

Synergistic Antitumor Activity of Gemcitabine and ABT-737 *In Vitro* and *In Vivo* through Disrupting the Interaction of USP9X and Mcl-1

Chong Zhang¹, Tian-yu Cai¹, Hong Zhu¹, Liu-qing Yang¹, Hai Jiang³, Xiao-wu Dong², Yong-zhou Hu², Neng-ming Lin⁴, Qiao-jun He¹, and Bo Yang¹

Abstract

The Bcl-2 antagonist ABT-737 targets Bcl-2/Bcl-xL, but not Mcl-1, which may confer resistance to this agent in various cancers with high levels of Mcl-1. Here, we showed that the combination of gemcitabine and ABT-737 exhibited synergistic cytotoxicity and induced significant apoptosis in multiple cancer types, including lung, renal, bladder, and prostate cancers. The enhanced apoptosis induced by gemcitabine plus ABT-737 was accompanied by the greater extent of mitochondrial depolarization, caspases-3 activation, and PARP cleavage in 95-D and 5637 cell lines. Importantly, in ABT-737-resistant cancer cells, the interaction between USP9X and Mcl-1, which was increased by ABT-737 treatment, could be disrupted by gemcitabine, thus resulting in enhanced ubiquitination and the subsequent degradation of Mcl-1 and ultimately in the synergism of these two drugs. Moreover, the increased anticancer efficacy of gemcitabine combined with ABT-737 was further validated in a human lung cancer 95-D xenograft model in nude mice. Taken together, our data first showed the synergistic anticancer capabilities achieved by combining gemcitabine and ABT-737 and, second, opened new opportunities to use antiapoptotic Bcl-2 family members, which drive tumor cell resistance in current anticancer therapies, therapeutically. *Mol Cancer Ther*; 10(7); 1264–75. ©2011 AACR.

Introduction

Antiapoptotic myeloid cell leukemia-1 (Mcl-1) is an essential modulator of survival during the development and maintenance of a variety of cell lineages (1, 2). Its turnover, believed to be mediated by the ubiquitin–proteasome system, facilitates apoptosis induction in response to cellular stress (3). Mcl-1 overexpression is associated with poor prognostic outcome and resistance to ABT-737 in several types of cancer cells (4). Recent reports revealed that Mcl-1 stabilization may also be mediated by the deubiquitinase ubiquitin-specific peptidase 9, X-linked (USP9X; refs. 5, 6). USP9X has been shown to regulate multiple cellular functions (7–10), and increased expression of USP9X in tumors is signifi-

cantly associated with poor prognosis for patients with multiple myeloma (4). USP9X does not affect all apoptosis signaling pathways but specifically modulates those regulated by Mcl-1: USP9X binds to Mcl-1 and removes the Lys48-linked polyubiquitin chain that normally marks Mcl-1 for proteasomal degradation. Thus, the interaction between USP9X and Mcl-1 could enhance the stability of Mcl-1, and human tumors overexpressing Mcl-1 might also overexpress USP9X (4).

Gemcitabine shows anticancer activity against a variety of solid tumors, including non-small-cell lung (11), pancreatic (12), bladder (13), and breast cancers (14). Gemcitabine is converted intracellularly to the active metabolites difluorodeoxycytidine di- and triphosphates (dFdCDP and dFdCTP). dFdCDP inhibits ribonucleotide reductase, thereby decreasing the deoxynucleotide pool available for DNA synthesis and causing DNA strand termination and apoptosis (15). Combining gemcitabine with a second chemotherapeutic agent may be a logical way to potentially enhance response rates and prolong survival times for patients (16–18).

ABT-737 is a small-molecule chemical that mimics the direct binding to the hydrophobic groove in Bcl-2, Bcl-xL, and Bcl-w and consequently prevents them from sequestering proapoptotic BH3-only proteins such as tBid, Bad, and Bim (2, 19, 20). However, ABT-737 binds with lower affinity to the Bcl-B, Mcl-1, and Bfl-1/A1 proteins. ABT-737 has shown preclinical anticancer activity as a single agent or in combination with other chemotherapeutic

Authors' Affiliations: ¹Institute of Pharmacology and Toxicology, ²Zhejiang University-Ecole Normale Supérieure Joint Laboratory of Medicinal Chemistry, College of Pharmaceutical Sciences, Zhejiang University; ³Department of Urology, First Affiliated Hospital, Zhejiang University School of Medicine; and ⁴Laboratory of Clinical Pharmacy, Zhejiang Cancer Hospital, Hangzhou, Zhejiang, China

Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

Corresponding Authors: Bo Yang and Qiao-jun He, #388 Yuhangtang Road, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, Zhejiang 310058, China. Phone: 86-571-882-08400; Fax: 86-571-882-08400; E-mail: yang924@zju.edu.cn, qiaojunhe@zju.edu.cn

doi: 10.1158/1535-7163.MCT-10-1091

©2011 American Association for Cancer Research.

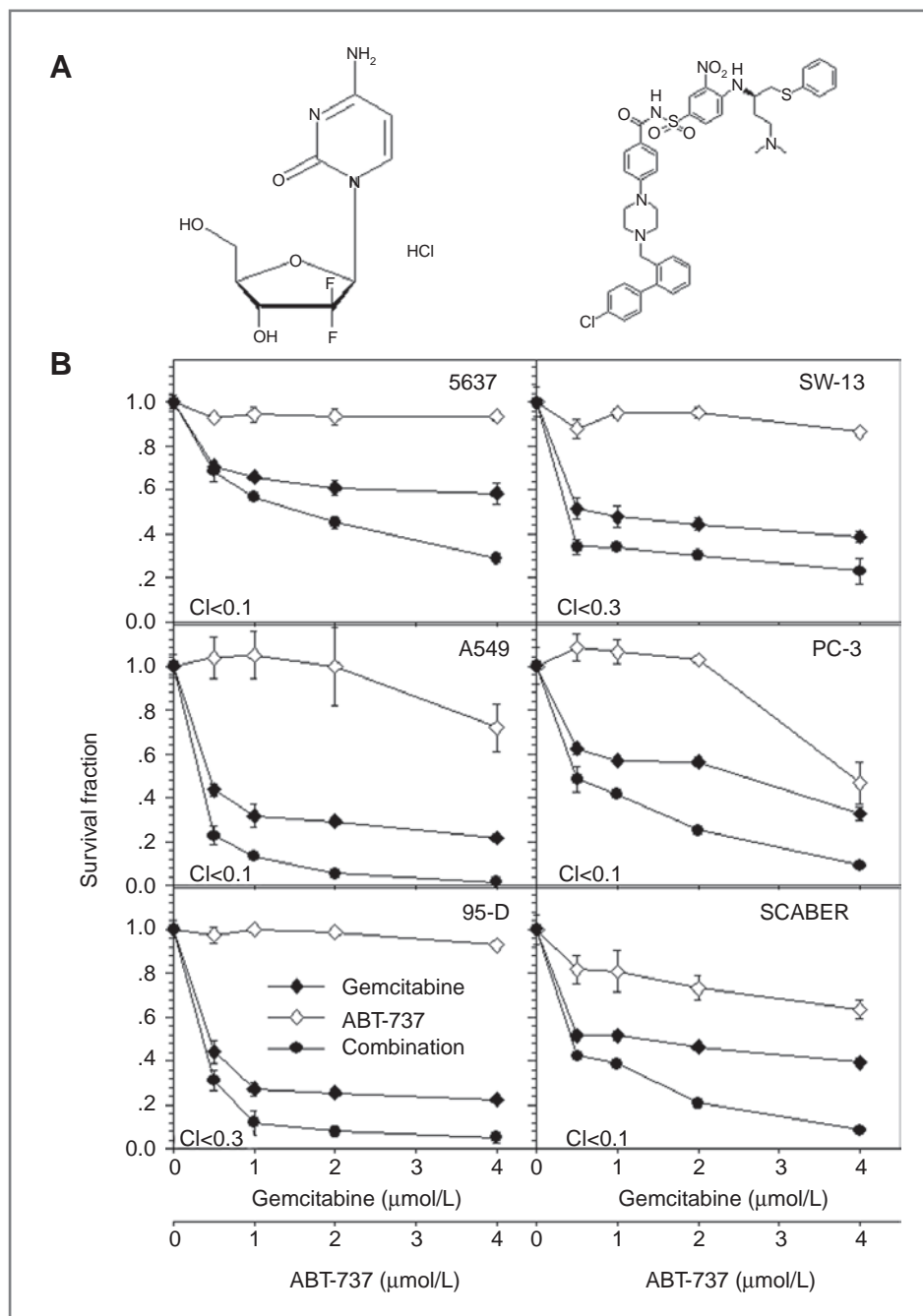


Figure 1. Combination cytotoxicity of gemcitabine and ABT-737. **A**, chemical structures of gemcitabine and ABT-737. **B**, the cells were incubated with compounds for 72 hours. Dose-response curves of human cancer cell lines to gemcitabine, ABT-737, or the combination. The concentrations applied for the cell lines were 0.5 to 4 $\mu\text{mol/L}$ for gemcitabine and ABT-737.

agents against acute myeloid leukemia (AML; refs. 20, 21), multiple myeloma (22), lymphoma (23, 24), chronic lymphocytic leukemia (25), small-cell lung cancer (19, 26), head and neck squamous cancer (27), and acute lymphoblastic leukemia (1, 28). Given that ABT-737 binds to Mcl-1 with low affinity, the high basal expressions of Mcl-1 in small-cell lung cancer cells (29, 30) and in other types of cancer cells (19, 31) have been validated to associate with the resistance to ABT-737 (24). In our study, we showed for the first time that gemcitabine and ABT-737 in combination had substantial synergistic antitumor efficacy

against human cancer cells both *in vitro* and *in vivo*. Chemical structures of the drugs are shown in Fig. 1A.

