Human non-small cell lung cancer cells can be sensitized to camptothecin by modulating autophagy

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Abstract. Lung cancer is a prevalent disease and is one of the leading causes of mortality worldwide. Despite the development of various anticancer drugs, the prognosis of lung cancer is relatively poor. Metastasis of lung cancer, as well as chemoresistance, is associated with a high mortality rate for patients with lung cancer. Camptothecin (CPT) is a well-known anticancer drug, which causes cancer cell apoptosis via the induction of DNA damage; however, the cytotoxicity of CPT easily reaches a plateau at a relatively high dose in lung cancer cells, thus limiting its efficacy. The present study demonstrated that CPT may induce autophagy in two human non-small cell lung cancer cell lines, H1299 and H460. In addition, the results of a viability assay and Annexin V staining revealed that CPT-induced autophagy could protect lung cancer cells from programmed cell death. Conversely, the cytotoxicity of CPT was increased when autophagy was blocked by 3-methyladenine treatment. Furthermore, inhibition of autophagy enhanced the levels of CPT-induced DNA damage in the lung cancer cell lines. Accordingly, these findings suggested that autophagy exerts a protective role in CPT-treated lung cancer cells, and the combination of CPT with a specific inhibitor of autophagy may be considered a promising strategy for the future treatment of lung cancer.

Introduction

Lung cancer is one of the leading causes of mortality worldwide. Even though numerous anticancer drugs have been developed, it remains a challenge to effectively treat lung cancer without increasing chemoresistance. Non-small cell lung cancer (NSCLC) is a type of lung cancer that is further classified into three histopathological subtypes: Adenocarcinoma, squamous cell carcinoma and large-cell carcinoma (LCC) (1). NSCLC accounts for 80% of lung cancer cases, and of these, ~10% are LCC (2). LCC occurs in the lungs and is characterized by poor differentiation, rapid growth and early metastasis (3,4). These cancers also have an aggressive pattern and are associated with a poorer prognosis. The 5-year survival rate for patients with stage III/IV LCC is <10% (5). Although LCC of the lung is a relatively uncommon type of lung cancer, a better understanding of the molecular and cellular biology of LCC, the mechanisms underlying LCC drug resistance and the possible molecular pathway associated with autophagy-mediated drug resistance may identify more effective NSCLC therapeutic strategies and targets. Therefore, the present study used two LCC cell lines, H1299 and H460.

Camptothecin (CPT), which is a natural compound originally derived from the Asian tree *Camptotheca acuminata*, was synthesized by Wall and Wani in 1966 (6). CPT is able to form a stable tertiary structure with DNA and topoisomerase I, thus resulting in formation of the topoisomerase I-CPT complex, which inhibits topoisomerase I and results in DNA damage.
damage and cell death (7). Recent studies have revealed that CPT and its derivatives exert broad antitumor activity against various tumors cells in vitro and in vivo. CPT and its derivatives have been reported to exhibit anticancer activities against various types of cancer cells, including lung cancer (8), gastric cancer (9), esophageal cancer (10), colorectal cancer (11) and breast cancer (12). Oral topotecan has been used as a second-line medication for the treatment of metastatic ovarian cancer (13) and small-cell lung cancer (14). Furthermore, irinotecan-based chemotherapy improves overall response rate, overall survival and progression-free survival, and has been recommended as a first-line treatment in Asian patients with stage IIIIB/IV NSCLC (15).

Although the clinical use of CPT derivatives has exhibited efficacy in the treatment of the aforementioned types of cancer, de novo and developed clinical resistance to these drugs is common. The mechanism underlying the resistance of cancer cells to CPT-based anticancer drugs remains to be fully elucidated, since the resistance and selectivity towards cancer cells is multifactorial. Sugimoto et al revealed that quantitative reduction of topoisomerase I content contributes to the most frequently occurring events in the development of resistance to CPT in various tumor cell lines (16). In addition, a previous study indicated that the increased expression of ATP-dependent drug transporters, such as ATP-binding cassette subfamily C member 4 and ATP-binding cassette subfamily G member 2 (ABCG2), is sufficient to confer resistance of lung cancer cells to the CPT-based anticancer drugs irinotecan and topotecan (17). Furthermore, it has been reported that breast cancer induces resistance to topotecan and irinotecan via regulation of the cell cycle and DNA repair activity (18). Although numerous novel therapies have been developed, the prognosis of patients has not significantly improved, and chemoresistance is one of the main reasons for the low survival of patients with lung cancer. CPT derivatives are also affected by chemoresistance during the treatment of lung cancer. For example, an increase in ABCG2 expression is often correlated with irinotecan and topotecan resistance, and may result in clinical failure in patients with advanced NSCLC (19). Therefore, the present study applied CPT as a model to determine the mechanisms underlying chemoresistance in NSCLC cells.

