Synergistic anti-tumor activity of gemcitabine and ABT-737 in vitro and in vivo through disrupting the interaction of USP9X and Mcl-1

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Running title: Combination therapy of gemcitabine and ABT-737

Keywords: gemcitabine, ABT-737, combination therapy, apoptosis, Mcl-1

Abbreviations list:

PBS phosphate buffer saline
Cl combination index
USP9X Ubiquitin-specific peptidase 9, X-linked
CHX cycloheximide
Mcl-1 myeloid cell leukemia-1
JC-1       5,5',6,6'tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide
PI         propidium iodide
MTT       3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Notes:

Grant support: Zhejiang Provincial Foundation of National Science for Outstanding Youths (QJH), Zhejiang Provincial Program for the Cultivation of High-level Innovative Health Talents (BY), Zhejiang Provincial Natural Science Foundation (NML), Program for New Century Excellent Talents in University (BY) and Science Research Foundation of Zhejiang Health Bureau (HZ).

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Conflicts of Interest: No potential conflicts of interest were disclosed.

Abstract: 180 words; Word count of main text: 5278 (excluding Title page and References); Number of Figures: 6; Supplementary Figures: 3
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 demonstrated 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, increased by ABT-737 treatment, could be disrupted by gemcitabine, thus enhanced the ubiquitination and the subsequent degradation of Mcl-1, and ultimately resulted in the synergism of these two drugs. Moreover, the increased anti-cancer efficacy of gemcitabine combined with ABT-737 was further validated on human lung cancer 95-D xenograft model in nude mice. Taken together, our data firstly demonstrated the synergistic anti-cancer capabilities achieved by combining gemcitabine and ABT-737, and moreover, opened new opportunities to exploit therapeutically where antiapoptotic Bcl-2 family members drive tumor cell resistance to current anticancer therapies.
Introduction

Anti-apoptotic 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) (5, 6). USP9X has been shown to regulate multiple cellular functions (7-10), and increased expression of USP9X in tumors is significantly 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 Lys 48-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 anti-cancer activity against a variety of solid tumors, including non-small cell lung (11), pancreatic (12), bladder (13) and breast cancer (14). Gemcitabine is converted intracellularly to the active metabolites difluorodeoxycytidine di- and triphosphate (dFdCDP, 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 anti-cancer activity as a single agent or in combination with other chemotherapeutic agents against acute myeloid leukemia (AML) (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 (ALL) (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 anti-tumor efficacy against human cancer cells both \textit{in vitro} and \textit{in vivo}. Chemical structures of the drugs were 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.1) (19). The primary antibodies against USP9X (5G-02), PARP (H250), procaspase-3 (E-8), Mcl-1 (22), Bax (2D2), Ub (P4D1), β-actin (C-11), GSK3β (H-76), p-GSK3β (Ser-9) and HRP-labeled secondary anti-goat, anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); cleaved-caspase-3 (D-175) from Cell Signaling Technology (Danvers, MA). Cycloheximide (CHX) was purchased from Sigma-Aldrich.

Cell Culture

Human renal carcinoma cell lines (SW-13), human lung cancer cell lines (95-D, A549), human prostate cancer cell line PC-3 and human bladder carcinoma cell lines (5637, 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 <6 months, and no authentication was done. All these cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum plus 2 mM of glutamine and 50 units/ml of 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 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (32). The inhibition rate on cell proliferation was calculated for each well as \((\frac{A570 \text{ control cells} - A570 \text{ treated cells}}{A570 \text{ control cells}}) \times 100\%\).

**Analysis of apoptosis by Annexin V and PI staining**

Apoptosis was quantified using the Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, San Diego, CA) via the manufacturer's instructions. Briefly, cells were then incubated with 5 μl Annexin V at room temperature for 15 minutes in the dark. Before flow cytometric analysis, 5 μl of 50 μg/ml PI stock solution was added to the samples. For each sample \(1 \times 10^4\) cells were collected and analyzed using an FACS-Calibur cytometer (Becton Dickinson, San Jose, CA).

**Determination of mitochondrial membrane depolarization**

Cells \(5 \times 10^5\)/well) were exposed to gemcitabine, ABT-737 or the combination for 12, 24 and 36 h, collected, and resuspended in fresh medium containing 10 μg/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 M urea, 10% glycerol, 10 mM Tris-HCl (pH 6.8), 1% SDS, 5 mM DTT, 0.5 mM phenylmethylsulfonyl, 1 μg/ml aprotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin] 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 1:500-1:1000) followed by horseradish peroxidase-labeled secondary antibodies at 1:2000 dilution. Antibody binding was then detected with the use of a chemiluminescent substrate and visualized on 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 min. After washing with phosphate-buffered saline, the binding of antibody was visualized with fluorescein isothiocyanate-conjugated anti-mouse IgG (1:200) (Sigma). Ten thousand cells were analyzed using Cell QuestTM software (BD Biosciences).