Materials and Methods

Materials

Gemcitabine hydrochloride was supplied by Shanghai Institute of Materia Medica, Chinese Academy of Sciences. ABT-737 was synthesized (>99% purity) according to the literature (Supplementary Materials and Methods; Supplementary Fig. S1; ref. 19). The primary antibodies against

USP9X (5G-02), PARP (H250), procaspase-3 (E-8), Mcl-1 (22), Bax (2D2), ubiquitin (P4D1), β -actin (C-11), GSK3 β (H-76), p-GSK3 β (Ser-9), and horseradish peroxidase-labeled secondary anti-goat, anti-mouse, and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology; cleaved caspase-3 (D-175) from Cell Signaling Technology. Cycloheximide (CHX) was purchased from Sigma-Aldrich.

Cell culture

Human renal carcinoma cell lines (SW-13), human lung cancer cell lines (95-D and A549), human prostate cancer cell line PC-3, and human bladder carcinoma cell lines (5637 and SCABER) were purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China); they were tested and authenticated for genotypes by DNA fingerprinting. The cell lines were passaged for less than 6 months, and no authentication was done. All these cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS plus 2 mmol/L glutamine and 50 units/mL penicillin, pH 7.4, in a humidified atmosphere of 95% air plus 5% CO₂ at 37°C.

Cytotoxicity assay

The antiproliferative activity of combination treatment with gemcitabine and ABT-737 was measured by MTT assay (32). The inhibition rate on cell proliferation was calculated for each well as [(A570 control cells–A570 treated cells)/A570 control cells] \times 100%.

Analysis of apoptosis by Annexin V and propidium iodide staining

Apoptosis was quantified using the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's instructions. Briefly, cells were then incubated with 5 μ L Annexin V at room temperature for 15 minutes in the dark. Before flow cytometric analysis, 5 μ L of 50 μ g/mL propidium iodide (PI) stock solution was added to the samples. For each sample, 1×10^4 cells were collected and analyzed using an FACSCalibur cytometer (Becton Dickinson).

Determination of mitochondrial membrane depolarization

Cells (5×10^5 /well) were exposed to gemcitabine, ABT-737, or the combination for 12, 24, and 36 hours, collected, and resuspended in fresh medium containing 10 μ g/mL 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1). After incubation at 37°C for 30 minutes, cells were analyzed by flow cytometry.

Protein preparation from tissue samples and Western blot analysis

Tumor samples were homogenized in extraction buffer (8 mol/L urea, 10% glycerol, 10 mmol/L Tris-HCl, pH 6.8, 1% SDS, 5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl, 1 μ g/mL aprotinin, 10 μ g/mL pepstatin, and 10 μ g/mL leupeptin) by using Dounce

tissue grinders. After centrifugation of tissue homogenates, the supernatants were transferred to a new tube and the protein concentration was determined. Proteins were fractionated on 10% to 15% Tris-glycine gels and then they were transferred to nitrocellulose membrane (Pierce Chemical) and probed with primary antibodies (dilution range: 1:500–1:1,000) followed by horseradish peroxidase-labeled secondary antibodies at 1:2,000 dilution. Antibody binding was then detected with the use of a chemiluminescent substrate and visualized on an autoradiography film.

Analysis of Bax conformational change by flow cytometry

Cells were fixed and permeabilized using FIX & PERM cell permeabilization reagents. Fixed cells were incubated with anti-Bax antibody on ice for 30 minutes. After washing with PBS, the binding of antibody was visualized with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:200; Sigma). A total of 10,000 cells were analyzed using Cell Quest software (BD Biosciences).

Real-time reverse transcriptase PCR

Total RNA was extracted from sample cells with TRIzol, precipitated with isopropyl alcohol, and rinsed with 70% ethanol. Single-strand cDNA was prepared from the purified RNA using oligo(dT) priming (Thermoscript RT Kit; Invitrogen), followed by SYBR-Green real-time PCR (Qiagen). The primers used are as follows: Mcl-1, 5'-GGGCAGGATTGTGACTCTCATT-3', 5'-GATGCAGCTTTCTTGGTTTATGG-3'; USP9X, 5'-CCTGCTGGTGCA-CCTCTGGC-3', 5'-AGGCCGGTGTCCCATGCAA-3'; and GAPDH, 5'-GAGTCAACGGATTTGGTCTG-3', 5'-TTGATTTTGGAGGGATCTCG-3'.

Immunoprecipitation

Cell lysates (250 μ g of total protein) were incubated with primary antibodies (1:50) at slow rotation for 4 hours, followed by Protein A/G-conjugated agarose for an additional 1 hour. The beads were washed 4 times with lysis buffer containing protease inhibitor and phosphatase inhibitors, and the supernatant was removed by centrifugation (3,000 \times g, 1 minute). The pellet was mixed with loading buffer and heated to 95°C for 5 minutes, followed by immunoblotting.

Mcl-1 and USP9X gene silencing by short interfering RNA

Cells (5×10^4) were incubated overnight in 6-well plates. On day 2, the medium was replaced with Opti-MEM I Reduced Serum Media (GIBCO) containing 20.0 nmol/L Mcl-1 or USP9X short interfering RNA (siRNA; GenePharma) and oligofectamine reagent (Invitrogen Corporation) according to the manufacturer's recommendations. The sense sequences of the Mcl-1 and USP9X siRNA were 5'-CGCCGAAUUCAUUAAUUUA-3' and 5'-AGAAAUCGCGUGUAUAAAUUU-3', respectively (4).

Animals and antitumor activity *in vivo*

Human lung cancer 95-D xenografts were established by injecting 5×10^6 cells subcutaneously into nude mice. When the tumor reached a volume of 50 to 150 mm³, the mice were randomized to control and treated groups and then received vehicle [1% DMSO, 7% Cremophor/ethanol (3:1), and 92% PBS, i.p. administration], gemcitabine (20 mg/kg, i.p. administration) once per week, ABT-737 (100 mg/kg, i.p. administration) twice per week for 20 days ($n = 10$ per group). Tumor volume (V) was calculated as $V = (\text{length} \times \text{width} \times \text{height})/2$. The tumor volume at day n was expressed as relative tumor volume (RTV) according to the following formula: $\text{RTV} = \text{TV}_n/\text{TV}_0$, where TV_n is the tumor volume at day n and TV_0 is the tumor volume at day 0. Therapeutic effects of treatment were expressed in terms of T/C%, using the following formula: $\text{T/C} (\%) = (\text{mean RTV of the treated group}/\text{mean RTV of the control group}) \times 100\%$ (33).

Statistical analyses

Two-tailed Student's t tests were used to determine the significance of differences between the experiment conditions. For *in vitro* experiments, combination index (CI) values were calculated for each concentration of gemcitabine, ABT-737, and the combination in cell proliferation assays by using CalcuSyn (Biosoft; refs. 34, 35). Different CI values were obtained when solving the equation for different effect levels, and the 80% effect was chosen for presentation. A CI value less than 0.9 indicated synergism; 0.1, very strong synergism; 0.1 to 0.3, strong synergism; 0.3 to 0.7, synergism; 0.7 to 0.85, moderate synergism; 0.85 to 0.9, slight synergism; 0.9 to 1.10, additive; and more than 1.10, antagonism.