Autophagy is a cellular degradation response to various types of stress, including starvation, hypoxia, reactive oxygen species (ROS) and DNA damage (20,21). Membrane receptors receive signals, which are communicated to the cell interior, thus resulting in activation of autophagy, which degrades dysfunctional proteins and organelles, in order to yield more energy for adaptation to adverse environments and avoid cell apoptosis (22). Therefore, cell fate depends on the association between apoptosis and autophagy. According to previous studies, when cells are treated with chemotherapy, autophagy serves a major role in chemoresistance (23). For example, celecoxib is able to suppress autophagic flux by preventing lysosome function, and strengthens the cytotoxicity of imatinib in imatinib-resistant myeloid leukemia cells (24). In addition, human epidermal growth factor receptor 2-overexpressing breast cancer cells exposed to trastuzumab exhibit increased autophagy, and protect breast cancer cells from the inhibitory effects of trastuzumab. Conversely, the blockade of autophagosome formation/function significantly enhances the growth inhibitory activity of trastuzumab in trastuzumab-refractory breast cancer cells (25). These findings suggest that inhibiting autophagy may be a novel target for increasing drug effects.

The present study aimed to examine the effects of CPT on cell viability, migration, apoptosis and autophagy in the H1299 NSCLC cell line. The results demonstrated that CPT exerted limited cytotoxic and anti-metastatic effects on H1299 cells. In addition, apoptosis and DNA damage were not increased following CPT dose accumulation. However, CPT induced the increased formation of autophagosomes in the H1299 NSCLC cell line in a dose-dependent manner. Furthermore, the present study revealed that the autophagy inhibitor, 3-methyladenine (3-MA), was able to suppress CPT-induced autophagy. The results demonstrated that 3-MA enhanced the cytotoxicity of CPT in CPT-resistant H1299 cells. Accordingly, 3-MA may serve as a novel agent to enhance the antitumor activity of conventional therapeutic agents in CPT-resistant H1299 cells.

Materials and methods

Cell culture. Human NSCLC cell lines H1299 and H460 were generously provided by Dr K. Fang (National Taiwan Normal University, Taipei, Taiwan). H1299 and H460 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 μg/ml streptomycin and 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.), at 37°C in a humidified incubator containing 5% CO₂. Cell morphology was observed under an inverted light microscope.

Source of CPT and 3-MA, and half maximal inhibitory concentration (IC₅₀) values of CPT. CPT and 3-MA were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). CPT was dissolved in dimethyl sulfoxide (DMSO) to form a stock concentration of 5 mM, and 3-MA was freshly dissolved in ddH₂O at 20 mM. Cells were treated with 0.5, 1, 2 and 5 μM CPT, and co-treated with 0.1, 0.5, 1 and 5 mM 3-MA for 24 h at 37°C in a humidified incubator containing 5% CO₂. Cell morphology was observed under an inverted light microscope.

Cell viability assay. A cell viability assay was performed as described previously with slight modifications (26). Briefly, 5x10⁴ H1299 and H460 cells/well were seeded in 12-well plates, and were treated with DMSO as a vehicle or with various concentrations of CPT for 24 h, respectively. Subsequently, the
cells were suspended in 0.2% trypan blue and counted using a Countess automated cell counter (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

**Cell migration assays.** The migration of H1299 and H460 cells was determined using wound-healing assays as previously described (27). Briefly, for wound-healing assays, 3x10^5 H1299 and H460 cells/well were seeded in 12-well plates, and were grown overnight until they reached ~95% confluence, after which wound gaps were generated using a sterile pipette tip. Cellular debris was removed with PBS and cells were incubated in medium containing 0.5, 1, 2, and 5 µM CPT. The wound-healing ability of the cells was documented after 16 h using the Nikon Eclipse TE2000U microscope (Nikon Corporation, Melville, NY, USA). The migration distance was assessed using TScratch software, version 1.0 (MathWorks Inc., Natick, MA, USA). The migration rate was calculated according to the relative cell migration area for each treatment.

**Annexin V staining.** H1299 and H460 cells (1x10^5 per well in a 6-well plate) were collected following incubation with CPT and/or 3-MA for 24 h. The cells were washed with PBS and were then resuspended in Annexin V binding buffer [140 mM NaCl, 10 mM HEPES-NaOH (pH 7.4) and 2.5 mM CaCl_2]. Following 300 x g centrifugation for 5 min, the cells were incubated with Annexin V binding buffer containing 1.25 ml fluorescein isothiocyanate (FITC)-conjugated Annexin V and PI (BD Pharmingen; BD Biosciences, San Jose, CA, USA) at room temperature for 15 min in the dark. Data acquisition and analysis were performed using the BD Accuri™ C6 flow cytometer with BD Accuri™ C6 software version 1.0.264.21 (both from BD Biosciences).

