

# Nedaplatin enhanced apoptotic effects of ABT-737 in human cancer cells via Mcl-1 inhibition

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**Abstract.** Platinum compounds, such as cisplatin, carboplatin, oxaliplatin and nedaplatin, are widely used to treat a number of solid malignancies. Nedaplatin is a second-generation platinum complex, based on its pronounced anti-cancer activities against several solid tumors being equivalent to that of cisplatin, but with lower nephrotoxicity. In this context, the present study aimed to investigate the potential anti-cancer effect by combining nedaplatin with ABT-737. It was found that nedaplatin greatly increased ABT-737-mediated apoptosis in A549 and 95-D cells, accompanied by enhanced cleavage of poly(ADP-ribose) polymerase and caspase-3. In addition, this enhancement was also paralleled by cytochrome *c* release and dissipation of mitochondrial membrane potential. Additional mechanistic investigations revealed that nedaplatin plus ABT-737 exerted a synergistic effect on cancer cells through their ability to accelerate the degradation of Mcl-1. The present study has revealed nedaplatin as a pertinent sensitizer to ABT-737, which opens up new avenues for this promising BH3-mimetic molecule in the clinic.

## Introduction

Nedaplatin, a platinum derivative synthesized to overcome problems of cisplatin resistance, was developed by Shionogi Pharmaceutical Company in 1983 with the aim to provide a treatment with effectiveness similar to cisplatin, but with less nephrotoxicity and gastrointestinal toxicities (1). Nedaplatin produces promising response rates in clinical trials as a monotherapy for the treatment of squamous cell carcinoma of the uterus, cervix, head and neck, ovary, lung and esophagus (2-4). Nedaplatin exerts anti-tumor effects following uptake into tumor cells by binding to DNA bases and inhibiting DNA replication, similar to cisplatin and carboplatin (5). However, the ability of cancer cells to become resistant to nedaplatin remains a notable obstacle to successful chemotherapy; resistance of numerous species of cancer cells to nedaplatin can be reversed by combining with chemotherapeutic agents, including 5-fluorouracil, docetaxel and vindesine (6-8). The identification of new chemotherapy regimens incorporating nedaplatin with other chemotherapeutic agents can enhance the knowledge of nedaplatin resistance and support the development of nedaplatin-based approaches to cancer therapy.

The B-cell lymphoma 2 (Bcl-2) family members serve as primary regulators of apoptosis and include both pro- and anti-apoptotic molecules (9). Overexpression of anti-apoptotic Bcl-2 family proteins has been associated with chemotherapy resistance in multiple human cancers (10). Myeloid cell leukemia 1 (Mcl-1), an anti-apoptotic protein in the Bcl-2 family, is frequently observed in numerous tumor types and contributes to chemotherapeutic resistance (11).

ABT-737 is a small molecule inhibitor of Bcl-2 family proteins. It can bind Bcl-2 and B-cell lymphoma-extra large (Bcl-xL) with high affinity. ABT-737 has shown single agent and combination therapy efficacy against multiple myeloma, acute myeloid leukemia, lymphoma and solid tumor cell lines (12). The resistance to ABT-737 correlates with the overexpression of Mcl-1 protein in several cancer cell lines (13,14). The present study exhibited for the first time that combining

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*Abbreviations:* Mcl-1, myeloid cell leukemia 1; SRB, sulforhodamine blue; JCl, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazol-carbocyanine iodide; PI, propidium iodide; CI, combination index

*Key words:* nedaplatin, ABT-737, combination, Mcl-1, cancer

nedaplatin and ABT-737 has substantial synergistic anti-cancer efficacy. These enhanced anti-tumor activities were accompanied by the promotion of apoptosis. The promotion of Mcl-1 degradation was involved in the synergistic anti-cancer effect by combining nedaplatin with ABT-737. These results indicated that combining nedaplatin with ABT-737 may be an effective therapeutic strategy to achieve synergistic anti-cancer activities.

## Materials and methods

**Materials.** In total, 3.01 mg nedaplatin from Selleck Chemicals (Houston, TX, USA) was dissolved in 1 ml dimethyl sulfoxide. ABT-737 was synthesized according to the literature (12), and its purity was determined to be >99% by high performance liquid chromatography. MG132 was purchased from Selleck Chemicals. All the following antibodies used for western blotting are rabbit anti-human: Anti-Mcl-1 polyclonal antibody (dilution, 1:500; catalog no., SC-819), anti-PARP polyclonal antibody (dilution, 1:500; catalog no., SC-7150) and anti-procaspase-3 polyclonal antibody (dilution, 1:500; catalog no., SC-7148) (Santa Cruz Biotechnology, Dallas, TX, USA); and anti-cleaved-caspase-3 monoclonal antibody (dilution, 1:1,000; catalog no., 9668; Cell Signaling Technology, Danvers, MA, USA). The mouse anti-human antibodies for western blotting are as follows: Anti-x-linked inhibition of apoptosis protein (XIAP) monoclonal antibody (dilution, 1:500; catalog no., SC-55550; Santa Cruz Biotechnology); and anti- $\beta$ -actin monoclonal antibody (dilution, 1:2,000; catalog no., BD-612656; BD Biosciences, Franklin Lakes, NJ, USA).

