MLN4924 neddylation inhibitor promotes cell death in paclitaxel-resistant human lung adenocarcinoma cells

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Received June 22, 2016; Accepted March 30, 2017

DOI: 10.3892/ol.2017.7314

Abstract. Acquired resistance to first-line chemotherapeutics, including paclitaxel (PTX), is a primary factor contributing to chemotherapy failure in non-small cell lung cancer (NSCLC) patients. Previous studies have identified that targeting NEDD8-activating enzyme (NAE) with MLN4924 effectively overcomes platinum resistance in preclinical models of ovarian cancer. However, the underlying mechanisms are yet to be fully elucidated. The present study demonstrates that the inhibition of the neddylation pathway with MLN4924 an NAE inhibitor inhibited protein neddylation, inactivated cullin-RING E3 ligase and exhibited a potent antiproliferative effect on PTX-resistant A549 and H460 cells (A549/PTX and H460/PTX). The application of MLN4924 promotes apoptosis and DNA damage in A549/PTX and H460/PTX cells. Additionally, MLN4924 abrogated the 3-dimensional growth potential of these cells and inhibited the formation of the A549/PTX and H460/PTX spheroids. Notably, combining MLN4924 with PTX did not exhibit synergy in PTX-resistant NSCLC cells. Taken together, the results of the current study suggest that MLN4924 may be utilized as an effective strategy for the treatment of PTX-resistant NSCLC.

Introduction

Lung cancer is a leading cause of cancer mortality globally, in which the predominant subtype of non-small cell lung cancer (NSCLC) represents ~80% of all cases (1). Despite advances in therapy, the overall 5-year survival rate is <15% in patients with NSCLC (2). Currently, conventional chemotherapy remains an important treatment option for patients with NSCLC. Anti-mitotic agents, including docetaxel and paclitaxel (PTX), are predominantly used as chemotherapy regimens for NSCLC (3). However, taxane-based therapy is disadvantaged by the rapid emergence of acquired resistance (4). Therefore, novel therapeutic strategies that overcome the resistance to taxane-based therapy are required.

Nedd8 (neural precursor cell expressed, developmentally downregulated 8), a 9-kDa small ubiquitin-like molecule, is involved in protein neddylation initiated by NEDD8 activating enzyme (NAE) (5). Nedd8 serves a role in the activation of the cullin-RING ligases (CRL), also termed SKP1-cullin-F-box (SCF) E3 ligases for its founding member (CRL/SCF) (6), which has been established to be involved in the regulation of multiple DNA replication and repair pathways (7,8). MLN4924 is a recently identified small molecule inhibitor of NAE and is currently in Phase I clinical trials (9,10). By inhibiting neddylation, MLN4924 promotes uncontrolled S-phase DNA replication as well as in the induction of DNA damage and subsequent cell death (11-13). Therefore, MLN4924 exhibits potent antitumor activity in numerous types of cancer (14). Notably, previous studies have indicated that MLN4924 overcomes platinum resistance in preclinical models of ovarian cancer (15,16), suggesting that inhibiting neddylation with MLN4924 may be a novel strategy to target drug resistance in cancer.

The focus of the present study was to investigate the effects of MLN4924 on PTX-resistant NSCLC cells. The results identified that MLN4924 suppresses the growth of PTX-resistant NSCLC cells by inducing apoptosis and DNA damage.

Materials and methods

Cell lines and cell culture. PTX-resistant H460 (H460/PTX) cells were cultured in RPMI 1640 (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 70 nM (60 ng/ml) PTX (Zhejiang University, Hangzhou, China) (17), PTX-resistant A549 (A549/PTX) cells, were kindly provided

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Key words: MLN4924, neddylation, paclitaxel, lung cancer, drug resistance, spheroids

by Dr Sang Kook Lee (Seoul National University, Seoul, Korea) (18) and cultured in RPMI 1640 containing 117 nM (100 ng/ml) PTX to maintain resistance at 37˚C in an atmosphere containing 5% CO_2. The cells were cultured in complete media without PTX for 3 days prior to performing experiments.