**Western blot analysis.** A total of 1x10^6 cells per 10 cm dish were treated with the indicated concentrations of CPT and/or 3-MA for 24 h. Cells were lysed with radioimmunoprecipitation assay lysis buffer (cat. no. 20-188; EMD Millipore, Billerica, MA, USA) containing 0.05 M Tris-HCl (pH 7.4), 0.15 M NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA and a protease inhibitor mixture (Sigma-Aldrich; Abcam), human β-actin (1:8,000, cat. no. 612656; BD Biosciences) and human GAPDH (1:8,000, cat. no. YH80536; Yao-Hong Biotechnology, Inc., New Taipei City, Taiwan). Each membrane was incubated with appropriate primary antibodies to 4°C overnight and washed with PBS containing Tween. After washing with PBS with 0.5% Tween, blots were incubated with a 1:10,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse (cat. no. #20102; Leadgene Biomedical, Inc., Taiwan, Taiwan) or goat anti-rabbit IgG (cat. no. #20202; Leadgene Biomedical, Inc.) antibodies for 1.5 h at room temperature. The signals of specific proteins were detected using a Western Bright ECL HRP substrate kit (Advansta, Inc., Menlo Park, CA, USA).

**Flow cytometric detection of γH2AX.** Briefly, cells were collected and fixed in 70% ethanol at -20°C overnight. Prior to flow cytometry, the ethanol was aspirated, and cells were rinsed with PBS and incubated with anti-γH2AX (1:100; cat. no. sc-101696; Santa Cruz Biotechnology, Inc.) dissolved in PBS containing 1% bovine serum albumin (BSA; cat. no. 101-9048-46-8; MDBio, Inc., Taipei, Taiwan) and 0.5% Triton (BSA-T-PBS) at 4°C overnight. The cells were then incubated with FITC-conjugated anti-mouse immunoglobulin G (1:500, cat. no. GTX26816; Genetex, Inc.) dissolved in BSA-T-PBS for 1 h at 4°C in the dark. The cells were then rinsed and suspended in 1 µl 20 µg/ml propidium iodide (PI) containing RNase for 20 min at 37°C, and FITC (γH2AX) and PI (DNA content) were quantified using BD Accuri™ C6 flow cytometer with BD Accuri™ C6 software (both from BD Biosciences).

**Immunofluorescence detection of γH2AX nuclear foci.** H1299 cells (3x10^4/well) grown on a 24-well plate were exposed to various concentrations of 3-MA in the presence of 0.5 µM CPT for 24 h at 37°C. The cells were then washed with PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. The fixed cells were washed twice with PBS, permeabilized with 0.5% Triton-PBS, blocked with 1% BSA-PBS and incubated for 2 h with anti-γH2AX mouse monoclonal antibodies (cat. no. sc-101696; Santa Cruz Biotechnology, Inc.). The antibodies were then washed off.
and cells were rinsed three times in 0.5% Triton-PBS and incubated for 1 h in the dark with a FITC-conjugated anti-mouse IgG secondary antibody (1:500, cat. no. GTX26816; Genetex, Inc.). Cells were then rinsed twice in 0.5% Triton-PBS and the cells were stained with 0.5 µg/ml DAPI (cat. no. 32670; Sigma-Aldrich; Merck KGaA) for 5 min. Images were captured using a fluorescence microscope (TE2000-U; Nikon Corporation).

Fluorescence microscopy. The green fluorescent protein (GFP) fluorescence-tagged LC3 (LC3-GFP) construct was kindly provided by Dr Wei-Pang Huang (Department of Life Science, National Taiwan University, Taipei, Taiwan) (29). H1299 and H460 cells were seeded onto 6-well microplates and transfected with 1 µg wild-type or mutant pEGFP-LC3 constructs using Lipofectamine® and PLUS™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to a previous study with minor modifications (29). A total of 48 h post-transfection, the medium was replaced with selective medium containing 0.4 mg/ml (for H460 cells) and 0.8 mg/ml (for H1299 cells) Geneticin selective antibiotic (G418 Sulfate; cat. no 10131035; Gibco; Thermo Fisher Scientific, Inc.). Surviving colonies were picked 2 weeks later and amplified. Stable transfectants were examined for LC3-GFP expression and used in relevant experiments. Cells were seeded onto a cover glass slide chamber (Nunc™ Lab-Tek™; Thermo Fisher Scientific, Inc.), and after the designated treatments, the cells were examined under a Nikon Eclipse TE2000U fluorescence microscope (Nikon Corporation). The GFP-LC3 puncta were quantified by counting the number of cells as described previously (29,30). Briefly, the GFP-LC3 puncta in a single cell were manually counted under a fluorescence microscope. For each group, 50 cells were randomly selected to determine the average number of GFP-LC3 puncta/cells. The presented results were selected from experiments performed at least three times.