Results

Cytotoxicity of the gemcitabine and ABT-737 combination in human cancer cell lines

We determined the cytotoxicity of gemcitabine and ABT-737 at clinically achievable concentrations ranging from 0.5 to 4 $\mu\text{mol/L}$ in 6 human carcinoma cell lines by using the MTT cytotoxicity assay. Survival curves to gemcitabine, ABT-737, and gemcitabine combined with ABT-737 are shown in Fig. 1B. The *in vitro* cytotoxicity of ABT-737 as a single agent was not concentration dependent; a 10-time greater concentration resulted in a less than 50% increase in cytotoxicity in all 6 human cancer cell lines. However, in combination with gemcitabine, ABT-737 showed strong synergy ($\text{CI} < 0.3$) in the carcinoma cell lines tested (Fig. 1B).

Gemcitabine synergized with ABT-737 to trigger apoptosis

Gemcitabine plus ABT-737 induced apoptosis and depolarization of mitochondrial membrane potential. We first detected apoptosis by Annexin V/PI staining in the 95-D and 5637 cell lines. 95-D cells were treated with 2

$\mu\text{mol/L}$ gemcitabine, 2 $\mu\text{mol/L}$ ABT-737, or the combination for 12 and 24 hours. As shown in Fig. 2A, the proportion of apoptotic 95-D cells was 7.70% in control cells, 19.60% with gemcitabine, 9.60% with ABT-737, and 34.30% in the combination treatment group (exposure time: 12 hours). As shown in Fig. 2A, combined treatment with gemcitabine and ABT-737 resulted in an increased percentage of mitochondrial membrane depolarized 95-D cells than either agent used alone (18.30% in combination treated cells, 3.30% in gemcitabine-treated cells, 3.40% in ABT-737-treated cells, and 2.90% in control group). Combination treatment with gemcitabine and ABT-737 resulted in increased apoptosis and mitochondrial membrane potential in a time-dependent manner in the 95-D and 5637 cell lines (Fig. 2B and C).

Combination therapy activated caspase cascades.

We observed that treatment of cells with gemcitabine plus ABT-737 for 24 hours caused a significantly greater activation of procaspase-3 than did either agent used alone (Fig. 2D). To further investigate whether caspase activation could play a role in the cytotoxicity induced by gemcitabine or ABT-737 singly or in combination, we pretreated 95-D cells with the pan-caspase inhibitor Boc-D-fmk before treatment with gemcitabine, ABT-737, or the combination. Only 5.54% of apoptotic cells pretreated with Boc-D-fmk for 1 hour, followed by incubation with gemcitabine and ABT-737, were detected compared with 33.86% in combination treatment group, suggesting that the cytotoxicity induced by combination treatment of 95-D cells was caspase dependent (Fig. 3A).

Gemcitabine combined with ABT-737 abolished the interaction between Mcl-1 and Bax. Several studies have shown that high levels of Mcl-1 confer resistance to ABT-737 (36). We were thus interested in examining the involvement of Mcl-1 in the gemcitabine and ABT-737 combination treatment. Interestingly, we found that ABT-737 increased the expression of Mcl-1 in ABT-737-resistant 95-D and 5637 cell lines whereas Mcl-1 expression was dramatically downregulated in gemcitabine plus ABT-737 combination treated cells compared with gemcitabine alone, indicating that Mcl-1 might be involved in the synergistic effect of combination treatment (Fig. 2D). Mcl-1 could heterodimerize with Bax to prevent mitochondrial membrane permeabilization (37). We determined the involvement of Bax in enhanced mitochondrial control of apoptosis induced by the combination of gemcitabine and ABT-737. Although the protein level of Bax remained unchanged in combination treated 95-D cells (Supplementary Fig. S2), a significant conformational change was observed using flow cytometric analysis with an antibody against the activated form of the Bax protein (Fig. 3B, left). To confirm the synergistic mechanism of combination, coimmunoprecipitation study of Mcl-1 with Bax was carried out on lysates from gemcitabine, ABT-737, combination, and vehicle-treated 95-D cells. As shown in Fig. 3B, right, there were large amounts of Bax immunoprecipitated with Mcl-1 in ABT-737 or vehicle-treated cells, indicating that Mcl-1 exerted

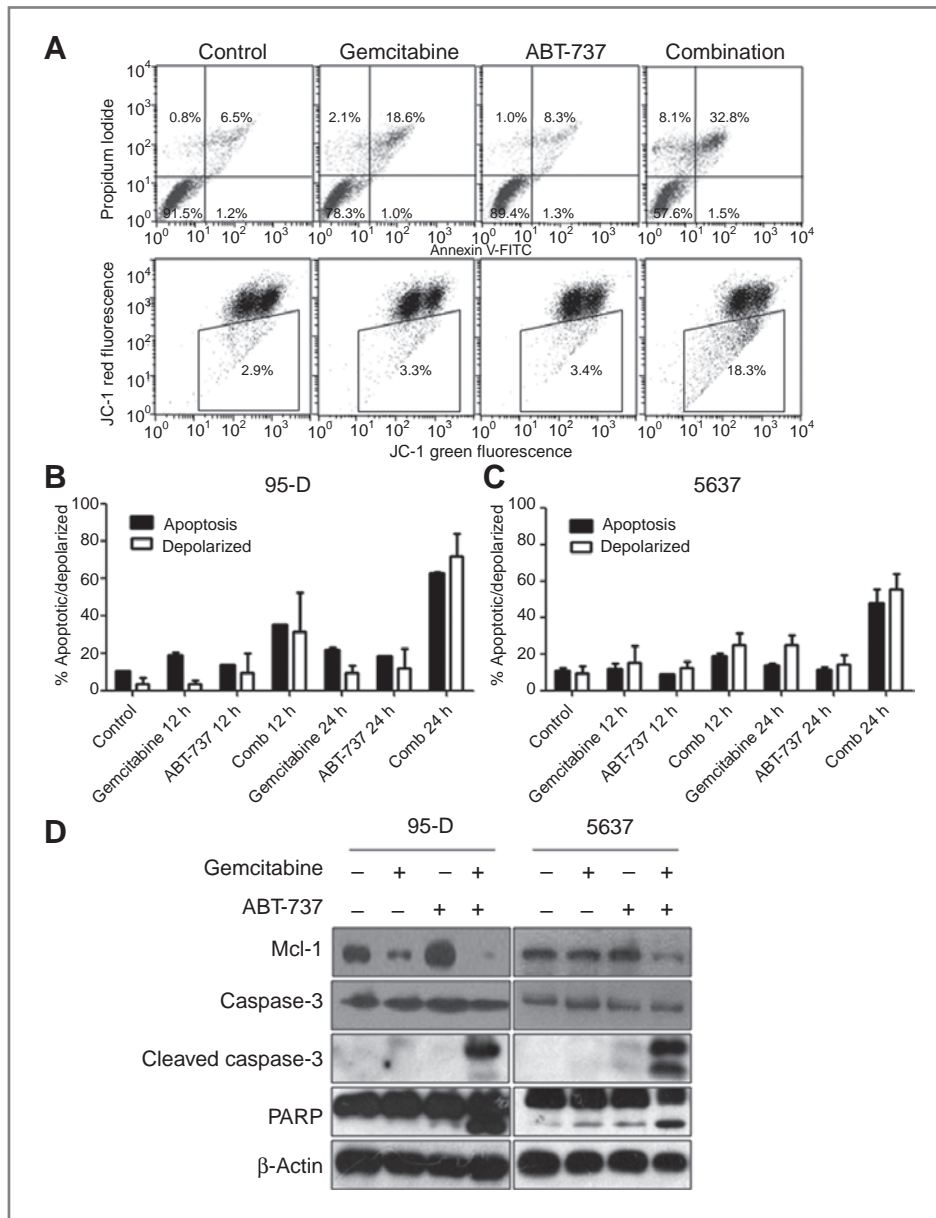


Figure 2. Gemcitabine plus ABT-737 caused enhanced apoptosis, mitochondrial membrane depolarization, and activation of various apoptosis-related proteins. A, 95-D cells were treated with gemcitabine (2 μ mol/L), ABT-737 (2 μ mol/L), or the combination for 12 hours, and then cells were incubated with Annexin V/PI (top) or JC-1 (bottom) and analyzed by flow cytometry. B and C, cells in 6-well plates were exposed to gemcitabine (2 μ mol/L), ABT-737 (2 μ mol/L), or the combination for 12 and 24 hours and then cells were exposed to Annexin V/PI (top) or JC-1 (bottom) and analyzed by flow cytometry. D, cells were exposed to gemcitabine (2 μ mol/L), ABT-737 (2 μ mol/L), or the combination for 24 hours, after which protein extracts were immunoblotted with specified antibodies for PARP, Mcl-1, caspase-3, and cleaved caspase-3.