Antibodies and reagents. The antibody against β-actin was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany, A5316). The antibody against regulated in development and DNA damage responses 1 (REDD1) was purchased from ProteinTech Group, Inc. (Chicago, IL, USA; cat. no. 10638-1-AP). The anti-checkpoint kinase 2 (CHK2; cat. no. ab109413), anti-Histone H2AX (H2AX) (cat. no. ab124781) and anti-p21 (cat. no. ab109199) antibodies were obtained from Abcam (Cambridge, UK). The anti-cullin1 (cat. no. sc-11384) and anti-chromatin licensing and DNA replication factor 1 (CDT1; cat. no. sc-36530) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The following antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA): Phospho-CHK2 (cat. no. 2661), phospho-H2AX (cat. no. 9718), Wee1 (cat. no. 4936), p27 (cat. no. 3686), caspase-3 (cat. no. 9662) and poly(ADP-ribose) polymerase (PARP; cat. no. 9532). The following agents were used: MLN4924 (Merck KGaA) and PTX (Sigma-Aldrich; Merck KGaA). Recombinant human epidermal growth factor (EGF), fibroblast growth factors (FGF; both from PeproTech, Inc., Rocky Hill, NJ, USA) and B-27 (Gibco; Thermo Fisher Scientific, Inc.) were used to culture spheroids. Drugs were dissolved in dimethyl sulfoxide (DMSO) and stored at -20˚C.

Bromodeoxyuridine (BrdU) labeling. H460/PTX and A549/PTX cells were labeled with 10 mmol/l BrdU (Sigma-Aldrich; Merck KGaA) in growth medium for 12 h at 37˚C. BrdU-labeled DNA was detected with mouse monoclonal anti-BrdU antibody (cat. no. RPN202; 1:50; GE Healthcare, Chicago, IL, USA), according to the manufacturer's protocol (19).

Colony formation assay. For clonogenic assay, cells were seeded into 6-well plates (300 cell/well) in triplicate and cultured for 14 days at 37˚C in an atmosphere containing 5% CO_2. The colonies on the plates were fixed with 4% paraformaldehyde at room temperature for 15 min and stained with crystal violet (0.2% in anhydrous ethanol) at room temperature for 30 min. Colonies with >50 cells were counted (19). Colonies were counted by eye and representative results of 3 independent experiments with similar results are presented.

Spheroid formation. Adherent cells were suspended in serum-free Dulbecco's modified Eagle's medium/F12 (Gibco; Thermo Fisher Scientific, Inc.) containing 20 ng/ml FGF, 20 ng/ml EGF and B-27 (B-27 and medium at a 1:50 volume ratio), plated ($1x10^3$ cell/well) onto a 96-well clear flat-bottomed ultra-low attachment micro plate (Corning Incorporated, Corning, NY, USA) at 37˚C and 5% CO_2 for 10 days. Spheroids with a diameter of ~50 μm were counted.

Cell death assay. A549/PTX and H460/PTX cells were suspended in complete medium for spheroids, and plated ($1x10^3$ cell/well) onto a 96-well clear flat-bottomed ultra-low attachment microplate (Corning Incorporated, Corning, NY, USA) at 37˚C and 5% CO_2 for 10 days. The spheroids were treated with 10 μM MLN4924 for the 24 and 72 h and stained with propidium iodide (PI) at 10 μg/ml. Subsequently, cells were visualized using fluorescence microscopy as previously described (20) where red-fluorescing cells were indicative of cell death.

Western blot analysis. A549/PTX and H460/PTX cells were plated in 60-mm dishes and treated with DMSO or 2.5, 5, 10, 20 and 0.1, 0.3, 1, 3, 10, 30 μM MLN4924 at 37˚C in an atmosphere containing 5% CO_2. After 12, 24 and 48 h, cells were placed on ice, washed with cold PBS, harvested using a scraper and lysed in lysis buffer (1% Triton X-100, 50 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 5 mmol/l sodium pyrophosphate, 25 mmol/l NaF, 0.5 mmol/l sodium orthovanadate, 1 mmol/l DTT, 1 μg/ml pepstanin, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1 mg/ml phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at 12,000 x g for 10 min at 4˚C and supernatants were subjected to western blot analysis. Concentration of protein was determined using a bicinchoninic acid assay kit (cat. no. 23225; Pierce, Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Protein (50 μg per lane) was loaded on SDS-PAGE (8-15% gels) and transferred onto a nitrocellulose membrane (Applygen Technologies, Inc., Beijing, China). The membranes were blocked with 5% non-fat milk in TBST at room temperature for 3 h and incubated with primary antibodies at 4˚C overnight. The antibodies for β-actin (cat. no. A5316; 1:10,000; Merck KGaA, Darmstadt, Germany,), REDD1 (1:2,000), CHK2 (1:2,000), H2AX (1:2,000), p21 (1:1,000), cullin1 (1:200), CDT1 (1:1,000), phospho-CHK2 (1:1,000), phospho-H2AX (1:1,000), Wee1 (1:1,000), p27 (1:1,000), caspase-3 (1:1,000) and PARP (1:1,000) were used. Following washing three times with TBS-Tween-20, the membranes were incubated at room temperature for 1 h with corresponding horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse, 1:1,000, cat. no. 62-6520; goat anti-rabbit, 1:10,000, cat. no. 65-6120; Invitrogen; Thermo Fisher Scientific, Inc.). The blots were detected using an ECL Western Blot Substrate kit (cat. no. 34580; Thermo Fisher Scientific, Inc.) according to the manufacturers protocol.