Statistical analysis. All experiments were performed at least in triplicate. Data were analyzed by multivariate analysis of variance. If a significant difference was found, a Holm-Sidak multiple comparison test was used to identify significant groups. Statistical analyses were conducted using SigmaPlot software version 12.0 (Systat Software, Inc., San Jose, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

IC50 values in NSCLC cell lines. The present study was conducted using two LCC cell lines, H1299 (p53-deleted, p53null) and H460 (wild-type p53-expressing, p53wt), since CPT is a recommended therapeutic treatment for advanced NSCLC (31). The IC50 values of CPT were initially determined in H1299 and H460 cells prior to examining the antitumor effects of CPT and the CPT-resistant effects of H1299 and H460 cells. The determined IC50 for CPT was 1 µM in H1299 cells and 1 µM in H460 cells (data not shown).

CPT failed to induce a cytotoxic effect on drug-resistant H1299 and H460 cell lines in a dose-responsive manner. Since CPT is able to inhibit tumor cell viability and metastasis of lung carcinoma cells (32) the present study aimed to determine its molecular/cellular mechanism. H1299 and H460 lung carcinoma cells were incubated for 24 h in the presence of various concentrations of CPT. As shown in Fig. 1A, the trypan blue assay demonstrated that CPT damaged cell viability of H1299
and H460 cells. In H1299 cells, the percentage of viable cells decreased from 100±1.8% in the untreated group to 49.2±3.9, 47.3±11.4 and 39.2±4.6% in the presence of 0.5, 1, 2 and 5 µM CPT, respectively. In H460 cells, the percentage of viable cells decreased from 100±0.0% in the untreated group to 47.4±4.9, 44.4±4.5, 47.0±3.3 and 39.9±0.5% in the presence of 0.5, 1, 2 and 5 µM CPT, respectively; therefore, the susceptibility of the two lung carcinoma cells to CPT was considered similar.

Most types of cancer harbor significant patterns of relapse following treatment due to evolved resistance. It has been reported that cancer cells exist due to their resistance to cell death and the loss of their ability to undergo apoptosis-induced death, thus leading to uncontrolled proliferation (33). Evasion of apoptosis may contribute to tumor development and progression, and to treatment resistance, since the majority of anticancer therapies that are currently available, including CPT-based chemotherapy, primarily act by activating the apoptotic pathway in NSCLC (8). A better understanding of the pathway underlying tumor resistance to apoptotic cell death is required, in order to provide understanding of the molecular mechanisms underlying development of resistance to targeted therapy. Therefore, the present study detected apoptosis and DNA-damage-related protein expression in H1299 cells treated with various concentrations of CPT. The phosphorylation of H2AX at Ser139, resulting in the formation of γH2AX puncta in the nuclei, is an early event in the cellular response to DNA damage. The expression levels of γH2AX were detected in order to evaluate DNA damage of H1299 cells. As shown in Fig. 1B, the results of western blotting indicated that, in the presence of 0.5 µM CPT, the expression levels of the DNA damage biomarker γH2AX were increased, as were the expression levels of cleaved caspase-9 and caspase-3. Furthermore, 1 µM CPT increased B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax) and cleaved caspase-3 expression (Fig. 1B). These results indicated that low dosages of CPT induced apoptosis and DNA damage of H1299 cells; however, the protein expression levels of γH2AX, Bax, cleaved caspase-9, cleaved caspase-3 and cleaved poly (ADP-ribose) polymerase (PARP) were not markedly increased by CPT in a dose-dependent manner. These observations suggested that H1299 NSCLC cells may eventually become resistant in response to higher concentrations of CPT. Therefore, 0.5 µM CPT may be considered a reasonable dose due to its significant effect on cell viability; cells were treated with 0.5 µM CPT in subsequent experiments.

The present study also determined the effects of various concentrations of CPT on the migration of H1299 and H460 cells using wound-healing assays. In the control group, the cells migrated into the wound area and the wound edges became indistinguishable; however, following the addition of CPT, cells exhibited slower wound healing (Fig. 2A). In response to various concentrations of CPT, H1299 cell migration was inhibited from 100.0±8.4% in the control group, to 63.5±9.9, 58.7±6.8, 55.8±4.0 and 54.6±4.6% in response to 0, 0.5, 1, 2 and 5 µM CPT, respectively (Fig. 2B). The percentage of cell viability and wound healing was significantly decreased in H1299 cells in the presence of 0.5-5 µM CPT compared with in the control group (P<0.05). However, there was no alteration in the percentage of cell viability and wound healing following CPT dosage accumulation, thus indicating that treatment did not affect growth and metastasis; in addition, the inhibitory effects of CPT on H1299 cells reached a plateau. Consistent with the results in H1299 cells, H460 cells were treated with the indicated concentrations of CPT for 24 h, after which the migration rate of cells was significantly reduced compared with in the control group; however, no change in migration was detected as the CPT dosage accumulated (Fig. 2). These observations supported the finding that higher concentrations of CPT (1, 2 and 5 µM) exhibited no further inhibitory effect on cell viability and metastasis compared with the lower CPT concentration (0.5 µM) on H1299 and H460 NSCLC cells.

