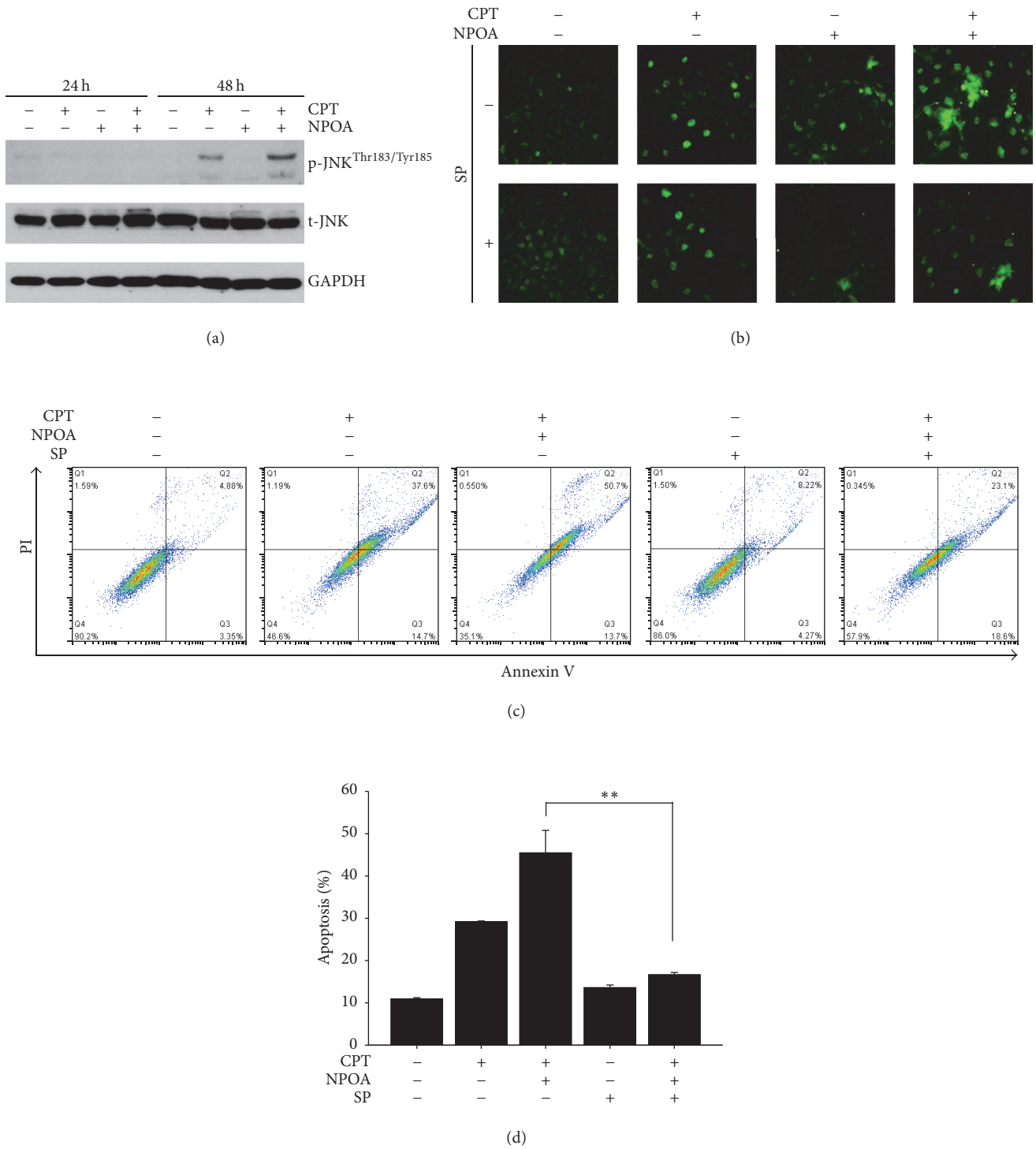


FIGURE 6: The CPT and NPOA cotreatment induced apoptosis through activating JNK activity. The cells were subject to treatment with vehicle control or the indicated concentration of CPT and NPOA alone or in combination for 24 h and 48 h, respectively. (a) Western blot showed that the CPT and NPOA cotreatment significantly increased levels of JNK phosphorylation (Thr¹⁸³/Tyr¹⁸⁵) for 48 h. (b) The photograph showed the JC-1 green fluorescence image and indicated that SP600125 (SP) rescued the loss of mitochondrial membrane potential of H1299 cells after CPT and NPOA cotreatment. (c) The effect of SP600125 on CPT and NPOA cotreatment-induced JNK activation in H1299 cells was determined using flow cytometer-based detection assay. The cells were pretreated with JNK inhibitor SP600125 for 3 h and then subject to the treatment of CPT and NPOA alone or their combination. (d) The quantitative analysis of (c). Data are presented as means ± SD (***p* < 0.001). Abbreviations: p-JNK indicates phosphorylation-ERK; t-JNK indicates total-ERK. SP indicates SP600125, a JNK inhibitor. GAPDH was measured as an internal control.