**Cell culture.** Human lung cancer A549, NCI-H1299 and 95-D cell lines, prostate cancer PC-3 cell line and ovarian cancer SKOV3 cell line were obtained from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). All cell lines were tested and authenticated for genotypes by DNA fingerprinting. The 95-D, NCI-H1299 and SKOV3 cells were maintained in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), and the PC-3 and A549 cells were grown in Ham's F12 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum. All the cell lines were maintained in a humidified atmosphere of 95% air plus 5% CO<sub>2</sub> at 37°C.

**Sulforhodamine blue (SRB) assay.** The anti-proliferative activity of nedaplatin plus ABT-737 was detected by SRB (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) assay. Briefly, cancer cells were fixed with 10% trichoroacetic acid solution. Subsequent to washing, 0.4% SRB solution (100  $\mu$ l per well) was added into each well. Following 20 min staining, wells were rinsed with 1% acetic acid to remove unbound dye, and then left to air dry. Subsequently, 100  $\mu$ l Tris-base lye (10 mmol/l; Biosharp, Hefei, China) was added, followed by 10-min oscillation. The absorbance was then recorded at 515 nm using a multi-scan spectrum (Thermo Scientific Multiskan Go 1510; Thermo Fisher Scientific, Inc.).

**Colony formation assay.** Cancer cells were plated at 500-1,000 cells per dish, and then cells were exposed to nedaplatin (1  $\mu$ M), ABT-737 (1  $\mu$ M) or a combination of the two. After 14 days, dishes were stained by crystal violet and colony numbers were counted.

**Propidium iodide (PI) staining.** Sub-G1 analysis following PI staining was used to detect apoptosis. A549 cells (3x10<sup>5</sup>/well) were seeded into 6-well plates and exposed to either nedaplatin (20  $\mu$ M) or ABT-737 (5  $\mu$ M), or the two agents together. 95-D cells (3x10<sup>5</sup>/well) were seeded into 6-well plates and exposed to either nedaplatin (10  $\mu$ M) or ABT-737 (10  $\mu$ M), or the two agents together. Cells were harvested and washed with phosphate-buffered saline (PBS) three times and fixed with pre-cooled 70% ethanol at -20°C overnight. Cells were washed and resuspended in 500  $\mu$ l PBS containing 50  $\mu$ g/ml RNase at 37°C for 30 min. The cells were then stained with 5  $\mu$ g PI at room temperature for 30 min. For each sample, 2x10<sup>4</sup> cells were collected and analyzed using a FACS-Calibur cytometer (Becton Dickinson, San Jose, CA, USA), and the data were analyzed using Cellquest Software (version 6.0; Becton Dickinson, San Jose, CA, USA).

**Determination of mitochondrial membrane depolarization.** Cells (3x10<sup>5</sup>/well) were treated with nedaplatin and/or ABT-737 for 48 h, collected, and resuspended in fresh medium containing 10  $\mu$ g/ml 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1; Sigma-Aldrich; Merck Millipore). After incubation at 37°C for 30 min, cells were analyzed by FACS-Calibur cytometer.

**Western blot analysis.** Proteins were extracted with lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, 0.1% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid, 0.5% deoxycholic acid, 1% NP-40, 2.0  $\mu$ g/ml aprotinin, 1 mM phenylmethylsulfonylfluoride and 0.02% sodium azide (Beyotime Institute of Biotechnology, Haimen, China). The lysates were centrifuged at 10,000 x g for 30 min at 4°C, then the concentrations protein were determined. Proteins were fractionated on 8-15% Tris-glycine gels, and then they were transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA) and probed with the aforementioned primary antibodies (dilution range 1:500-1:1,000). The proteins were visualized with peroxidase-coupled secondary antibodies horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, catalog no., GAM007; HRP-conjugated goat anti-rabbit IgG, catalog no., GAR007; MultiSciences, Hangzhou, China) at a dilution of 1:5,000. Finally, proteins were visualized using the enhanced chemiluminescence detection system (PerkinElmer, Waltham, MA, USA).

**Reverse transcription-quantitative PCR (RT-qPCR).** RNA was isolated from A549 and 95-D cells using the TRIzol system (Thermo Fisher Scientific, Inc.), and the concentration of RNA was determined using NanoDrop 2000 (Thermo Fisher Scientific, Inc). Single-strand cDNA was prepared from the purified RNA using oligo (dT) priming (Thermoscript RT kit; Invitrogen; Thermo Fisher Scientific, Inc.), followed by SYBR-Green qPCR (Qiagen, Hilden, Germany). The sequences of PCR primers were as follows: Mcl-1 forward,

5'-GGGCAGGATTGTGACTCTCATT-3' and reverse, 5'-GATGCAGCTTCTTGGTTTATGG-3'; glyceraldehyde 3-phosphate dehydrogenase forward, 5'-GAGTCAACGGATTG GTCGT-3' and reverse, 5'-TTGATTTTGGAGGGATCTCG-3' (Sangon Biotech, Shanghai, China).