Flow cytometric analysis of apoptosis. A549/PTX and H460/PTX cells were treated with DMSO or 1, 5 and 10 μM MLN4924 for 24, 47 and 72 h at 37˚C in an atmosphere containing 5% CO_2, and then the cells were harvested with 0.25% trypsin without EDTA, washed twice with ice-cold PBS and resuspended in 500 μl binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Subsequently, cells were incubated with 5 μl annexin V-fluorescein isothiocyanate (40 μg/ml; BD Biosciences, Franklin Lakes, NJ, USA) and 5 μl PI (40 μg/ml; BD Biosciences) in the dark for 10 min at room temperature and detected using flow cytometry. The percentage of apoptotic cells was determined in 3 independent experiments.

Statistical analysis. The results are expressed as means ± standard deviation. Statistical significance was evaluated using the Student's t-test. Group comparisons were

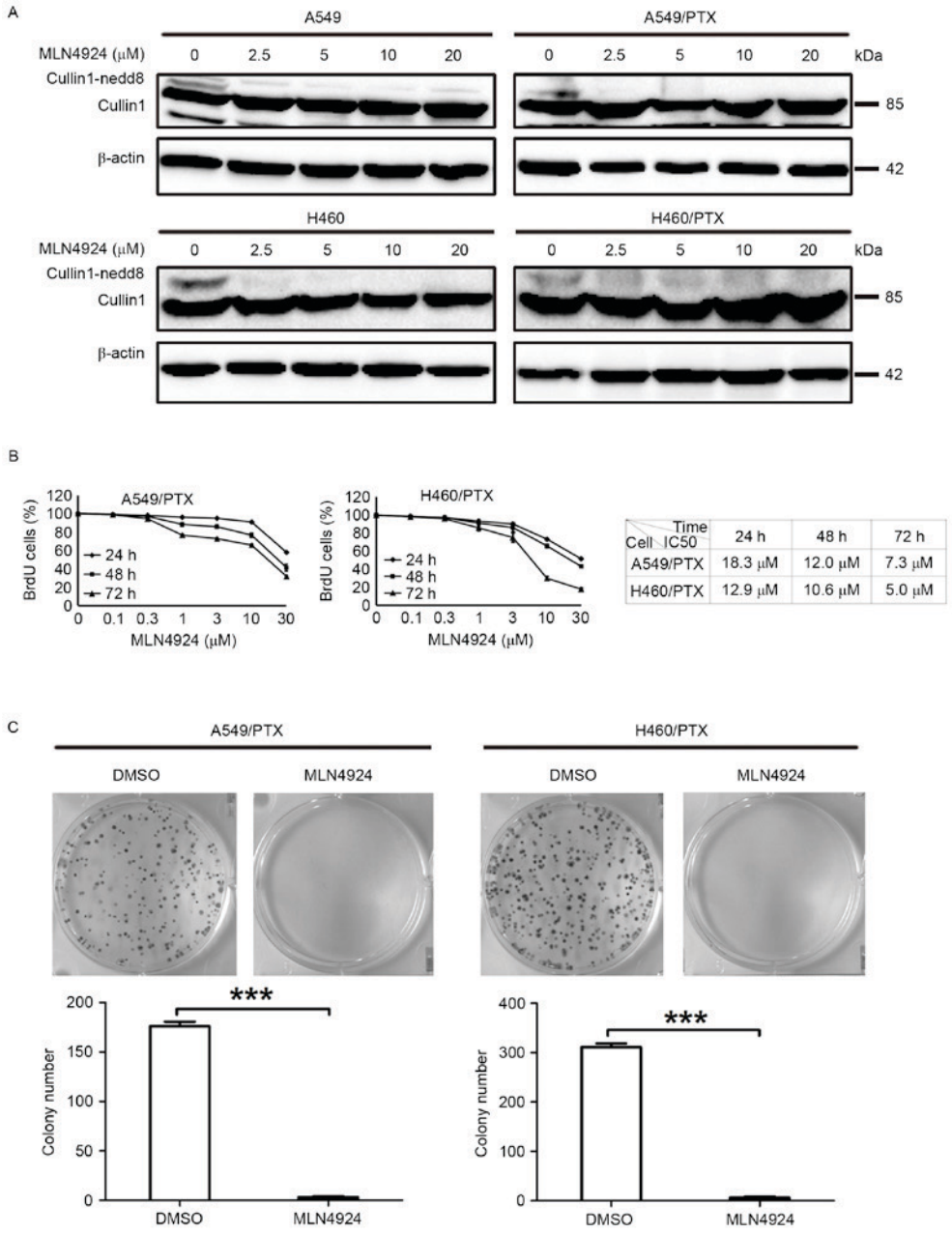

Figure 1. MLN4924 suppresses the growth of PTX-resistant NSCLC cells. (A) The A549 and H460 NSCLC cells and PTX-resistant A549 (A549/PTX) and H460 (H460/PTX) were treated with vehicle or numerous concentrations (0, 2.5, 5, 10 and 20 μM) of MLN4924 for 24 h. (A) Cell lysates were examined for cullin1 using western blot analysis. β-actin was used as a loading control. (B) The A549/PTX and H460/PTX cells were treated with vehicle or the indicated concentrations (0, 0.1, 0.3, 1, 3, 10 and 30 μM) of MLN4924 for 24, 48 and 72 h, the cell viability was determined