Downloaded from <http://aascjournals.org/mcl/article-pdf/10/7/1264/2320065/1284.pdf> by guest on 14 April 2024

its antiapoptotic function by heterodimerizing with Bax. In contrast, levels of Bax coimmunoprecipitated with Mcl-1 were markedly decreased in the combination treated cells compared with the gemcitabine treatment group, indicating that gemcitabine could sensitize cells to ABT-737 by abrogating the interaction between Mcl-1 and Bax in 95-D cells.

The combination of gemcitabine and ABT-737 promoted the degradation of Mcl-1

The ubiquitin-proteasome system was activated by the combination of gemcitabine and ABT-737. To determine whether the synergistic reduction of Mcl-1 protein by gemcitabine and ABT-737 combination treatment was

the result of transcriptional inhibition, Mcl-1 mRNA levels were evaluated by real-time reverse transcriptase PCR (RT-PCR) in 95-D cells treated with 2 μ mol/L gemcitabine, 2 μ mol/L ABT-737, or the combination. As predicted, ABT-737 alone resulted in overexpression of Mcl-1 mRNA whereas no apparent synergistically inhibitory effects on Mcl-1 mRNA levels were observed in the combination treated group in 95-D cells (data not shown). Like all proteins, the equilibrium between production and degradation determines the protein level of Mcl-1 and the stability of Mcl-1 may be critically important in many physiologic and pathologic situations (3). We thus hypothesized that the putative ubiquitination of Mcl-1 in response to gemcitabine and ABT-737 combination

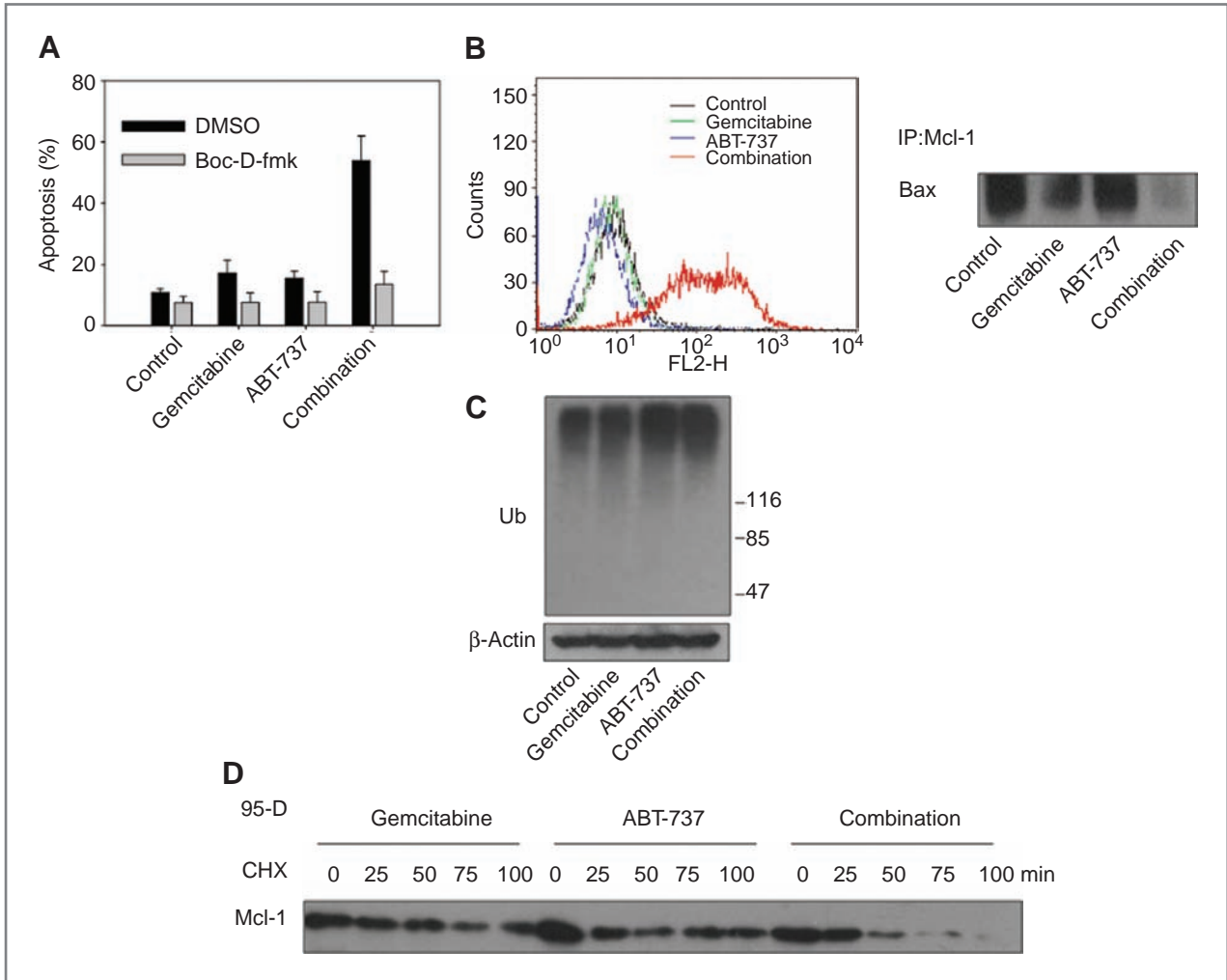


Figure 3. Gemcitabine plus ABT-737 caused enhanced caspase-dependent apoptosis and degradation of Mcl-1. **A**, 95-D cells were pretreated with pan-caspase inhibitor Boc-D-fmk (10 μ mol/L) for 1 hour and then treated with 2 μ mol/L gemcitabine and/or ABT-737 for 24 hours. The cells were analyzed for apoptosis by flow cytometry. **B**, left, 95-D cells in 6-well plates were exposed to gemcitabine (2 μ mol/L), ABT-737 (2 μ mol/L), or the combination for 24 hours and then Bax conformational change was detected in the FL2-H channel by flow cytometry. Right, 95-D cells were harvested in the presence of gemcitabine, ABT-737, or the combination followed by immunoprecipitation with Mcl-1 (1:50). The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted using Bax (1:1,000). **C**, 95-D cells were exposed to gemcitabine (2 μ mol/L), ABT-737 (2 μ mol/L), or the combination for 18 hours, after which protein extracts were immunoblotted with the ubiquitin (Ub). **D**, cells were treated with CHX (200 μ g/mL) to block new protein synthesis, and the degradation of Mcl-1 in the presence of 20 μ mol/L gemcitabine and/or ABT-737 at 25, 50, 75, and 100 minutes was detected by Western blotting.

treatment might play a key role in the synergistic effect. Indeed, Fig. 3C shows that gemcitabine plus ABT-737 caused a large increase in the amount of ubiquitination. To further investigate this hypothesis, we treated 95-D cells with CHX (200 μ g/mL) to block new protein synthesis and observed Mcl-1 degradation in the presence of 20 μ mol/L gemcitabine and/or ABT-737. We compared the half-life of Mcl-1 in 95-D cells treated with CHX in the presence of gemcitabine, ABT-737, or the combination. Figure 3D shows that Mcl-1 protein levels decreased more rapidly in the combination treatment group than that in single-agent groups, indicating that a promotion of Mcl-1 degradation was involved in the synergistic effect of gemcitabine and ABT-737.

Increased Mcl-1 levels led to resistance to ABT-737.