A recent study suggested that when treated with chemotherapeutic agents, certain tumor cells fail to undergo

Figure 2. (A) Migration of H1299 and H460 cells following CPT treatment for 16 h was determined using a wound-healing assay. Magnification, x100. (B) Quantification of the wound-healing assay results. Data are presented as the means ± standard deviation of at least three independent experiments. a vs. a, P>0.05; a vs. b, P<0.05. CPT, camptothecin.
apoptosis and instead undergo autophagy followed by delayed cell death (34). To determine whether autophagy is associated with the suppression of CPT-induced apoptotic cell death, the autophagic marker phosphatidylethanolamine-LC3 (LC3-II) was detected in H1299 cells in the presence of 0.5 μM CPT. As shown in Fig. 3A and B, 1.4±0.5 and 6.0±0.9 GFP-LC3 puncta were detected in H1299 cells in the presence of 0 and 0.5 μM CPT, respectively. These findings indicated that supplementation with 0.5 μM CPT may lead to an enrichment of GFP-LC3 protein in H1299 cells (P<0.05), thus suggesting that H1299 cells respond to CPT by activating autophagy. Similar effects were detected on H460 NSCLC cells (Fig. 3C and D).

Since the present study demonstrated that CPT increased autophagy in H1299 cells, it was hypothesized that alterations in cell survival may be due to autophagy. The protein expression levels of mammalian target of rapamycin (mTOR), Atg3, LC3B, SQSTM/p62 and LAMP2, which are involved in the autophagic process, were also examined by western blot analysis (Fig. 4). The expression levels of Atg3 were slightly increased in response to 0.5, 1 and 2 μM CPT, and appeared the same in the the control and 5 μM CPT groups. Decreases in the expression levels of mTOR and p62 were detected in response to various CPT concentrations. LAMP2 was increased in response to 0.5 and 1 μM CPT, and was only slightly decreased in response to 5 μM CPT. Furthermore, the expression levels of the protein LC3B-I (an unprocessed form of LC3) and the cleaved protein LC3B-II (lipidated and autophagosome-associated form of LC3) were markedly increased in H1299 cells following CPT treatment at various concentrations compared with in the control group (Fig. 4).

These findings were consistent with detection of the increased formation of autophagosomes, and suggested that CPT may enhance autophagy in an H1299 NSCLC cell lines in a dose-dependent manner.

3-MA blocks CPT-induced autophagy in H1299 lung cancer cells in a dose-dependent manner. To determine the role of
autophagy in CPT-treated NSCLC, the present study cotreated H1299 cells with various concentrations of the autophagy inhibitor 3-MA and 0.5 µM CPT, in order to determine whether 3-MA blocked CPT-induced autophagy. As shown in Fig. 5, 3-MA had an effect on CPT-induced autophagy; treatment with CPT alone resulted in 5.91±0.9 GFP-LC3 puncta, whereas 3-MA induced a significant dose-dependent decrease in autophagy in H1299 cells (P<0.05). To further validate the inhibitory effects of 3-MA on CPT-induced autophagy, activation of LC3B and the expression of autophagic proteins were analyzed by western blotting. As shown in Fig. 6, 3-MA treatment reduced the accumulation of LC3B induced by 0.5 µM CPT, but exhibited no marked effects on SQSTM/P62 and LAMP2 (Fig. 6). The ratio of LC3BII/I was decreased in a dose-dependent manner; the ratio of LC3BII/I was 3.8 and 2.3 in response to 0.1 and 5 mM 3-MA, respectively. These findings indicated that 3-MA blocked CPT-induced autophagy in H1299 lung carcinoma cells.