**Plasmid transfection.** The pTOPO-Mcl-1 plasmid from Addgene (15) (Plasmid 21605; Cambridge, MA, USA) or the empty vector (pTOPO) was transfected into A549 cells by Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

**Statistical analysis.** Two-tailed Student's *t*-test was used to detect the significance of differences between the experimental conditions.  $P < 0.05$  was considered to indicate a statistically significant difference. Combination index (CI) was used to quantify drug synergism, based on the multiple drug-effect equation of Chou-Talalay (16). For *in vitro* experiments, CI values were calculated for each concentration of nedaplatin, ABT-737 and the combination of the two in SRB assays using CalcuSyn (version 2.0; Biosoft, Cambridge, UK), and the mean CI values were presented.

## Results

**Nedaplatin plus ABT-737 inhibited the proliferation of human cancer cell lines.** The sensitivities of 5 human cancer cell lines to nedaplatin, ABT-737, or nedaplatin combined with ABT-737 were detected by SRB assay, and the survival curves are shown in Fig. 1. The fixed-ratio concentrations of nedaplatin and ABT-737 were used and CI values were calculated using CalcuSyn Software to assess combination activity (95-D, nedaplatin vs. ABT-737, 2:1; other cell lines, nedaplatin vs. ABT-737, 1:1). Nedaplatin plus ABT-737 showed synergistic effects in 5 human cancer cell lines, with the CI values  $< 0.7$ . Nedaplatin at  $1 \mu\text{M}$  and ABT-737 at  $1 \mu\text{M}$  alone had limited effects on suppressing A549 cell colony formation; however, the combination almost eliminated colony formation in the colony formation assay (Fig. 2). Thus, combination was much more effective than either single agent in inhibiting the proliferation of cancer cells.

**Nedaplatin plus ABT-737 induced enhanced apoptosis in A549 and 95-D cells.** To investigate whether the cytotoxic effects of treatments were linked to the enhancement of apoptosis, PI staining was used to detect apoptosis in A549 and 95-D cells, which showed strong synergistic anti-cancer effects in cytotoxicity assay. As shown in Fig. 3A (top panel), the percentage of apoptotic A549 cells was markedly increased in the combination treatment group (66.4%) compared to ABT-737 (10.18%) or nedaplatin (19.81%) alone (ABT-737 vs. combination,  $P = 0.004$ ; nedaplatin vs. combination,  $P = 0.005$ ). Similarly, the apoptotic 95-D cells were significantly higher in the combination treatment group compared with the single treatment group (ABT-737 vs. combination,  $P = 0.008$ ; nedaplatin vs. combination,  $P = 0.009$ ; Fig. 3B and C).

To further assess whether increased ABT-737-mediated apoptosis by nedaplatin-induced caspase activation, immunoblot analysis was performed. As shown in Fig. 4, combined

treatment markedly activated caspase-3 and PARP in both A549 and 95-D cells. In addition, combination treatment also resulted in potentiation of XIAP downregulation in A549 and 95-D cells. Overall, nedaplatin and ABT-737 showed a synergistic effect by inducing apoptosis in cancer cells.

**Apoptosis induced by nedaplatin plus ABT-737 was through mitochondrial pathway.** Rapid loss of mitochondrial membrane potential and the release of cytochrome *c* are considered to be the hallmarks of mitochondrial dysfunction, which can induce the activation of caspases and lead to apoptosis (10). Thus, the present study investigated whether or not apoptosis induced by nedaplatin plus ABT-737 was triggered by mitochondrial dysfunction. As shown in Fig. 3A (bottom panel), nedaplatin plus ABT-737 resulted in an increased percentage of mitochondrial membrane depolarized A549 cells than compared with a single agent used alone (51.76% in combination treated cells, 23.04% in nedaplatin-treated cells, 18.00% in ABT-737-treated cells and 8.08% in control group; ABT-737 vs. combination,  $P = 0.03$ ; nedaplatin vs. combination,  $P = 0.04$ ). Combination treatment with nedaplatin and ABT-737 resulted in increased mitochondrial membrane potential in A549 and 95-D cell lines (Fig. 3D and E).