using a BrdU assay. The results are presented as the mean ± standard deviation. (C) The A549/PTX and H460/PTX cells were treated with vehicle or 10 μM MLN4924 for 14 days in complete medium and colony formation assay was performed. The number of colonies was counted and presented as the mean ± standard deviation. All experiments were repeated 3 times. ***P<0.001. NSCLC, non-small cell lung cancer; PTX, paclitaxel; BrdU, bromodeoxyuridine; DMSO, dimethylsulfoxide; IC50, half-maximal inhibitory concentration.

evaluated using a one-way analysis of variance. All statistical tests were performed with Prism software (version 5; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

MLN4924 exhibits a potent antiproliferative effect on PTX-resistant NSCLC cells. To investigate the effect of MLN4924 on A549/PTX and H460/PTXPTX-resistant NSCLC cells, the cells were treated with MLN4924 and used for several cell-based assays. As presented in Fig. 1A, MLN4924 induced the loss of NEDD8-conjugated (neddylated) cullins in A549/PTX and H460/PTX cells. In accordance with previous studies, cullin neddylation was substantially blocked in parent A549 and H460 cells following MLN4924 treatment (Fig. 1A) (21,22). BrdU assay analysis demonstrated that MLN4924 treatment led to a reduction in cell viability of A549/PTX and H460/PTX cells in a dose- and time-dependent manner (Fig. 1B). The half-maximal inhibitory concentration (IC50) values of A549/PTX were