3-MA enhances the anticancer effect of CPT via autophagy inhibition. As shown in Fig. 7A, the percentage of viable H1299 cells was 56.8±3.1, 47.3±4.3 and 22.3±5.3% following treatment with 0, 0.5 and 1 mM 3-MA, in the presence of 0.5 µM CPT, respectively, as opposed to 100.0±0.2% in the control group and 94.2±4.0 % in the 1 mM 3-MA group. Once CPT-induced autophagy was blocked, the cytotoxic effects of CPT were enhanced in H1299 cells. Furthermore, suppressing autophagy increased apoptosis, as determined using the Annexin V/PI staining assay. Following treatment with vehicle control, or 0, 0.5, 1 and 5 mM 3-MA in the presence of 0.5 µM CPT for 24 h, apoptosis was detected (Fig. 7B). In response to CPT, the percentage of apoptotic H1299 cells was 4.0±0.1%; however, in response to CPT and 3-MA cotreatment, the percentage of apoptotic cells was 19.0±1.13, 18.8±0.35 and 15.4±0.35% following 3-MA accumulation (Fig. 7B).

The present study indicated that there was a slight increase in apoptosis in the CPT group; however, in cells receiving CPT and 3-MA cotreatment apoptosis was significantly increased compared with in those receiving CPT only (P<0.05; Fig. 7B). These results suggested that, following cotreatment with 3-MA, CPT may efficiently induce apoptosis of CPT-resistant H1299 cells and CPT-induced autophagy may be suppressed. Since the major mechanism underlying CPT-induced cellular cytotoxicity and apoptosis is induction of DNA damage, the present study examined whether blocking autophagy affected the anti-lung cancer effects of CPT. As shown in Fig. 8, the expression levels of γH2AX were increased in response to CPT and 3-MA cotreatment compared with in cells treated with CPT or 3-MA alone. In addition, the expression levels of the apoptosis-associated protein caspase-9 were detected by western blotting. The results indicated that activation of caspase-9 was enhanced in response to the suppression of CPT-induced autophagy by 3-MA.
Effects of a pan-caspase inhibitor. H1299 NSCLC cells were pretreated with or without the pan-caspase inhibitor Z-VAD-FMK for 2 h prior to treatment with CPT/3-MA for 24 h. Subsequently, cell viability was analyzed; the results indicated that pretreatment with the caspase inhibitor did not significantly affect the 3-MA-enhanced cytotoxic effects on CPT-treated H1299 cells (Fig. 9). These results indicated that 3-MA-enhanced cell death of NSCLC cell lines may not predominantly occur via a caspase-dependent pathway.

CPT-induced DNA damage is increased following autophagy blockade. To further demonstrate that the observed DNA-damaging effects were caused by blocking CPT-induced autophagy, γH2AX foci formation was analyzed in H1299 cells (Fig. 10A), and a γH2AX/PI double staining assay was conducted in H1299 and H460 cells (Fig. 10B). γH2AX foci are generally regarded as markers of DNA double-strand breaks (35); therefore, increases in γH2AX foci indicate CPT-induced DNA damage in H1299. Notably, DNA damage was enhanced in H1299 and H460 cells in response to CPT and 3-MA cotreatment, and the effects of 3-MA on DNA damage were concentration-dependent.

Discussion

Drug resistance or chemoresistance is a phenomenon whereby cells undergo adaptive mutation under environmental stress (36). Chemoresistance disrupts anticancer drug actions and renders therapy ineffective. Chemoresistance is induced by numerous factors, including inhibition of drug absorption (37), epigenetic modifications (38), cell cycle alterations (39) and inhibition of apoptosis (40). Accordingly, one of the critical factors mediating chemoresistance is inhibition of cell apoptosis. To understand whether inhibition of apoptosis is attributed to a plateau of the cytotoxic effects of CPT, apoptotic factor expression was detected by western blot analysis. Notably, low doses CPT increased caspase activation, decreases in the activation of caspase-3 and caspase-9 were detected in cells following treatment with high doses of CPT. In addition, other apoptotic factors, and the DNA damage marker γH2AX, were also similarly reduced following treatment with increasing doses of CPT. These data indicated that when lung cancer H1299 cells were treated with a relatively
Despite the initial high responsiveness of cells to CPT and its derivatives (8-14), cancer cells often develop acquired resistance following treatment (41), which significantly limits the therapeutic efficacy of CPT. Methods for reversing CPT resistance are currently lacking; therefore, a better understanding of the mechanisms underlying such resistance is essential. A reduction in apoptotic cell death may be one of the factors that mediate chemoresistance, and the present study revealed that high concentrations of CPT may diminish apoptotic death and DNA damage in H1299 and H460 NSCLC cell lines.

In our previous studies, it was demonstrated that some chemosensitizers could reduce antagonism and sensitize cancer cells to CPT, thus enhancing CPT-induced anticancer effects under the IC₅₀ concentration (0.5 µM) (8,42). In the

Figure 10. 3-MA treatment enhances γH2AX foci formation in CPT-resistant H1299 and H460 cells. (A) γH2AX foci (green fluorescence) formation was detected in H1299 cells following 24-h exposure to various concentrations of 3-MA in the presence of 0.5 µM CPT. Nuclei were stained blue by DAPI. Magnification, x200. (B) Analysis of γH2AX intensity in H1299 (left panel) and H460 (right panel) cells stained with anti-γH2AX-fluorescein isothiocyanate antibody and propidium iodide in the presence or absence of 5 µM 3-MA and 0.5 µM CPT, as determined by flow cytometry. Percentage of γH2AX-positive cells was quantified. Data are presented as the means ± standard deviation of at least three independent experiments. *P<0.05.