**Combination of nedaplatin and ABT-737 promoted the degradation of Mcl-1.** It has been reported that high levels of Mcl-1 may confer resistance to ABT-737 in several solid tumors (17). Thus, the present study examined the involvement of Mcl-1 in nedaplatin and ABT-737 combination treatment. Notably, it was found that treatment with nedaplatin or ABT-737 alone increased the expression of Mcl-1 in A549 and 95-D cells, whereas Mcl-1 expression was markedly decreased in the nedaplatin plus ABT-737 combination group, suggesting that Mcl-1 may be involved in the synergistic effect (Fig. 4). To determine whether the synergistic reduction of Mcl-1 protein by nedaplatin and ABT-737 combination treatment was the result of transcriptional inhibition, the level of Mcl-1 mRNA expression was assessed by RT-PCR in A549 and 95-D cells treated with ABT-737, nedaplatin, or a combination of the two for 12 h. As shown in Fig. 5A, no apparent synergistic inhibitory effects on Mcl-1 mRNA expression levels were observed in the nedaplatin plus ABT-737 group in A549 and 95-D cells, whereas the Mcl-1 mRNA expression level was upregulated in nedaplatin plus ABT-737 combination-treated cells. Therefore, these data revealed that the synergistic reduction of Mcl-1 protein by nedaplatin and ABT-737 combination treatment was not the result of transcriptional inhibition. Thus, it was hypothesized that the putative ubiquitination of Mcl-1 in response to nedaplatin and ABT-737 combination treatment may play a key role in the synergistic effect. To further investigate this hypothesis, A549 cells were treated with CHX (200 mg/ml) to block new protein synthesis and observed Mcl-1 degradation in the presence of  $5 \mu\text{M}$  ABT-737 and/or  $20 \mu\text{M}$  nedaplatin. The half-life of Mcl-1 was compared in A549 cells treated with CHX in the presence of nedaplatin, ABT-737, or a combination of the two. Fig. 5B showed that the level of Mcl-1 protein decreased more rapidly in the nedaplatin plus ABT-737 group compared with either of the single-agent groups. Addition of the proteasome inhibitor MG132 attenuated combination-mediated Mcl-1 degradation (Fig. 5C). These data suggested that a promotion