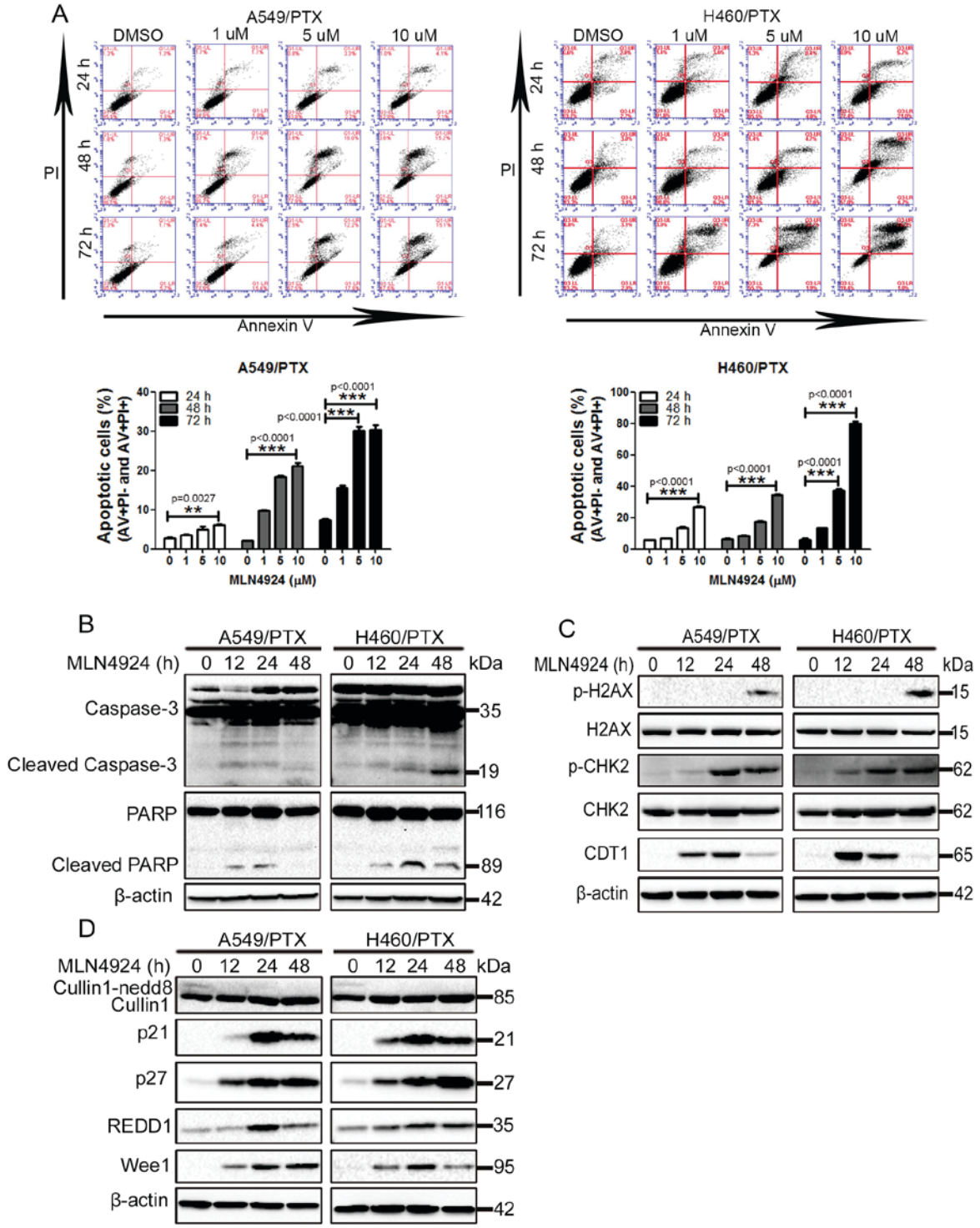

Figure 2. MLN4924 induces apoptosis and DNA damage in PTX-resistant non-small cell lung cancer cells. (A) A549/PTX and H460/PTX cells were treated with DMSO or 1, 5 and 10 μM MLN4924 for 24, 48 and 72 h. The cells were double-stained with AV and PI, apoptosis was analyzed using flow cytometry. The percentage of apoptotic cells were presented as the mean ± standard deviation. The A549/PTX and H460/PTX cells were treated with vehicle or 10 μM MLN4924 for 12, 24 and 48 h. The cell lysates were collected and detected using western blot analysis. (B) Western blot analysis for caspase-3, PARP and β-actin. (C) Western blot analysis for p-H2AX, H2AX, p-CHK2, CHK2, CDT1 and β-actin. (D) Western blot analysis for cullin1, p21, p27, REDD1, Wee1 and β-actin. All experiments were performed 3 times. **P<0.01, ***P<0.001. PI, propidium iodide; AV, annexin V; PTX, paclitaxel; PARP, poly(ADP-ribose) polymerase; p-, phosphorylated; H2AX, histone 2AX; CHK2, checkpoint kinase 2; CDT1, chromatin licensing and DNA replication factor 1; REDD1, regulated in development and DNA damage responses 1.