To explore the mechanism of differing ABT-737 sensitivity among cell lines, we first examined the cytotoxicity of ABT-737 in 4 human carcinoma cell lines (Supplementary Fig. S3). In addition, we explored the relationship between ABT-737 cytotoxicity and the expression of Mcl-1 and USP9X. Next, we selected 2 highly resistant ABT-737 cell lines (95-D and 5637) and 1 ABT-737-sensitive cell line (SCABER) and examined the Mcl-1 and USP9X protein and mRNA levels after treatment with ABT-737 for 12 and 18 hours (Fig. 4A and B). As shown in Fig. 4A and B, Mcl-1 mRNA and protein levels increased with exposure to ABT-737 in the ABT-737-resistant cell lines (Mcl-1 mRNA level fold increased after 18 hours

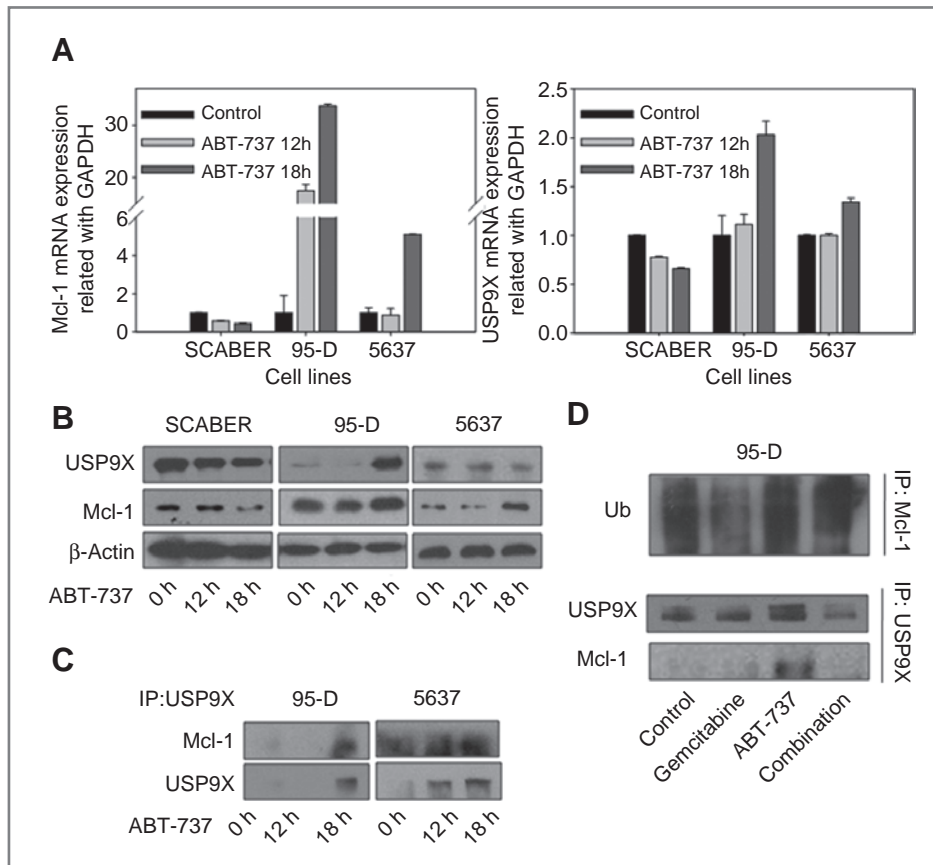


Figure 4. Gemcitabine plus ABT-737 disrupted the interaction of USP9X and Mcl-1. A, SCABER, 95-D, and 5637 cells in 6-well plates were exposed to ABT-737 (2 μ mol/L) for 12 and 18 hours. The mRNA levels of Mcl-1 and USP9X were detected by RT-PCR. B, the protein levels of Mcl-1 and USP9X were determined by Western blotting in SCABER, 95-D, and 5637 cell lines. C, 95-D and 5637 cells treated with ABT-737 for 12 and 18 hours were used to prepare lysates for immunoprecipitation analysis. D, the interaction between USP9X and Mcl-1 was detected by immunoprecipitation in 95-D cells treated with gemcitabine and/or ABT-737 for 18 hours.

ABT-737 treatment compared with vehicle control for 5637 and 95-D, which were 4.3-fold and 33.7-fold, respectively). In contrast, we observed that Mcl-1 mRNA and protein levels decreased with increased exposure to ABT-737 in SCABER cells.

GSK3 β has been shown to increase Mcl-1 turnover and thereby promote apoptosis (38). However, we did not observe a change in p-GSK3 β (Ser-9) expression with gemcitabine and ABT-737 combination treatment (Supplementary Fig. S2). The USP9X–Mcl-1 interaction stabilizes Mcl-1 and thereby promotes cell survival, which implicates overexpression of Mcl-1 and USP9X (4). Our results showed that USP9X protein and mRNA increased in ABT-737-resistant cell lines but decreased in the ABT-737-sensitive cell line, implicating the relationship between sensitivity to ABT-737 and the overexpression of USP9X (Fig. 4A and B). Therefore, we next examined the interaction between USP9X and Mcl-1 in 95-D and 5637 cells treated with 2 μ mol/L ABT-737 by immunoprecipitation and found that ABT-737 could increase the binding of USP9X to Mcl-1 in 2 ABT-737-resistant cell lines (Fig. 4C).

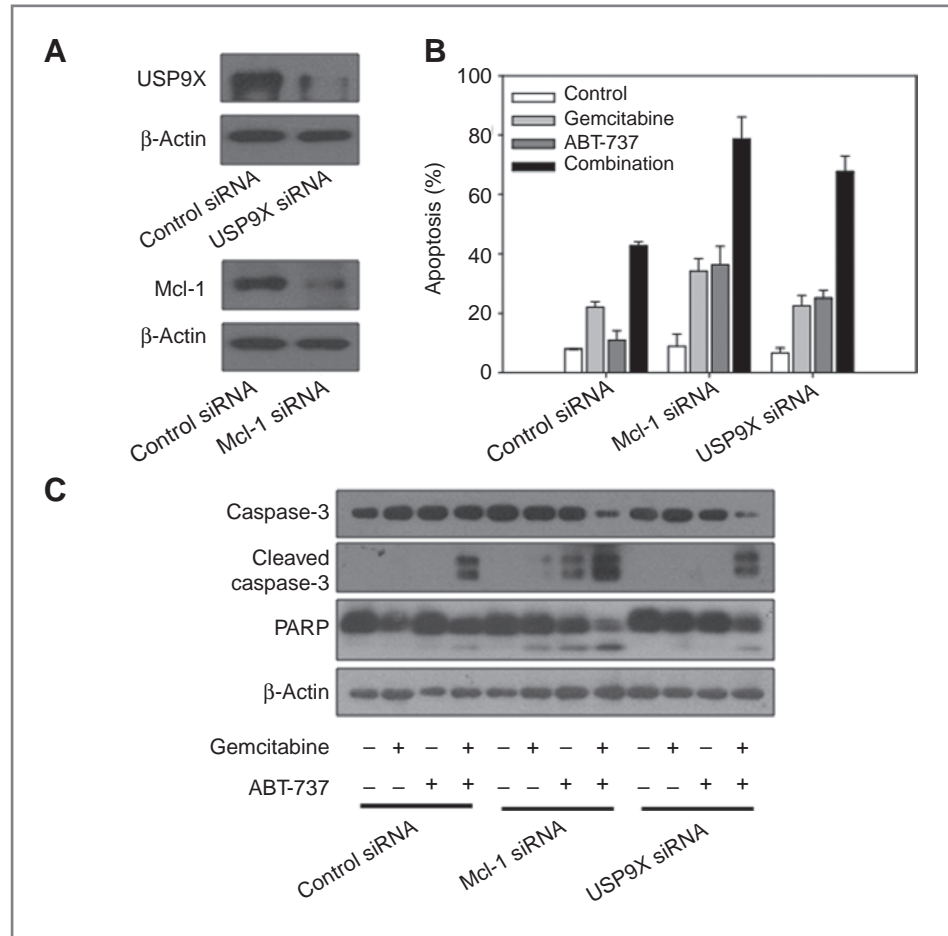
Gemcitabine disrupted the enhanced interaction of USP9X and Mcl-1 by ABT-737. The above data implied that the ABT-737 resistance was likely due to the interaction of USP9X and Mcl-1. To explore mechanisms

underlying the ABT-737 sensitivity that was induced by gemcitabine, we first examined Mcl-1–ubiquitin conjugation in gemcitabine- and/or ABT-737-exposed cells by immunoprecipitation. As shown in Fig. 4D (top), gemcitabine plus ABT-737 treatment could increase the ubiquitination of Mcl-1 compared with single-agent treatment, indicating that Mcl-1 degradation by ubiquitination was involved in the synergistic effect of gemcitabine and ABT-737. Next, we tested whether gemcitabine plus ABT-737 treatment could affect the interaction between USP9X and Mcl-1 in 95-D cells. Intriguingly, we observed that gemcitabine could disrupt the increased interaction between USP9X and Mcl-1 caused by ABT-737 treatment (Fig. 4D, bottom). Thus, gemcitabine might attenuate the deubiquitination of Mcl-1, which would agree with our observation that the combination treatment could increase the ubiquitination of Mcl-1 in 95-D cells. These results showed that gemcitabine could sensitize cells to ABT-737 via disrupting the interaction between USP9X and Mcl-1 to inhibit the expression of Mcl-1.