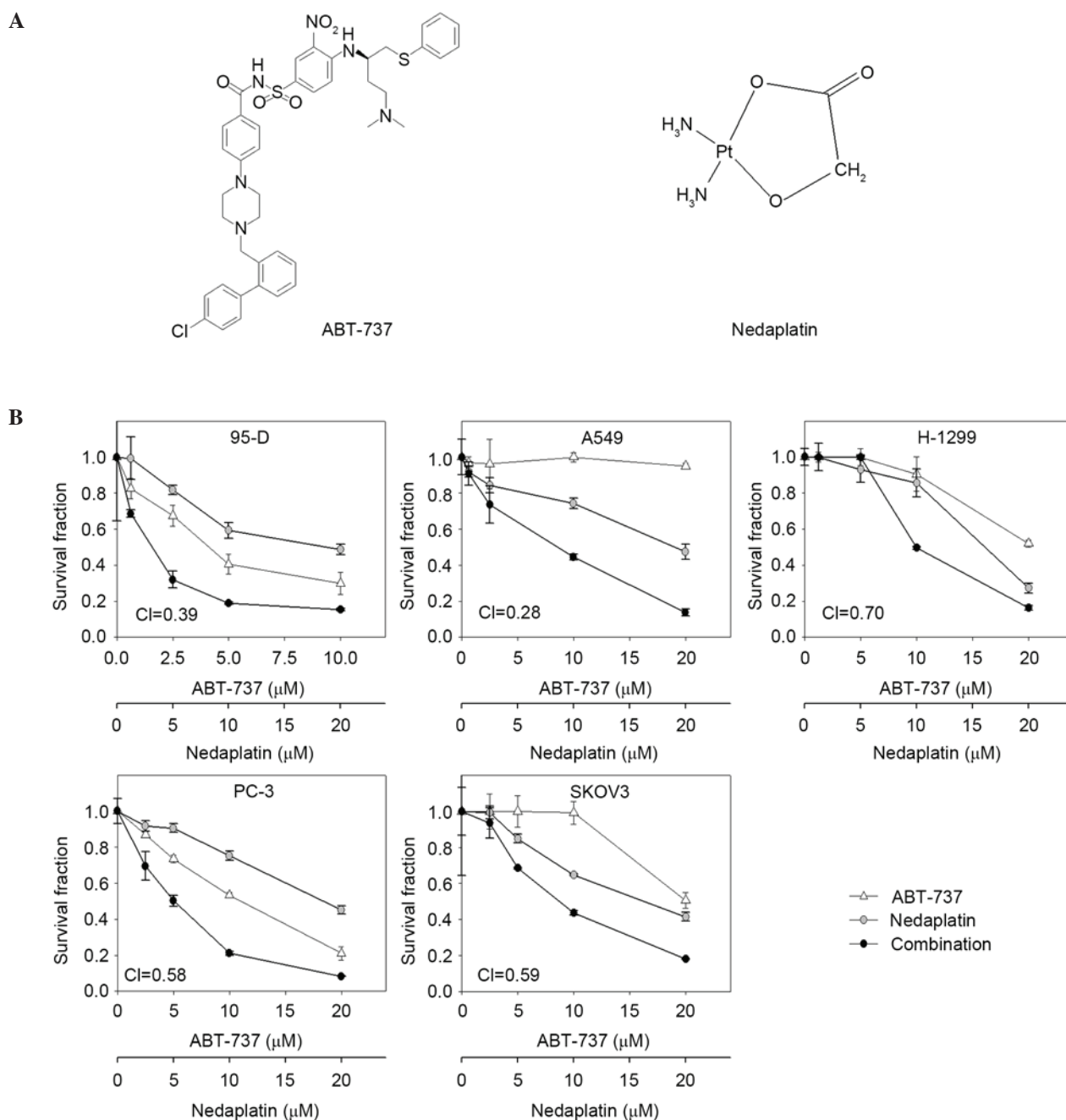


Figure 1. Combination cytotoxicity of nedaplatin and ABT-737. (A) Chemical structures of nedaplatin and ABT-737. (B) The cells were incubated with compounds for 72 h. Dose-response curves of human cancer cell lines to nedaplatin, ABT-737, or a combination of the two. A combination index <0.9 indicated synergism; 0.1, extremely strong synergism; 0.1-0.3, strong synergism; 0.3-0.7, synergism; 0.7-0.85, moderate synergism; 0.85-0.9, slight synergism; 0.9-1.10, additive; and <1.10, antagonism.

of Mcl-1 degradation was involved in the synergistic effect of nedaplatin and ABT-737.

*Overexpression of Mcl-1 rescued cells from synergistic killing by the combination of nedaplatin and ABT-737.* To further evaluate whether the downregulation of Mcl-1 was required for nedaplatin plus ABT-737-induced apoptosis, the expression of Mcl-1 protein was successfully increased via transfecting pTOPO-Mcl-1 plasmid to A549 cells (Fig. 5D). Mcl-1 overexpression significantly decreased apoptosis in A549 cells treated with nedaplatin plus ABT-737 (Fig. 5E). The present results indicated that downregulation of Mcl-1 may contribute to the

synergistic killing of cells by the combination of nedaplatin and ABT-737.

## Discussion

Platinum compounds are widely used in the treatment of a number of solid malignancies. Platinum compounds exhibit individual characteristics, although they share similar chemical structures and cytotoxic mechanisms (18). As single agents, high response rates have been observed in first-line chemotherapy, but the majority of patients will relapse and subsequently prove resistant to platinum compounds (19).

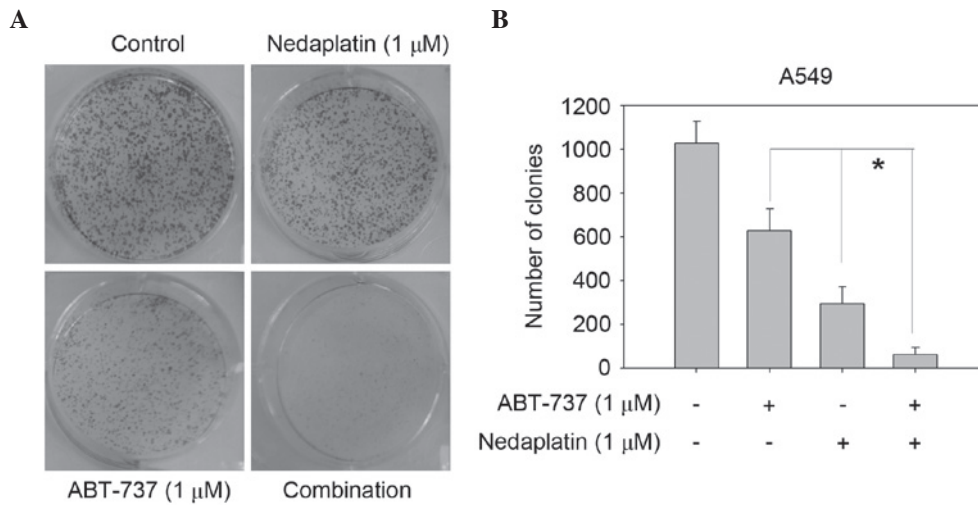


Figure 2. Combination treatment with nedaplatin and ABT-737 inhibited the colony formation in A549 cells. (A) A549 cells were treated with nedaplatin (1.0 μM), ABT-737 (1.0 μM) or a combination of the two for 14 days. Dishes were stained by crystal violet and colony numbers were counted. (B) Changes in the number of colonies formed by A549 cells following treatments. \*P<0.05, mono-treatment vs. combination treatment.

In addition, severe nephrotoxicity and gastrointestinal toxicity also limit their clinical application (20). Thus, it is urgent to develop a new anti-tumor combination therapy with a lower concentration of platinum compounds and a high anti-cancer efficacy. Nedaplatin is a second-generation platinum complex that is ~10 times as soluble in water as cisplatin (1). Additionally, nedaplatin is considered to have more pronounced anti-cancer activity, but less nephrotoxicity and gastrointestinal toxicity (21). Thus, high anti-cancer efficacy and limiting toxicity have made nedaplatin an attractive compound for combination therapy.