18.3, 12.0 and 7.3 μM for 24, 48 and 72 h, respectively, and the IC50 values of H460/PTX were 12.9, 10.6 and 5.0 μM for 24, 48 and 72 h, respectively (Fig. 1B). Additionally, 10 μM MLN4924 significantly decreased the clonogenic survival of A549/PTX and H460/PTX cells (P=0.0003; Fig. 1C). Therefore 10 μM MLN4924 was used throughout the current study. Taken together, these data indicate that MLN4924 potently inhibits the growth of A549/PTX and H460/PTX cells.

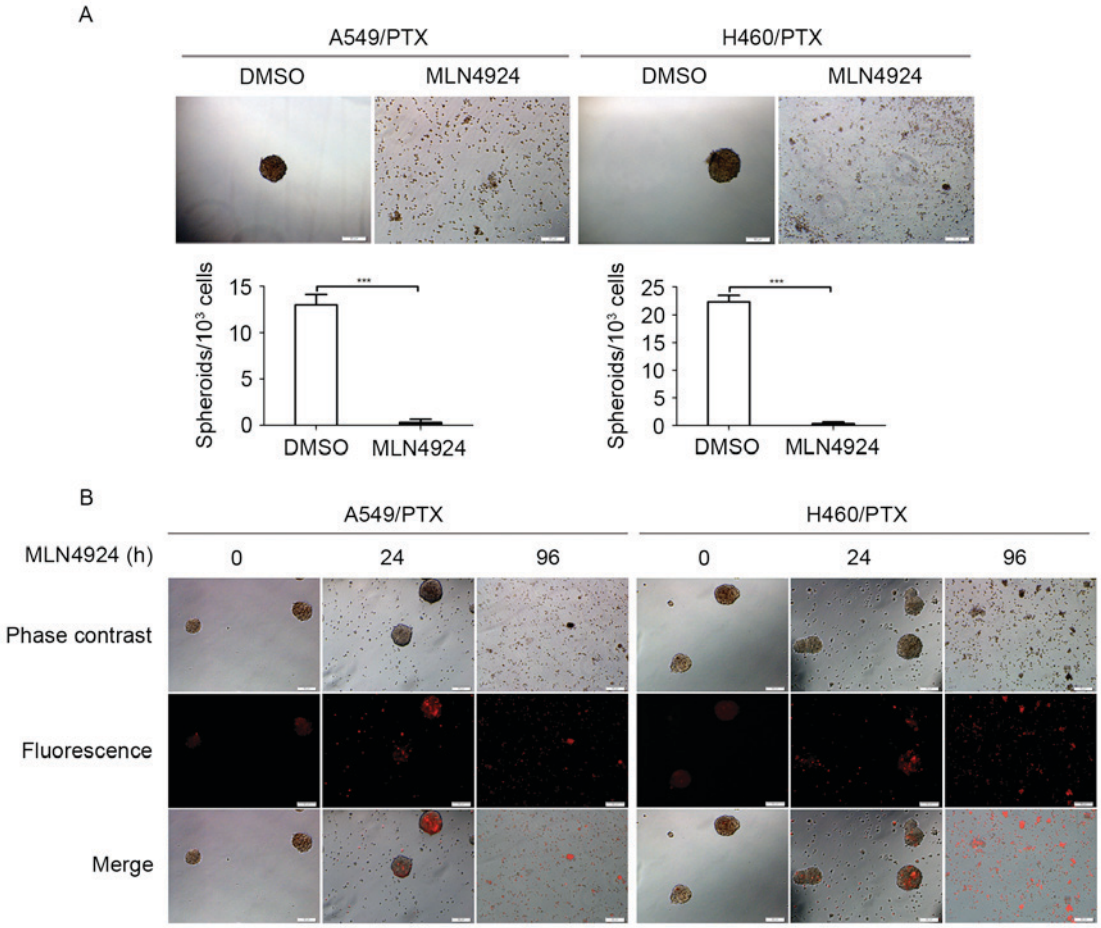

Figure 3. MLN4924 inhibits the growth of PTX-resistant non-small cell lung cancer cells in 3-dimensional cultures. (A) A549/PTX and H460/PTX cells were treated with DMSO or 10 μM MLN4924 for 24 h, and then cultured in ultra-low attachment plates for 10 days. The number of spheroids (diameter ≥50 μm) was counted and the results are expressed as the mean ± standard deviation from three independent experiments. (B) A549/PTX and H460/PTX cells were cultured in ultra-low attachment plates for 10 days to form spheroids, the spheroids were treated with vehicle or 10 μM MLN4924 for 24 and 96 h then stained with propidium iodide. The cells were captured using phase contrast and red fluorescence microscopy, and merged images were created. Scale bar, 50 μm. All experiments were performed 3 times. ***P<0.001. PTX, paclitaxel; DMSO, dimethylsulfoxide.