RNA interference of Mcl-1 and USP9X sensitized cells to combination gemcitabine and ABT-737 treatment

To validate the contribution of the modulation on USP9X and Mcl-1 by the gemcitabine and ABT-737

Figure 5. Mcl-1 and USP9X were involved in synergistic induction of apoptosis by gemcitabine and ABT-737 treatment. A, 95-D cells were transfected with Mcl-1 or USP9X siRNA according to the manufacturer's recommendations. Forty-eight hours after transfection, cell lysates were prepared for Western blot analysis. The ratio of apoptosis (B) and the expression of caspase-3 and PARP (C) in 95-D cells that had been transfected with Mcl-1 or USP9X siRNA and treated with 2 μmol/L gemcitabine, either alone or in combination with 2 μmol/L ABT-737, for 18 hours were examined.



combination treatment, we depleted USP9X and Mcl-1 with siRNA in 95-D cells, respectively. As shown in Fig. 5A, Mcl-1 siRNA successfully abolished the Mcl-1 protein level and knockdown of USP9X reduced the expression of USP9X in 95-D cells.

Mcl-1 depletion increased gemcitabine plus ABT-737-induced apoptosis. Next we aimed to determine whether Mcl-1 could affect the synergistic effect of gemcitabine and ABT-737. We examined apoptotic ratio and caspase-3 and PARP expression in 95-D cells with or without siRNA transfection targeting Mcl-1, upon the treatment with 2 μmol/L gemcitabine, either alone or in combination with 2 μmol/L ABT-737. As shown in Fig. 5B, Mcl-1 depletion restored the sensitivity to ABT-737 in ABT-737-resistant 95-D cells, consistent with the reported data (4). Furthermore, gemcitabine plus ABT-737 induced only 48.78% apoptosis in 95-D cells treated with control siRNA for 18 hours, compared with 75.75% apoptosis in the Mcl-1 siRNA group (Fig. 5B). In addition, Fig. 5C showed that Mcl-1 depletion resulted in greater activation of caspase-3 and PARP cleavage in 95-D cells treated with gemcitabine plus ABT-737. These data collectively suggested that Mcl-1 participated in the syner-

gistic effect of gemcitabine and ABT-737 combination treatment.

USP9X knockdown amplified the apoptosis induced by gemcitabine and ABT-737. Figure 5B shows that USP9X knockdown sensitized 95-D cells to the apoptosis induced by gemcitabine plus ABT-737 combination, but it had little effect on either gemcitabine or ABT-737 single-treatment groups. Furthermore, the caspase cascade was significantly activated when USP9X-depleted 95-D cells were treated with gemcitabine plus ABT-737 (Fig. 5C), as indicated by the decreased levels of pro-caspase-3 and PARP. These results showed the vital role of USP9X downregulation in the apoptosis caused by the combination of gemcitabine and ABT-737 in 95-D cells.

The antitumor activity of gemcitabine and ABT-737 combination therapy against human 95-D xenografts

The combination of gemcitabine and ABT-737 arrested tumor growth. To further characterize the anticancer efficacy of gemcitabine and ABT-737 combination treatment, the *in vivo* activity of gemcitabine and ABT-737 was

Downloaded from <http://aacrjournals.org/mcl/article-pdf/10/7/1264/2320065/1264.pdf> by guest on 14 April 2024

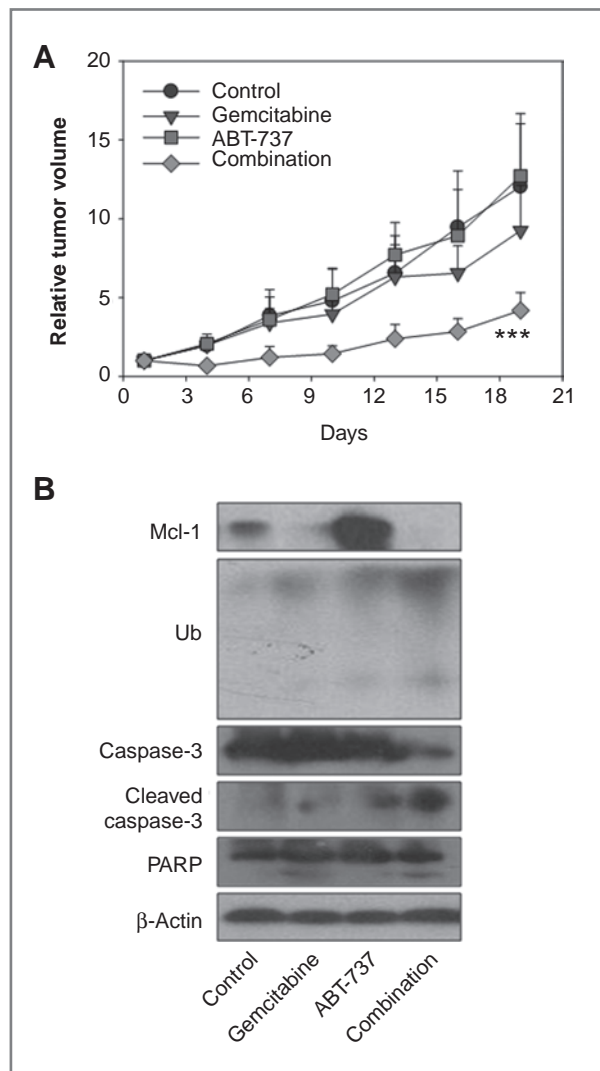


Figure 6. Efficacy of gemcitabine combined with ABT-737 treatment regimen *in vivo*. **A**, the mice transplanted with 95-D human xenografts were randomly divided into 4 groups and given injection of gemcitabine (20 mg/kg), ABT-737 (100 mg/kg), combination, or vehicle for a period of 20 days. RTVs are expressed as mean \pm SD ($n = 10$ per group). **B**, expression of the apoptosis-related proteins extracted from tumor tissues in the animal experiment was detected by Western blotting.

tested in a lung cancer 95-D xenograft model in nude mice. As shown in Fig. 6A, the i.p. administration of ABT-737 at a dose of 100 mg/kg twice per week for 20 days produced no significant difference in mean RTV compared with that of the control group (mean RTV, ABT-737 vs. control: 12.0 vs. 12.7; $P > 0.05$). However, with the dosage of 20 mg/kg every week for 20 days, gemcitabine exerted a moderate tumor growth inhibitory effect (mean RTV, gemcitabine vs. control: 9.3 vs. 12.0; $P < 0.05$). As predicted, gemcitabine plus ABT-737 caused marked tumor growth inhibition (T/C value: 33.0%), significantly greater than gemcitabine (T/C value: 81.3%) or ABT-737 treatment alone (T/C

value: 104.6%; mean RTV, combination vs. gemcitabine: 4.2 vs. 9.3; $P < 0.01$). Furthermore, compared with the initial body weights, combination treated mice showed no significant body weight loss on day 20. Thus, the synergistic effect of gemcitabine and ABT-737 was further validated *in vivo* on 95-D xenografts.

The combination therapy induced apoptosis and down-regulated Mcl-1 in tumor tissues. We next explored the effect of gemcitabine and ABT-737, alone and in combination, on the expression of the apoptosis-related proteins in tumor tissues from drug-administrated mice. As shown in Fig. 6B, caspase activation was triggered with the combination therapy in nude mice. Importantly, the expression of Mcl-1 was consistent with the aforementioned cell culture data (Fig. 2D), highlighting the involvement of these proteins in the tumor growth inhibitory effects exerted by gemcitabine and ABT-737 *in vivo*.

Discussion

ABT-737, a potent small-molecule inhibitor of anti-apoptotic members of the Bcl-2 family, is a promising therapeutic agent for multiple malignancies. However, the majority of the cell lines derived from solid tumors seem to be resistant to ABT-737 treatment (28, 39). Mcl-1 is the major factor that causes resistance to ABT-737 in cancer cells derived from diverse solid tumors. Gemcitabine has been widely used for patients with relapsed or refractory solid tumors, especially when combined with chemotherapeutic agents (40–43), aiming to improve the anticancer efficiency with lower toxicity. Downregulation of Mcl-1 was observed with treatments of gemcitabine combined with other chemotherapeutic agents, thus the combination of gemcitabine (Mcl-1-downregulating agents) with ABT-737 could be potent therapeutic regimens for patient with ABT-737-resistant solid tumors (22).