γH2AX, phosphorylated-H2A histone family, member X (Ser¹³⁹); 3-MA, 3-methyladenine; CPT, camptothecin.
present study, there was no marked alteration in H1299 and H460 cell viability, wound closure, DNA damage and apoptotic cell death between 0.5 and 1 µM CPT treatment. Conversely, CPT-induced formation of autophagosomes was increased and regulation of the expression of autophagy marker proteins was affected in H1299 and H460 lung carcinoma cells. As indicated by the reduction in apoptotic protein expression and the increase in autophagy at higher CPT concentrations, it was suggested that H1299 and H460 cells may eventually become less sensitive to higher concentrations of CPT. Since higher concentrations of CPT (>IC$_{50}$; 1 µM) did not exhibit an increased anticancer effect, a lower dose of CPT (0.5 µM) was considered a reasonable dosage for subsequent experiments.

Numerous studies have reported that autophagy induces chemoresistance against anticancer drugs by inhibiting apoptosis of cancer cells (23,43). One suggested strategy to overcome acquired resistance to CPT and its derivatives is the inhibition of autophagosome formation (44,45). A recent study demonstrated that defective autophagosome formation in p53$^{mut}$ colorectal cancer may enhance drug-induced apoptosis (46). In the present study, the antitumor effects of CPT were detected on both H460 p53$^{wt}$ and H1299 p53$^{null}$ lung cancer cell lines. A similar pattern was detected in p53$^{null}$ H1299 and p53$^{wt}$ H460 cells with regards to cell death, cell migration and autophagy in the presence of CPT; therefore, it may be suggested that CPT-mediated antitumor effects occur independently of p53 expression. On the basis of these results, further studies are required to verify the essential role of defective autophagosome formation in CPT-induced apoptosis in other NSCLC lines, such as p53$^{wt}$ A549, and p53-mutated CL1-0 and CL1-5 lung adenocarcinoma cells.

To understand the role of autophagy in CPT-treated NSCLC cells, in the present study H1299 cells were cotreated with various concentrations of 3-MA and 0.5 µM CPT, and the effects of 3-MA on the suppression of CPT-induced autophagy were confirmed. 3-MA is an inhibitor of phosphatidylinositol 3-kinase (PI3K), which serves an essential role in controlling the activation of mTOR and the regulation of autophagy. The group of PI3K inhibitors includes 3-MA, wortmannin and LY294002. Among them, wortmannin is able to suppress autophagy regardless of nutrient status; however, 3-MA has been revealed to promote autophagy flux when used under nutrient-rich conditions for a prolonged period of time, whereas it is still capable of suppressing starvation-induced autophagy (47). Due to the dual roles of 3-MA in autophagy, it has been widely used as an autophagy inhibitor in various types of cancer therapy (48-50). The present study confirmed that 3-MA significantly inhibited cytoprotective autophagy in H1299 cells in a dose-dependent manner. The results of western blotting further revealed a marked decrease in LC3B expression in response to 3-MA, which may be associated with the reduction in autophagosome formation.

Since CPT-induced autophagy was suppressed by 3-MA, inhibition of H1299 cells was enhanced; in particular, apoptosis of H1299 cells was increased, as determined by trypan blue dye exclusion assay and Annexin V/PI staining. To further confirm that the observed apoptotic effects were produced by blocking CPT-induced autophagy, the expression levels of mitochondrial
apoptotic proteins, including Bax, Bcl-2, caspase-3, caspase-9 and PARP, were detected via western blotting. The results demonstrated that activation of caspase-9 was enhanced in response to inhibition of CPT-induced autophagy by 3-MA. However, there was no significant alteration in the expression levels of Bax, caspase-3 and PARP in response to cotreatment with CPT and 3-MA (data not shown). Conversely, a marked decrease in the protein expression levels of Bcl-2 was detected following 3-MA dosage accumulation in the presence of 0.5 µM CPT (data not shown). Therefore, the elevated Bax/Bcl-2 ratio indicated that 3-MA enhanced the susceptibility of H1299 cells to autophagy-inhibited apoptosis. These results suggested a significant improvement in CPT sensitivity following 3-MA cotreatment; this effect is most likely due to the suppression of autophagy and enhanced apoptosis of H1299 NSCLC cells.