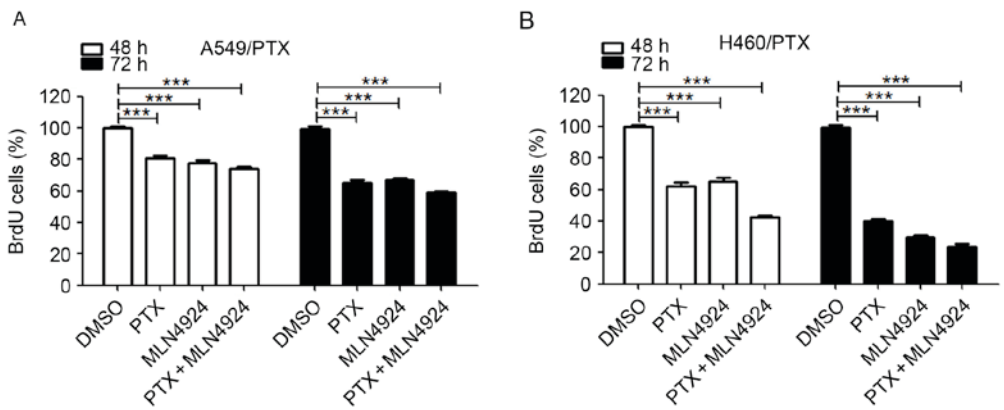

Figure 4. MLN4924 does not function synergistically with PTX in PTX-resistant non-small cell lung cancer cells. (A) A549/PTX and H460/PTX cells were treated with DMSO, 1 μM PTX, 10 μM MLN4924 or a combination of PTX and MLN4924 agents for 48 and 72 h, and the cell viability was assessed using (B) BrdU assay. The results were quantified and presented as the mean ± standard deviation. Experiments were performed 3 times. ***P<0.001. PTX, paclitaxel; DMSO, dimethylsulfoxide; BrdU, bromodeoxyuridine.

MLN4924 promotes apoptosis and DNA damage in PTX-resistant NSCLC cells. To investigate the underlying mechanism of how MLN4924 suppresses the growth of PTX-resistant NSCLC cells, MLN4924-treated A549/PTX and H460/PTX cells were analyzed for the induction of apoptosis using flow cytometry with annexin V and PI double staining, as MLN4924 has been established to induce apoptosis in a number of cancer cells (11,15,22,23). As presented

in Fig. 2A, exposure to MLN4924 led to a significant increase in apoptosis in A549/PTX and H460/PTX cells in a time- and dose-dependent manner. Furthermore, MLN4924 treatment promoted caspase-3 processing and PARP cleavage, 2 hallmarks of apoptosis, in A549/PTX and H460/PTX cells during a 48 h period (Fig. 2B). These results indicate that apoptosis is induced by MLN4924 in A549/PTX and H460/PTX cells.

In addition to inducing apoptosis, MLN4924 initiates the DNA damage response (DDR) in cancer cells (8,11,13,23). Increased phosphorylation of histone H2AX and CHK2, 2 classical markers of DDR, was observed in A549/PTX and H460/PTX cells following MLN4924 treatment (Fig. 2C). In addition, MLN4924-mediated accumulation of the DNA replication licensing factor, CDT1 in these cells (Fig. 2C). Taken together, these results indicate that MLN4924 promotes the DDR in PTX-resistant NSCLC cells.

It has been established that the effect of the induction of apoptosis and DDR by MLN4924 is due to its inactivation of CRL/SCF (13,22-24). Therefore, the levels of numerous CRL/SCF substrates were examined in MLN4924-treated cells. As presented in Fig. 2D, MLN4924 treatment led to increased levels of p21, p27 and Wee1 in A549/PTX and H460/PTX cells, whereas cullin neddylation was inhibited. Increased levels of REDD1 were observed in MLN4924-treated A549/PTX and H460/PTX cells (Fig. 2D). These data indicate that MLN4924 efficiently inactivates CRL/SCF in A549/PTX and H460/PTX cells.