In our preliminary *in vivo* experiment, mice were administrated with 100 mg/kg gemcitabine and/or 100 mg/kg ABT-737 once weekly for 19 days (the dosing regimens in the literature; refs. 40, 44), but severe toxicity was observed in mice administrated with gemcitabine. Although gemcitabine decreased tumor weight by 77%, 1 of 5 mice died of gemcitabine-related toxicity, and for remainder, toxicity became unacceptable, as the body weight loss was more than 30%. Thus, in the present study, gemcitabine was administrated at the dosage of 20 mg/kg once weekly and tumor growth inhibitory effect was observed from day 7 whereas gemcitabine plus ABT-737 synergistically arrested tumor growth from day 4. The synergistic effect was observed from the 95-D xenograft nude mice model (Fig. 6A). As single agents, gemcitabine and ABT-737 merely displayed insignificant activities against 95-D xenograft model, respectively; in contrast, the coadministration of gemcitabine and ABT-737 apparently arrested tumor growth by 56.5%. Moreover,

there was no difference in body weight loss between combination and gemcitabine treatment groups. These results suggested that gemcitabine and ABT-737 combination synergistically inhibited tumor growth and had minimal toxicity *in vivo*.

In our study, the synergistic anticancer effects *in vitro* and *in vivo* achieved by gemcitabine plus ABT-737 were observed in human cancer cells. Our results showed that the cytotoxicity of gemcitabine as a single agent and the combination cytotoxicity of gemcitabine plus ABT-737 in the 95-D and 5637 cell lines occurred via caspase-dependent apoptosis. In addition, loss of mitochondrial membrane potential was significantly greater with gemcitabine plus ABT-737 than with either drug used alone. Cytosolic Bax is unable to induce apoptosis, and blocking Bax translocation inhibits cell death (45). We found that Bax conformational change occurred only in 95-D cells exposed to both gemcitabine and ABT-737. We also detected changes in p53, NF- κ B, and Bcl-2 family proteins (i.e., Bcl-xL, Bid, and Bcl-2) in 95-D and 5637 cell lines treated with gemcitabine and/or ABT-737 for 12 and 24 hours. However, no synergistic effect was observed for these proteins, indicating that they were not involved in the cytotoxicity induced by the combination of gemcitabine and ABT-737 (data not shown).

The basal expression level of Mcl-1, to which ABT-737 has a low affinity, has been associated with resistance to ABT-737 in AML (20), lymphoma (23), CLL (25), and many other cancer types (22, 26, 46). ABT-737 was less efficient in killing tumor cells exhibiting relatively high levels of Mcl-1 (47), and downregulation of Mcl-1 was observed in cancer cell sensitization to ABT-737. We found that the basal expression of Mcl-1 in 5 human solid tumor cell lines was not associated with sensitivity to ABT-737 (data not shown). However, overexpression of the proapoptotic protein Bax was observed in the ABT-737-sensitive cell line SCABER, indicating that the basal expression of Bax may be involved in ABT-737 sensitivity of solid tumors (data not shown). This phenomenon needed to be further investigated in other solid tumor cell lines. Although the basal expression of Mcl-1 had no correlation with ABT-737 sensitivity in solid tumors, we found that the resistance to ABT-737 was associated with an increase in Mcl-1 protein expression in response to ABT-737 in the cell lines tested. In the meanwhile, we found that gemcitabine and ABT-737 exerted synergistic effect on cancer cells with decreased expression of Mcl-1 protein (Fig. 2D). Thus, we hypothesized that low expression of Mcl-1 contributed to the synergistic effect in 95-D cell lines. To further confirm the role of Mcl-1 in gemcitabine and ABT-737 combination, we designed Mcl-1 siRNA experiment and found that knockdown of Mcl-1 by siRNA could significantly sensitize 95-D cells to apoptosis induced by gemcitabine and ABT-737 combination. The low level of Mcl-1 could make Bax

to release more easily and induce more apoptosis (23). Therefore, our observations suggested that Mcl-1 might be involved in the synergistic effect of gemcitabine and ABT-737 combination.

In recent years, more and more studies have revealed the involvement of deubiquitinating enzymes in cancers as well as in other diseases (8, 48). USP9X, a ubiquitin-specific protease family member, is shown to regulate ubiquitination of different signal transduction pathway proteins, including AMPK (48), TGF β (8), ErbB2/HER2 (49), and ASK1 (7). It has been reported that deubiquitinase USP9X stabilizes Mcl-1 and promotes tumor cell survival. Knockdown of USP9X increases Mcl-1 polyubiquitination, which enhances Mcl-1 turnover and cytotoxicity by ABT-737 (4). In the present study, we observed that knockdown of USP9X in 95-D cells increased gemcitabine plus ABT-737-induced apoptosis but not apoptosis was induced by gemcitabine or ABT-737 alone (2 μ mol/L). In addition, a significant increase in ABT-737 (4 μ mol/L)-triggered apoptosis was observed in the cells transfected with USP9X siRNA compared with that of the control siRNA group (data not shown). We also showed that the increase in Mcl-1 protein expression might be due to the enhanced interaction between USP9X and Mcl-1 in ABT-737-resistant solid tumor cell lines upon treatment with ABT-737, and this interaction could be disrupted by gemcitabine, which might contribute to the sensitization to ABT-737.

In conclusion, we present evidence showing better therapeutic activity of gemcitabine when combined with ABT-737 both *in vitro* and *in vivo*. Our results also show that gemcitabine plus ABT-737 combination treatment synergistically induced caspase-dependent apoptosis via disrupting the interaction between USP9X and Mcl-1. Therefore, a combination chemotherapy regimen incorporating a small-molecule BH3-mimetic with gemcitabine warrants clinical investigation in solid tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This study was supported by the Zhejiang Provincial Foundation of National Science for Outstanding Youths (R2080326; Q.J. He), Zhejiang Provincial Program for the Cultivation of High-level Innovative Health Talents (B. Yang), Zhejiang Provincial Natural Science Foundation (Y2100682; N.M. Lin), Program for New Century Excellent Talents in University (B. Yang), and Science Research Foundation of Zhejiang Health Bureau (2010QNA009; H. Zhu).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 5, 2010; revised April 15, 2011; accepted April 26, 2011; published OnlineFirst May 12, 2011.