The experimental strategies under investigation aimed at overcoming platinum compounds resistance include introduction of functional genes (p53 and p21), or of genes that interfere with apoptotic pathways, such as Bcl-XL and Bcl-2 pathways. These are likely to contribute to tumor treatment, particularly in combination with regimens using platinum compounds (22). Regimens containing cisplatin/carboplatin together with the Bcl-2 inhibitor ABT-737 show high anti-cancer efficacy by modulation of the Mcl-1/Noxa axis (23). To the best of our knowledge, the present results indicated for the first time that the synergistic anti-tumor effect observed *in vitro* by combining nedaplatin with ABT-737 may be observed in several human cancer cell lines. The significant decline of the survival curves in the nedaplatin plus ABT-737 group strongly demonstrated that combination of the agents showed synergy in 5 human solid tumor cell lines, consisting of the PC-3, SKOV3, A549, NCI-H1299 and 95-D cell lines.

The present results also suggested that synergism achieved by combining nedaplatin with ABT-737 was accompanied by enhanced apoptosis. Mitochondria play a key role in apoptosis, mitochondrial outer membrane permeabilization leads to cytochrome *c* release and Bcl-2-associated X protein translocation (24). The current results suggested that loss of mitochondrial membrane potential was significantly greater in the nedaplatin plus ABT-737 group compared with the single agent group, indicating that nedaplatin plus ABT-737 may activate the mitochondrial-mediated apoptosis pathway in 95-D and A549 cells. Marked overexpression

of cleaved-caspase-3 and PARP was observed in 95-D/A549 cells following nedaplatin and ABT-737 combination treatment. XIAP, plays a critical role in the regulation of apoptosis through inhibiting caspases (25). The present data suggested that the synergistic effect on apoptosis induced by nedaplatin plus ABT-737 was accompanied by a large reduction of XIAP.

The Bcl-2 family members control the vital step in the intrinsic apoptotic pathway (13). ABT-737 and its orally active analog ABT-263 are the most potent Bcl-2/Bcl-XL inhibitors, but they have a much lower affinity for Mcl-1. Thus, they are not effective in certain cancer types with high levels of Mcl-1 as single agents (26). The up-regulation of Mcl-1, which is inducible upon treatment with ABT-737 in resistant cancer cells, has been confirmed to be involved in the resistance to ABT-737 (27). Platinum derivatives, such as cisplatin and carboplatin, have been described to reduce expression of Mcl-1 (23,28). Whether the potentiating effect of nedaplatin on ABT-737 response was also due to an inhibition effect on Mcl-1 expression was assessed. The current results showed that ABT-737-treatment increased the expression of Mcl-1 in ABT-737-resistant A549 and 95-D cells. However, nedaplatin plus ABT-737 exerted a synergistic anti-cancer effect by decreasing the expression of Mcl-1 protein. Thus, it was hypothesized that low expression of Mcl-1 contributed to the synergistic effect in cancer cell lines. As in all proteins, the equilibrium between production and degradation determines the protein level of Mcl-1, and the stability of Mcl-1 could be critically important in numerous physiologic and pathologic situations (17). Firstly, it was postulated that the synergistic reduction of Mcl-1 protein by nedaplatin plus ABT-737 was the result of transcriptional inhibition. However, no apparent inhibitory effects on Mcl-1 mRNA levels were observed in the combination treated group, suggesting that the synergistic decrease of Mcl-1 protein by nedaplatin plus ABT-737 was not the result of transcriptional inhibition. Subsequently, the present data indicated that Mcl-1 protein level decreased more rapidly in the combination treatment group than that in single-agent groups. Furthermore, addition of the proteasome