**Real-Time reverse transcription – polymerase chain reaction**

Total RNA was extracted from sample cells with trizol, precipitated by 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 are as follows: Mcl-1, 5’-GGGCAGGATTGTGACTCTCATT-3’, 5’-GATGCAGCTTTCTTGGTTTATGG-3’; USP9X, 5’-CCTGCTGGTGCACCTCTGGC-3’, 5’-AGGCCGGTGTCCTCGATGCA-3’;
GAPDH, 5'-GAGTCAACGGATTTGGTCGT-3, 5'- TTGATTTTGAGGGATCTCG-3'.

**Immunoprecipitation**

Cell lysates (250 µg of total protein) were incubated with slow rotation for 4 h with primary antibodies (1:50) followed by Protein A/G-conjugated agarose for an additional 1 h. The beads were washed four times with lysis buffer containing protease inhibitor and phosphatase inhibitors and the supernatant was removed by centrifugation (3000 g, 1 min). The pellet was mixed with loading buffer and heated to 95 °C for 5 min followed by immunoblotting.

**Mcl-1 and USP9X gene silencing by small 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 nM Mcl-1 or USP9X siRNA (GenePharma, China) and oligofectamine reagent (Invitrogen Corporation) according to manufacturer recommendations. The sense sequences of the Mcl-1 and USP9X siRNA were 5’-CGCCGAAUUCAUUAAUUUA-3’ and 5’-AGAAAUCGCUGGUAUAAAUU-3’, respectively (4).

**Animals and anti-tumor 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 50-150 mm^3, the mice were randomized to control and treated groups, and received vehicle (1%
DMSO, 7% Cremophor/ethanol (3:1), and 92% PBS, i.p. administration), gemcitabine (20mg/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 \( V = \frac{\text{length} \times \text{width} \times \text{height}}{2} \). The tumor volume at day \( n \) was expressed as RTV according to the following formula: \( \text{RTV} = \frac{\text{TV}_n}{\text{TV}_0} \), where \( \text{TV}_n \) was the tumor volume at day \( n \) and \( \text{TV}_0 \) was the tumor volume at day 0. Therapeutic effects of treatment were expressed in terms of \( T/C \% \) using the calculation formula \( \% = \frac{\text{mean RTV of the treated group}}{\text{mean RTV of the control group}} \times 100\% \) (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, CI values were calculated for each concentration of gemcitabine, ABT-737 and the combination in cell proliferation assays using CalcuSyn (Biosoft, Cambridge, United Kingdom) (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 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 μM to 4 μM in 6 human carcinoma cell lines using the MTT cytotoxicity assay. Survival curves to gemcitabine, ABT-737, and gemcitabine combined with ABT-737 were shown in Fig. 1B. The *in vitro* cytotoxicity of ABT-737 as a single agent was not concentration-dependent; a 10-times 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 (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 μM gemcitabine, 2 μM ABT-737 or the combination for 12 and 24 h. As shown in Fig. 2A, the percentage 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 h). As demonstrated in Fig. 2A, combined treatment with gemcitabine and ABT-737 resulted in an increased percentage of mitochondrial membrane depolarized 95-D cells than either single agent (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, C).

**Combination therapy activated caspase cascades.** We observed that treatment of cells with gemcitabine plus ABT-737 for 24 h caused a significantly greater activation of procaspase-3 than did either single agent (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 h followed by incubation of 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.
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. 2), a significant conformational change was observed using flow cytometric analysis with an antibody against the activated form of the Bax protein (Fig. 3B-1). To confirm the synergism 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-2, there were large amounts of Bax immunoprecipitated with Mcl-1 in ABT-737 or vehicle-treated cells, indicating that Mcl-1 exerted its anti-apoptotic 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-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 RT-PCR in
95-D cells treated with 2 μM gemcitabine, 2 μM 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 physiological and pathological situations (3). We thus hypothesized that the putative ubiquitination of Mcl-1 in response to gemcitabine and ABT-737 combination treatment might play a key role in the synergistic effect. Indeed, Fig. 3C showed 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 μg/ml) to block new protein synthesis and observed Mcl-1 degradation in the presence of 20 μM 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. Fig. 3D showed 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 four human carcinoma cell lines (Supplementary Fig. 3). Additionally, we explored the relationship between ABT-737 cytotoxicity and the expression of Mcl-1 and USP9X. Next, we selected two ABT-737-highly-resistant cell
lines (95-D and 5637) and one 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 h (Fig. 4A, 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 h ABT-737 treatment compared with vehicle control for 5637 and 95-D 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.