References

- Kang MH, Wan Z, Kang YH, Spoto R, Reynolds CP. Mechanism of synergy of *N*-(4-hydroxyphenyl)retinamide and ABT-737 in acute lymphoblastic leukemia cell lines: Mcl-1 inactivation. *J Natl Cancer Inst* 2008;100:580–95.
- Chen S, Dai Y, Harada H, Dent P, Grant S. Mcl-1 down-regulation potentiates ABT-737 lethality by cooperatively inducing Bak activation and Bax translocation. *Cancer Res* 2007;67:782–91.
- Zhong Q, Gao W, Du F, Wang X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. *Cell* 2005;121:1085–95.
- Schwickart M, Huang X, Lill JR, Liu J, Ferrando R, French DM, et al. Deubiquitinase USP9X stabilizes MCL1 and promotes tumour cell survival. *Nature* 2010;463:103–7.
- Nijman SM, Luna-Vargas MP, Velds A, Brummelkamp TR, Dirac AM, Sixma TK, et al. A genomic and functional inventory of deubiquitinating enzymes. *Cell* 2005;123:773–86.
- Ovaa H, Kessler BM, Rolen U, Galardy PJ, Ploegh HL, Masucci MG. Activity-based ubiquitin-specific protease (USP) profiling of virus-infected and malignant human cells. *Proc Natl Acad Sci U S A* 2004;101:2253–8.
- Nagai H, Noguchi T, Homma K, Katagiri K, Takeda K, Matsuzawa A, et al. Ubiquitin-like sequence in ASK1 plays critical roles in the recognition and stabilization by USP9X and oxidative stress-induced cell death. *Mol Cell* 2009;36:805–18.
- Dupont S, Mamidi A, Cordenonsi M, Montagner M, Zacchigna L, Adorno M, et al. FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitination. *Cell* 2009;136:123–35.
- Mouchantaf R, Azakir BA, McPherson PS, Millard SM, Wood SA, Angers A. The ubiquitin ligase itch is auto-ubiquitinated *in vivo* and *in vitro* but is protected from degradation by interacting with the deubiquitylating enzyme FAM/USP9X. *J Biol Chem* 2006;281:38738–47.
- Jolly LA, Taylor V, Wood SA. USP9X enhances the polarity and self-renewal of embryonic stem cell-derived neural progenitors. *Mol Biol Cell* 2009;20:2015–29.
- Hussain SA, Palmer DH, Swinson DE, Riley P, Wills A, Brown C, et al. A phase II clinical trial of gemcitabine and split dose cisplatin in advanced non-small cell lung cancer in an outpatient setting. *Oncol Rep* 2008;20:233–7.
- Michael M, Moore M. Clinical experience with gemcitabine in pancreatic carcinoma. *Oncology* 1997;11:1615–22.
- Cohen MH, Rothmann M. Gemcitabine and cisplatin for advanced, metastatic bladder cancer. *J Clin Oncol* 2001;19:1229–31.
- Airoldi M, Cattel L, Passera R, Pedani F, Delprino L, Micari C. Gemcitabine and oxaliplatin in patients with metastatic breast cancer resistant to or pretreated with both anthracyclines and taxanes: clinical and pharmacokinetic data. *Am J Clin Oncol* 2006;29:490–4.
- Bergman AM, Eijk PP, Ruiz van Haperen VW, Smid K, Veerman G, Hubeek I, et al. *In vivo* induction of resistance to gemcitabine results in increased expression of ribonucleotide reductase subunit M1 as the major determinant. *Cancer Res* 2005;65:9510–6.
- Valle JW, Wasan H, Johnson P, Jones E, Dixon L, Swindell R, et al. Gemcitabine alone or in combination with cisplatin in patients with advanced or metastatic cholangiocarcinomas or other biliary tract tumours: a multicentre randomised phase II study—The UK ABC-01 Study. *Br J Cancer* 2009;101:621–7.
- Benekli M, Yildiz R, Uner A, Er O, Yamac D, Alkis N, et al. Gemcitabine plus capecitabine combination in metastatic breast cancer patients previously treated with anthracyclines and taxanes. *Oncology* 2007;72:308–13.
- Mom CH, Verweij J, Oldenhuis CN, Gietema JA, Fox NL, Miceli R, et al. Mapatumumab, a fully human agonistic monoclonal antibody that targets TRAIL-R1, in combination with gemcitabine and cisplatin: a phase I study. *Clin Cancer Res* 2009;15:5584–90.
- Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 2005;435:677–81.
- Konopleva M, Contractor R, Tsao T, Samudio I, Ruvalo PP, Kitada S, et al. Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell* 2006;10:375–88.
- Kojima K, Konopleva M, Samudio IJ, Schober WD, Bornmann WG, Andreeff M. Concomitant inhibition of MDM2 and Bcl-2 protein function synergistically induce mitochondrial apoptosis in AML. *Cell Cycle* 2006;5:2778–86.
- Chauhan D, Velankar M, Brahmandam M, Hideshima T, Podar K, Richardson P, et al. A novel Bcl-2/Bcl-X(L)/Bcl-w inhibitor ABT-737 as therapy in multiple myeloma. *Oncogene* 2007;26:2374–80.
- van Delft MF, Wei AH, Mason KD, Vandenberg CJ, Chen L, Czabotar PE, et al. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* 2006;10:389–99.
- Yecies D, Carlson NE, Deng J, Letai A. Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. *Blood* 2010;115:3304–13.
- Del Gaizo Moore V, Brown JR, Certo M, Love TM, Novina CD, Letai A. Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. *J Clin Invest* 2007;117:112–21.
- Tahir SK, Yang X, Anderson MG, Morgan-Lappe SE, Sarthy AV, Chen J, et al. Influence of Bcl-2 family members on the cellular response of small-cell lung cancer cell lines to ABT-737. *Cancer Res* 2007;67:1176–83.
- Li R, Zang Y, Li C, Patel NS, Grandis JR, Johnson DE. ABT-737 synergizes with chemotherapy to kill head and neck squamous cell carcinoma cells via a Noxa-mediated pathway. *Mol Pharmacol* 2009;75:1231–9.
- Kang MH, Kang YH, Szymanska B, Wilczynska-Kalak U, Sheard MA, Harned TM, et al. Activity of vincristine, L-ASP, and dexamethasone against acute lymphoblastic leukemia is enhanced by the BH3-mimetic ABT-737 *in vitro* and *in vivo*. *Blood* 2007;110:2057–66.
- Wesarg E, Hoffarth S, Wiewrodt R, Kröll M, Biesterfeld S, Huber C, et al. Targeting BCL-2 family proteins to overcome drug resistance in non-small cell lung cancer. *Int J Cancer* 2007;121:2387–94.
- Hauack P, Chao BH, Litz J, Krystal GW. Alterations in the Noxa/Mcl-1 axis determine sensitivity of small cell lung cancer to the BH3 mimetic ABT-737. *Mol Cancer Ther* 2009;8:883–92.
- Lin X, Morgan-Lappe S, Huang X, Li L, Zakula DM, Verneti LA, et al. "Seed" analysis of off-target siRNAs reveals an essential role of Mcl-1 in resistance to the small-molecule Bcl-2/Bcl-XL inhibitor ABT-737. *Oncogene* 2007;26:3972–9.
- Zhu H, Ding WJ, Wu R, Weng QJ, Lou JS, Jin RJ, et al. Synergistic anti-cancer activity by the combination of TRAIL/APO-2L and celastrol. *Cancer Invest* 2010;28:23–32.
- Luo P, He Q, He X, Hu Y, Lu W, Cheng Y, et al. Potent antitumor activity of 10-methoxy-9-nitrocamptothecin. *Mol Cancer Ther* 2006;5:962–8.
- Chou TC TP. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984;22:29.
- Chou TC TP. Generalized equations for the analysis of inhibitions of Michaelis-Menten and higher-order kinetic systems with two or more mutually exclusive and nonexclusive inhibitors. *Eur J Biochem* 1981;115:10.
- Huang S, Sinicrope FA. BH3 mimetic ABT-737 potentiates TRAIL-mediated apoptotic signaling by unsequestering Bim and Bak in human pancreatic cancer cells. *Cancer Res* 2008;68:2944–51.
- Marrriott HM, Bingle CD, Read RC, Braley KE, Kroemer G, Hellewell PG, et al. Dynamic changes in Mcl-1 expression regulate macrophage viability or commitment to apoptosis during bacterial clearance. *J Clin Invest* 2005;115:359–68.
- Maurer U, Charvet C, Wagman AS, Dejardin E, Green DR. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Mol Cell* 2006;21:749–60.
- Bhat UG, Pandit B, Gartel AL. ARC synergizes with ABT-737 to induce apoptosis in human cancer cells. *Mol Cancer Ther* 2010;9:1688–96.

40. Csoka K, Liliemark J, Larsson R, Nygren P. Evaluation of the cytotoxic activity of gemcitabine in primary cultures of tumor cells from patients with hematologic or solid tumors. *Semin Oncol* 1995;22:47–53.
41. Hensley ML, Larkin J, Fury M, Gerst S, Tai DF, Sabbatini P, et al. A phase I trial of pemetrexed plus gemcitabine given biweekly with B-vitamin support in solid tumor malignancies or advanced epithelial ovarian cancer. *Clin Cancer Res* 2008;14:6310–6.
42. van Moorsel CJ, Veerman G, Ruiz van Haperen VW, Bergman AM, Jensen PB, Sehested M, et al. Differential effects of gemcitabine on nucleotide pools of 19 solid tumor cell lines. *Adv Exp Med Biol* 1998;431:661–5.
43. Bergman AM, Kuiper CM, Noordhuis P, Smid K, Voorn DA, Comijn EM, et al. Antiproliferative activity and mechanism of action of fatty acid derivatives of gemcitabine in leukemia and solid tumor cell lines and in human xenografts. *Nucleosides Nucleotides Nucleic Acids* 2004;23:1329–33.
44. Kim KW, Moretti L, Mitchell LR, Jung DK, Lu B. Combined Bcl-2/mammalian target of rapamycin inhibition leads to enhanced radiosensitization via induction of apoptosis and autophagy in non-small cell lung tumor xenograft model. *Clin Cancer Res* 2009;15:6096–105.
45. Deng Y, Wu X. Peg3/Pw1 promotes p53-mediated apoptosis by inducing Bax translocation from cytosol to mitochondria. *Proc Natl Acad Sci U S A* 2000;97:12050–5.
46. Witham J, Valenti MR, De-Haven-Brandon AK, Vidot S, Eccles SA, Kaye SB, et al. The Bcl-2/Bcl-XL family inhibitor ABT-737 sensitizes ovarian cancer cells to carboplatin. *Clin Cancer Res* 2007;13:7191–8.
47. Cory S, Adams JM. Killing cancer cells by flipping the Bcl-2/Bax switch. *Cancer Cell* 2005;8:5–6.
48. Thomson DM, Hansen MD, Winder WW. Regulation of the AMPK-related protein kinases by ubiquitination. *Biochem J* 2008;411:e9–10.
49. Marx C, Held JM, Gibson BW, Benz CC. ErbB2 trafficking and degradation associated with K48 and K63 polyubiquitination. *Cancer Res* 2010;70:3709–17.