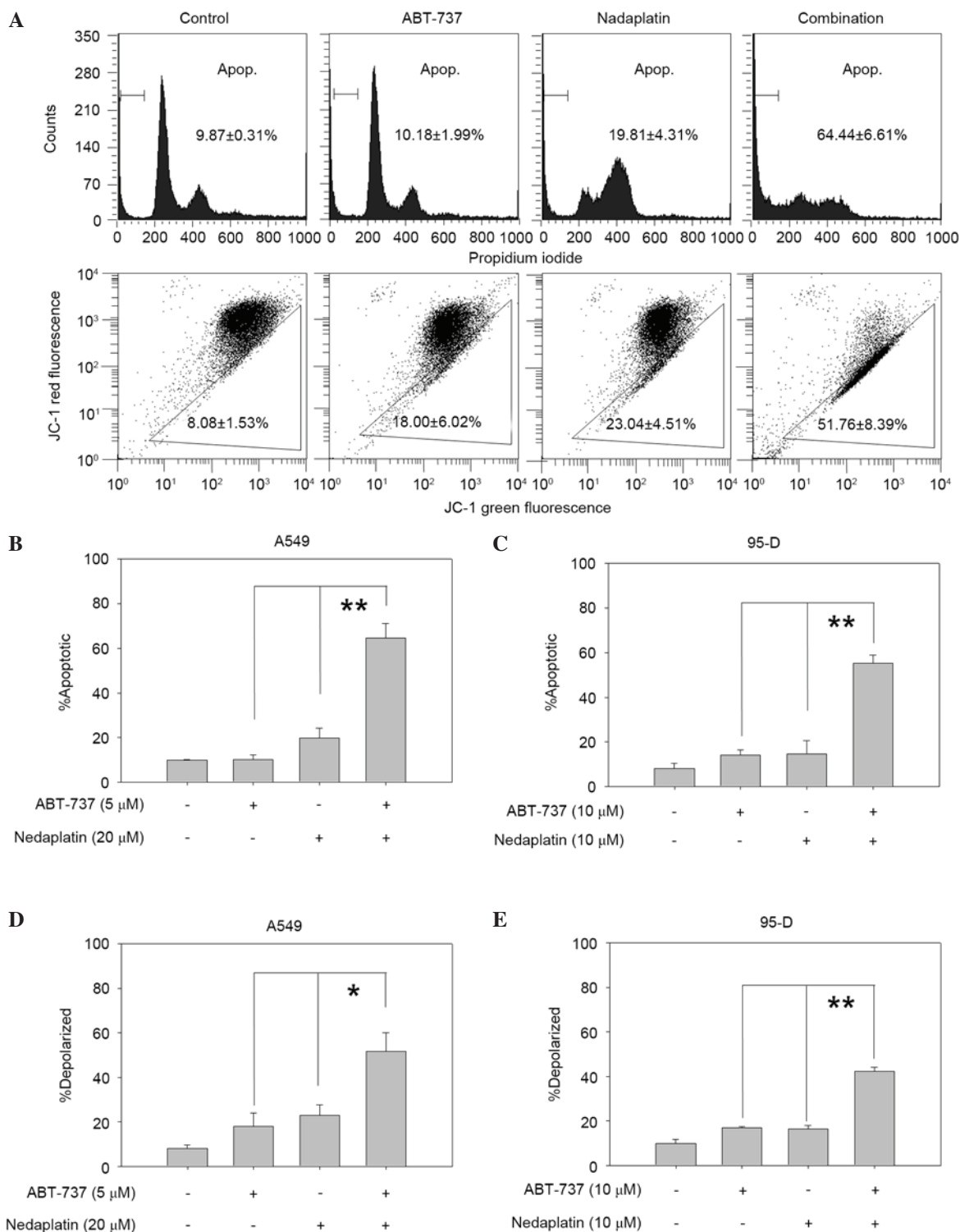


Figure 3. Nedaplatin plus ABT-737 caused enhanced apoptosis. (A) A549 cells were treated with ABT-737 (5  $\mu$ M), nedaplatin (20  $\mu$ M), or a combination of the two for 48 h, and then cells were incubated with PI (top) or JC-1 (bottom) and analyzed by flow cytometry. (B) A549 and (C) 95-D cells in 6-well plates were exposed to the compounds for 48 h and then cells were analyzed by flow cytometry after PI staining. (D) A549 and (E) 95-D cells were exposed to compounds for 48 h and then cells were analyzed by flow cytometry after JC-1 staining. The experiments were repeated three times and error bars represented the standard deviation. \* $P$ <0.05, \*\* $P$ <0.01. JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide; PI, propidium iodide.

inhibitor MG132 attenuated combination-mediated Mcl-1 degradation. These data suggested that a promotion of Mcl-1 degradation was involved in the synergistic effect of nedaplatin and ABT-737. Additionally, Mcl-1 overexpression significantly decreased apoptosis in A549 cells treated with nedaplatin plus ABT-737. These results indicated that Mcl-1 may be involved

in the synergistic anti-cancer effect of nedaplatin and ABT-737 combination.

In conclusion, the present study reports evidence showing apoptosis induction is strongly reinforced when ABT-737 is combined with nedaplatin. In addition, nedaplatin plus ABT-737 exerts a synergistic effect on cancer cells through the

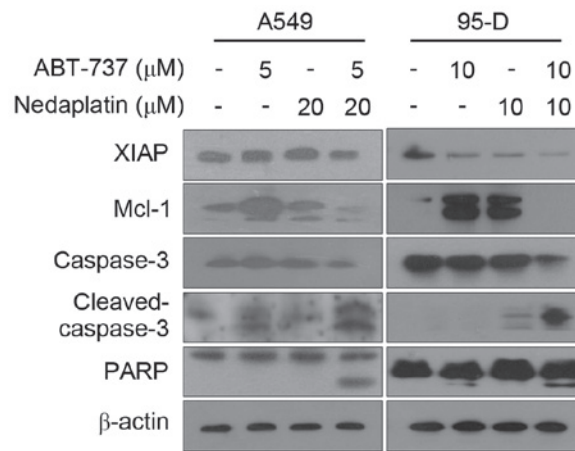


Figure 4. Combination treatment with nedaplatin and ABT-737 caused activation of various apoptosis-associated proteins. Cells were exposed to compounds for 48 h, following which protein extracts were immunoblotted with specified antibodies for XIAP, Mcl-1, caspase-3, cleaved caspase-3, PARP and  $\beta$ -actin. XIAP, x-linked inhibition of apoptosis protein; Mcl-1, myeloid cell leukemia 1; PARP, poly (ADP-ribose) polymerase.