Caspases are cysteine proteases that have critical roles in apoptosis (51,52). The present study demonstrated that treatment with CPT and 3-MA activated caspase-9. However, pretreatment with a pan-caspase inhibitor did not significantly suppress CPT/3-MA-induced cell death, thus suggesting that CPT/3-MA-induced apoptosis was not associated with caspase-dependent pathways; this finding differs from previous reports. It has previously been reported that the apoptotic effects of CPT/3-MA are usually associated with the activation of caspase-3, caspase-8 and caspase-9 in colon, liver (53) and lung cancer cells (54). The different pathways associated with CPT/3-MA-induced apoptosis may be due to various cell types, treatment duration and CPT/3-MA concentration.

Although it is widely believed that anticancer agents inhibit the viability of cancer cells through inducing apoptosis, accumulating evidence has revealed that other apoptosis-independent cell death modalities, such as autophagic cell death, may also contribute to cancer cell death, thus resulting in the inhibitory effects of anticancer drugs on cancer cells. For example, voacamine, which is a bisindolic alkaloid, induces apoptosis-independent autophagic cell death of the multidrug-resistant human osteosarcoma cell line U-2 OS (55). Similarly, autosis, a newly identified non-apoptotic form of cell death, may contribute to cell death during autophagy (56). In the present study, 3-MA significantly enhanced CPT-induced cell death and increased its inhibitory effect on the viability of NSCLC cells; furthermore, apoptosis was markedly increased in CPT/3-MA-treated cells compared with in cells treated with CPT alone, thus confirming that 3-MA increased apoptosis of CPT-treated cells. Pretreatment with the pan-caspase inhibitor Z-VAD-FMK did not significantly affect the viability of NSCLC cells, thus suggesting that apoptosis may not be fully responsible for NSCLC cell death induced by CPT and 3-MA cotreatment. Additionally, non-apoptotic cell death, such as autosis or other forms of autophagic cell death, may contribute to the enhancing effects of 3-MA on CPT-induced proliferation inhibition and death of NSCLC cells.

Accumulating evidence has revealed that autophagy is capable of attenuating DNA damage by decreasing generation of ROS and modulating DNA repair activity (57,58). Mitochondria, which are the major source of ROS, cause ROS production accompanied with DNA damage (59). Autophagy inhibits ROS production, in order to protect cells from DNA damage (60,61); therefore, blocking autophagy may induce an excess generation of ROS, consequently leading to more severe DNA damage (62). When cells encounter DNA damage, they initiate DNA repair mechanisms to reduce it. Notably, autophagy is also involved in the process of DNA repair; autophagy induces the generation of ATP and recycles dNTP to improve DNA repair activity, whereas suppression of autophagy leads to decreased levels of checkpoint kinase 1 and a markedly diminished ability to repair DNA double-strand breaks (63).

According to previous results, the present study aimed to determine whether inhibiting autophagy affected the degree of DNA damage. Following inhibition of CPT-induced autophagy, the present study detected γH2AX through immunofluorescence and flow cytometry. The present study demonstrated that suppression of CPT-induced autophagy significantly increased γH2AX foci formation. These findings suggested that inhibition of autophagy increased DNA damage and γH2AX expression. In addition, DNA damage was only slightly increased in response to treatment with 3-MA alone. Therefore, it may be suggested that inhibition of autophagy by 3-MA promotes ROS generation leading to DNA damage; this may explain why γH2AX is upregulated in response to treatment with 3-MA alone.

In conclusion, the present study provided detailed insights into the CPT-resistant mechanisms of NSCLC cells. The results demonstrated that CPT failed to further induce cell cytotoxicity, metastasis-inhibiting effects, DNA damage and apoptotic death following dosage accumulation, and the inhibitory effects of CPT on H1299 and H460 cells reached an early plateau. Conversely, CPT increased the formation of autophagosomes in NSCLC H1299 cells in a dose-dependent manner, and the present study indicated that CPT-induced autophagy may serve a protective role in NSCLC with regards to DNA damage and apoptosis. Cotreatment with the autophagy inhibitor 3-MA blocked CPT-induced autophagy, and activated caspase-9 and γH2AX, thereby enhancing induction of apoptosis and DNA damage in NSCLC cells. Taken together, 3-MA may serve as a promising clinical adjuvant to enhance CPT-based chemotherapies for the future treatment of lung cancer (Fig. 11).

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All data generated or analyzed during this study are included in this published article.

Authors' contributions
CCC initiated the work. YHC, SHH and CCC designed experiments. HWH, KCH and WL performed most of the assays. CYW, WPH, JYFC and BHC helped to acquire data and conduct statistical analysis. WPH helped conduct the transfection assay and analysed the autophagic puncta. YHC and CCC wrote the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate
Not applicable.

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Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