NPOA combination, and the low cytotoxicity of NPOA could be a promising chemosensitizer of CPT-based lung cancer treatment.

Because the synergistic effect of CPT/NPOA on the anti-proliferation and apoptosis can be observed in both A549 (*wild type-p53*) and H1299 (*null-p53*) cells (Figure 1), we suggest that the synergistic anti-lung-cancer induced by CPT/NPOA combination may be p53-independent. On the contrary, apoptosis can be induced by two typical signal cascades: the intrinsic (mitochondria-mediated) and the extrinsic (death receptor-mediated) pathways [39]. Fas/Apo-1 (CD95) is mainly involved in the pathway of extrinsic apoptosis; we conducted Western blot assay to determine whether the extrinsic pathway played a role in CPT/NPOA combination-induced apoptosis of lung cancer cells. The results of Western blot assay showed that neither protein level nor cleavage of caspase 8, an apical caspase of extrinsic apoptosis, was affected in both H1299 (Figure 2(c)) and A549 (data not shown). In contrast, CPT/NPOA combination caused the proteolytic activation of caspase-9, an apical caspase of mitochondria-mediated apoptosis in both NSCLC cells (Figure 2(c)). Accordingly, our present results suggest that CPT/NPOA combination-induced apoptosis of lung cancer cells seems to be through mainly mitochondria-mediated pathways. However, the results of our study should not rule out the possibility that other factors might be associated with extrinsic pathways such as caspase 10 being involved in CPT/NPOA-induced apoptosis.

CPT has been shown to inhibit topoisomerase and to induce DNA damage, causing the accumulation of cells at S phase. Therefore, the level of S phase arrest could be an index for the efficacy of CPT treatment [40]. Otherwise, CPT does not majorly arrest cell cycle at S phase, instead at G₁/S or G₂/M phases, resulting in attenuation of therapeutic outcome [41]. Our results showed that the accumulation of S phase for vehicle control, CPT alone, NPOA alone, and their combination were $9.03 \pm 0.71\%$, $10.95 \pm 0.49\%$, $11.85 \pm 0.92\%$, and $20.2 \pm 1.28\%$, respectively (Supplementary Figure 1), which were concordant with the activation of γ -H₂AX, a marker of DNA damage. The above-mentioned results suggest that NPOA might synergistically enhance the DNA damage induced by CPT through causing more S phase arrest compared to the treatment of CPT alone.

A previous study found that higher levels of endogenous ROS were detected in many chemoresistant cancer cells [39, 42]. Furthermore, chemoresistant cancer cells have been reported to exert a higher apoptotic threshold by anticancer drugs [43]. Moreover, these chemoresistant cancer cells seem to be more tolerant to increased endogenous ROS by upregulating antioxidant capacity [44]. Additionally, one of the promising strategies for treating chemoresistant cancer cells is through lowering the ROS-induced apoptosis threshold [45]. Therefore, the accumulation of endogenous ROS-generating cells could sensitize cancer cells towards antiproliferation and apoptosis. For example, doxorubicin synergistically enhances CPT-induced apoptosis of cervical carcinoma SiHa cells through upregulating ROS production [46]. It is well known that CPT and its derivatives also increase endogenous ROS of cancer cells, including lung

cancer [47] and breast cancer [48]. Besides, a previous study demonstrated that combining topotecan and vorinostat induced apoptosis of two small cell lung cancer (SCLC) cell lines (H209 and H526 cell) through increasing endogenous ROS [49].

Following this, we examined whether NPOA synergistically affected CPT-induced apoptosis of NSCLC cells through modulating ROS production. In this study, we found that NPOA significantly enhanced CPT-induced ROS production from $13.17 \pm 1.01\%$ to $39.44 \pm 0.09\%$ in H1299 cells (Figure 4). In contrast, our results confirmed that NAC, ROS scavenger, significantly attenuated CPT and NPOA cotreatment-induced ROS production and apoptosis of H1299 cells (Figures 4 and 5), suggesting that the synergistic effect of NPOA on CPT-induced apoptosis of H1299 cells might be through upregulating ROS production.