MLN4924 suppresses the growth of PTX-resistant NSCLC cells in 3-dimensional (3D) cultures. The 3D multicellular tumor spheroids are typically used as *in vitro* surrogates of tumorigenesis (25). Therefore, the growth inhibitory effect of MLN4924 was investigated in PTX-resistant NSCLC cells using 3D cultures. The A549/PTX and H460/PTX cells formed spheroids (with diameter of ~50 μm) under appropriate condition for 3D cultures (Fig. 3A). However, MLN4924-treated A549/PTX and H460/PTX cells did not form spheroids compared with the DMSO-treated cells (Fig. 3A), indicating that MLN4924 has the ability to abrogate the 3D growth potential of the PTX-resistant NSCLC cells. Furthermore, following exposure to MLN4924 for 96 h, the A549/PTX and H460/PTX spheroids had collapsed and there were increased levels of cell debris (Fig. 3B), indicating that prolonged treatment of MLN4924 promotes lysis of the A549/PTX and H460/PTX spheroids.

Combining MLN4924 with PTX does not exhibit synergy in PTX-resistant NSCLC cells. To investigate whether combining MLN4924 treatment with PTX results in an additive efficacy, the cell viability was evaluated using the BrdU assay. As presented in Fig. 4A, compared with each drug alone, the combination of MLN4924 and PTX treatment for numerous durations did not result in any significant alteration in cell viability in A549/PTX or H460/PTX cells. These data suggest that there is no synergistic effect between MLN4924 and PTX in the PTX-resistant NSCLC cells.

Discussion

Resistance to docetaxel or PTX remains a primary obstacle in the treatment of NSCLC. The current study demonstrated that the neddylation inhibitor, MLN4924, potently suppresses the growth of PTX-resistant NSCLC cells by inducing apoptosis and DNA damage. Furthermore, in addition to inducing clonogenic and spheroid formation, MLN4924 promotes the disassembly of PTX-resistant NSCLC cell spheroids. As MLN4924 is a first-in-class inhibitor of NAE that is being evaluated in multiple phase I clinical trials (9,10). Soucy *et al* (14) identified that MLN4924 suppressed the growth of human tumor xenografts in mice, suggesting that NAE inhibitors may have potential as a treatment for cancer. The results of the present study provide a rationale for the clinical investigation of protein neddylation inhibition as a novel strategy for the treatment of PTX-resistant NSCLC.

An increasing number of studies have demonstrated that MLN4924 promotes a DNA damage response, cell cycle arrest, apoptosis and senescence in a number of cancer cell types (8,11-13,15,22,23). In accordance with these previous studies, the current study observed that MLN4924 promotes apoptosis and the DDR in PTX-resistant NSCLC cells. Notably, MLN4924 suppresses the growth of PTX-resistant NSCLC cells in 3D culture. As the multicellular spheroids are an effective 3D cell culture model that is able to mimic *in vivo* microenvironments compared to 2D cell cultures (26), the results of the present study suggest a potential *in vivo* effect of MLN4924 on PTX-resistant NSCLC cells.

Consistent with previous studies (13,21-24), the effect of MLN4924 in PTX-resistant NSCLC cells was due to its inactivation of CRL/SCF demonstrated by the increased levels of a number of CRL substrates, including p21, p27, REED1 and Wee1. These data and the previous studies suggest an antineoplastic mechanism of action for MLN4924.

Previous studies have established that targeting NAE with MLN4924 effectively overcomes platinum resistance in preclinical models of ovarian cancer (15,16). Additionally, a synergic cytotoxic effect between cisplatin and MLN4924 was observed in platinum-sensitive and -resistant ovarian cancer cells (15,16). However, in the present study, combination treatment of MLN4924 and PTX did not result in synergistic cytotoxicity in the PTX-resistant NSCLC cells.

In conclusion, to the best of our knowledge, the current study is the first to demonstrate the growth inhibitory effect of MLN4924 in PTX-resistant NSCLC cells. The results of the present study support further investigation of NAE targeting with MLN4924 as an effective strategy for the treatment of PTX-resistant NSCLC.

Acknowledgements

The authors would like to thank Professor Songshu Meng (Institute of Cancer Stem Cell, Dalian Medical University of China, Liaoning, China) for critical discussions and suggestions. This study was supported in part by grants from the National Natural Science Foundation of China (grant nos. 81502674 to K.J. and 81301720 to W.C.).

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