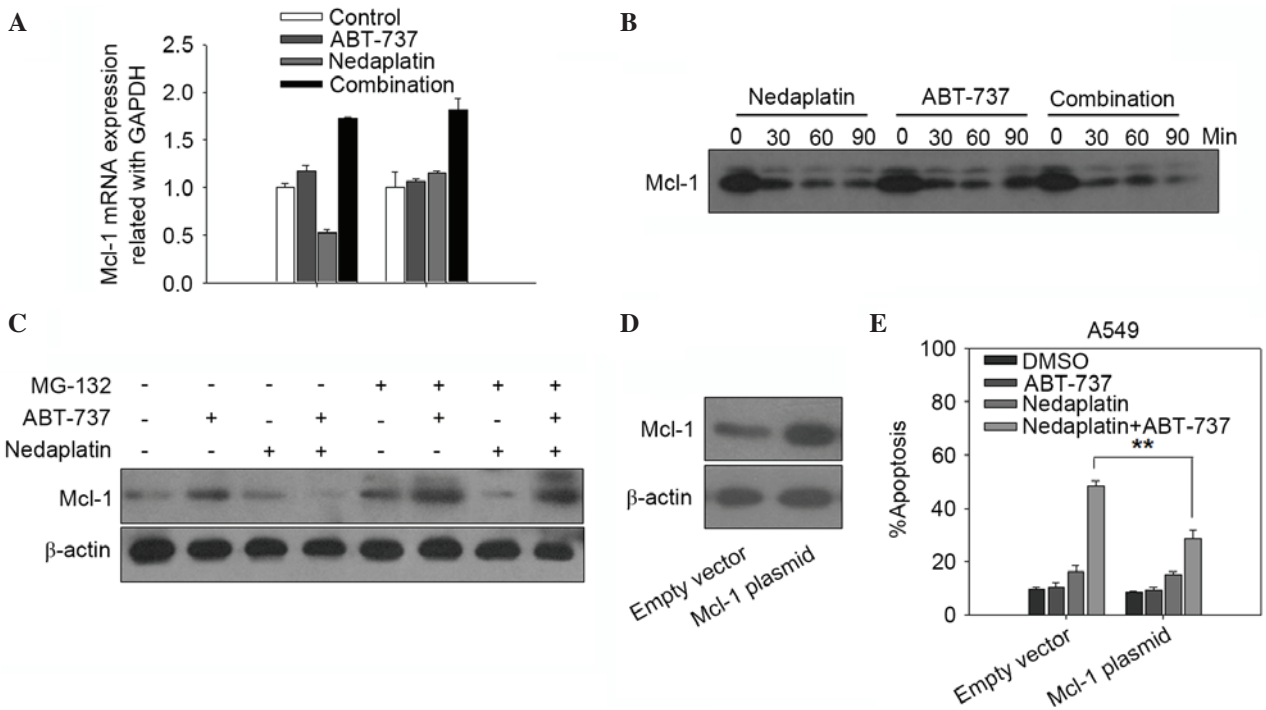


Figure 5. The involvement of Mcl-1 in the enhanced apoptosis synergistically induced by nedaplatin and ABT-737. (A) Mcl-1 mRNA levels were evaluated by RT-PCR in A549 and 95-D cells treated with ABT-737, nedaplatin, or a combination of the two for 12 h. (B) Cells were treated with CHX (200 mg/ml) to block new protein synthesis, and the degradation of Mcl-1 in the presence of 5  $\mu$ M ABT-737 and/or 20  $\mu$ M nedaplatin at 30, 60 and 90 min was detected by western blotting. (C) A549 cells were pretreated with or without 1  $\mu$ M MG132 for 30 min, then the cells were treated with ABT-737 (5  $\mu$ M), nedaplatin (20  $\mu$ M), or a combination of the two for 24 h. Cell lysates were prepared for western blot analysis. (D) A549 cells were transfected with Mcl-1 plasmid and empty vector according to manufacturer's protocol. A total of 48 h after transfection, cell lysates were prepared for western blot analysis. (E) The ratio of apoptosis in A549 cells that had been transfected with Mcl-1 plasmid or empty vector and then treated with 20  $\mu$ M nedaplatin, either alone or in combination with 5  $\mu$ M ABT-737 for 48 h were examined. Quantification of the apoptotic cells by PI staining was repeated three times, and the standard deviation was represented as error bars. \*\* $P < 0.01$ . GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Mcl-1, myeloid cell leukemia 1; DMSO, dimethyl sulfoxide; PI, propidium iodide.

ability of the agents to downregulate Mcl-1, and combination of the agents accelerates the proteasome-mediated degradation of Mcl-1. The present study has revealed nedaplatin as a pertinent sensitizer to ABT-737 through inhibition of Mcl-1, which opens up new avenues for this promising BH3-mimetic molecule in the clinic. Furthermore, the strategy of combining ABT-737 with nedaplatin appears to be an attractive option for reversing resistance to nedaplatin.

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