Endocytosis is a crucial form of active transport in which a cell uptakes molecules such as proteins and anticancer drugs including CPT [50]. Furthermore, previous studies reported that ion-base nanoparticles enhance the oxidative stress and apoptosis by increasing the uptake of ion-based nanoparticles possibly through endocytosis-dependent pathways [51, 52]. For example, zinc oxide nanomaterials enhanced ROS production and apoptosis in two colon cancer cells DLD-1 and SW480 through regulating the pathway of endocytosis [53]. Likewise, a silica nanoparticle of camptothecin was also suggested to enhance apoptosis of colon cancer HT-29 cells via the modulation of endocytosis [54]. Accordingly, we suggest that NPOA might improve the uptake of CPT through endocytosis to sensitize NSCLC H1299 cell towards CPT-induced oxidative stress and apoptosis.

The mitogen-activated protein kinases (MAPK) family is considered to be one of the major ROS-induced signal pathways and is involved in cellular survival, proliferation, and cell death [55]. Among the members of the MAPK family, c-Jun N-terminal protein kinase (JNK) is believed to regulate proapoptotic pathways and lead to cell death [56]. Conversely, the blockade of JNK may cause the resistance of cancer cells towards chemotherapy. For example, Suzuki et al. found that the JNK blockade attenuates two anticancer drugs 5-fluorouracil- (5-FU-) and gemcitabine- (GEM-) induced apoptosis of pancreatic cancer cells [57], suggesting the proapoptotic role of JNK. Therefore, we ought to examine whether the effect of CPT and NPOA cotreatment on apoptosis of NSCLC cells is through upregulating JNK activation (phosphorylation at sites Thr¹⁸³ and Tyr¹⁸⁵). In our result, JNK was moderately phosphorylated in CPT-treated H1299, whereas CPT and NPOA cotreatment dramatically increased the phosphorylation of JNK in H1299 cells (Figure 6(a)). Otherwise, the blockage of JNK largely decreased CPT and NPOA cotreatment-induced apoptosis (Figure 6(b)), suggesting that the sensitizer role of NPOA in CPT-induced apoptosis of H1299 cells was through modulating the activation of JNK.

5. Conclusions

Taken together, our present results suggest that NPOA sensitizes NSCLC cells towards CPT treatment via upregulating both endogenous ROS and JNK activation, resulting in the

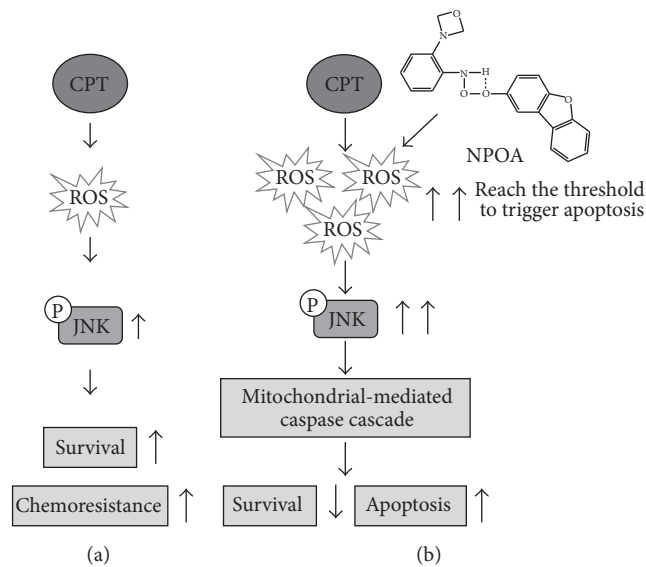


FIGURE 7: The proposed model shows NPOA sensitized CPT-induced apoptosis of NSCLC cells. (a) With CPT treatment alone, the increased level of ROS does not reach the threshold of apoptosis, whereas CPT-induced JNK activation favors prosurvival, causing an acquired chemoresistance. (b) CPT and NPOA cotreatment dramatically increases the endogenous ROS, JNK activation, and the upregulated expression of pro-apoptotic protein Bax, eventually initiating the mitochondrial-mediated caspase cascade and enhancing the apoptotic population of NSCLC cells.

increase of S phase accumulation and lowering the threshold of apoptosis (Figure 7). Accordingly, the acetamide derivative NPOA might be a promising sensitizer to CPT-based lung cancer therapy in the future and is worthy of further investigation.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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