GSK3β has been shown to increase Mcl-1 turnover and thereby promotes apoptosis (38). However, we did not observe a change in p-GSK3β (Ser-9) expression with gemcitabine and ABT-737 combination treatment (Supplementary Fig. 2). The USP9X-Mcl-1 interaction stabilizes Mcl-1 and thereby promotes cell survival, which implicates overexpression of Mcl-1 and USP9X (4). Our results demonstrated 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, B). Therefore, we next examined the interaction between USP9X and Mcl-1 in 95-D and 5637 cells treated with 2 μM ABT-737 by immunoprecipitation and found that ABT-737 could increase the binding of USP9X to Mcl-1 in two 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 (upper panel), 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 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, lower panel). Thus gemcitabine might attenuate the deubquitination 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 demonstrated 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 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 2μM gemcitabine, either alone or in combination with 2μM 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 h, 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 was participated in the synergistic effect of gemcitabine and ABT-737 combination treatment.

**USP9X knockdown amplified the apoptosis induced by gemcitabine and ABT-737.** Fig. 5B showed that USP9X knockdown sensitized 95-D cells to the apoptosis induced by gemcitabine plus ABT-737 combination, but 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 of pro-caspase-3 and PARP. These results demonstrated the vital role of USP9X down-regulation in the apoptosis caused by the combination of gemcitabine and ABT-737 in 95-D cells.
The anti-tumor 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 anti-cancer efficacy of gemcitabine and ABT-737 combination treatment, the in vivo activity of gemcitabine and ABT-737 was 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 downregulated 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, has been shown to be a promising therapeutic agent for multiple malignancies. However, the majority of the cell lines derived from solid tumors appear 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 at improve the anti-cancer 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) (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 since the body weight loss was over 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-treatment 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 anti-cancer 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 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-κB, Bcl-2 family proteins (ie, Bcl-xL, Bid, Bcl-2) in 95-D and 5637 cell lines treated with
gemcitabine and/or ABT-737 for 12 and 24 h. However, no synergistic effect was observed for those 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 down-regulation 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 pro-apoptotic 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 knock down 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 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 (USP) family member, is shown to regulate ubiquitination of different signal transduction pathway proteins, including AMPK (48), TGFβ (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 induced by gemcitabine or ABT-737 alone (2 μM). In addition, a significant increase of ABT-737 (4 μM)-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 the treatment with ABT-737, and this interaction could be disrupted by gemcitabine, which might contribute to the sensitization to ABT-737.

In conclusion, we presented evidence showing better therapeutic activity of gemcitabine when combined with ABT-737 both in vitro and in vivo. Our results also showed 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.
Acknowledgements: This work was supported by Zhejiang Provincial Foundation of National Science for Outstanding Youths (R2080326), Zhejiang Provincial Program for the Cultivation of High-level Innovative Heath Talents, Zhejiang Provincial Natural Science Foundation (Y2100682), Program for New Century Excellent Talents in University and Science Research Foundation of Zhejiang Health Bureau (2010QNA009).
Reference


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Figure Legends

Fig. 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 μM for gemcitabine and ABT-737.

Fig. 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 μM), ABT-737 (2 μM) or the combination for 12 h, then, cells were incubated with Annexin V/PI (top panel) or JC-1 (bottom panel) and analyzed by flow cytometry. (B, C) Cells in 6 well plates were exposed to gemcitabine (2 μM), ABT-737 (2 μM) or the combination for 12 and 24 h, then, cells were incubated with Annexin V/PI (top panel) or JC-1 (bottom panel) and analyzed by flow cytometry. (D) Cells were exposed to gemcitabine (2 μM), ABT-737 (2 μM) or the combination for 24 h, after which protein extracts were immunoblotted with specified antibodies for PARP, Mcl-1, caspase-3, and cleaved-caspase-3.

Fig. 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 μM) for 1 h and then treated with 2 μM
Gemcitabine and/or ABT-737 for 24 h. The cells were analyzed for apoptosis by flow cytometry. (B-1) 95-D cells in 6 well plates were exposed to gemcitabine (2 μM), ABT-737 (2 μM) or the combination for 24 h, and Bax conformational change was detected in the FL2-H channel by flow cytometry. (B-2) 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 analysed by SDS/PAGE and immunoblotted using Bax (1:1000). (C) 95-D cells were exposed to gemcitabine (2 μM), ABT-737 (2 μM) or the combination for 18 h, after which protein extracts were immunoblotted with the Ubiquitin. (D) Cells were treated with CHX (200 μg/ml) to block new protein synthesis and the degradation of Mcl-1 in the presence of 20 μM gemcitabine and/or ABT-737 at 25, 50, 75 and 100 min were detected by western blot.

Fig. 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 μM) for 12 and 18 h. The mRNA levels of Mcl-1 and USP9X were detected by real-time RT-PCR. (B) The protein levels of Mcl-1 and USP9X were determined by western blot in SCABER, 95-D and 5637 cell lines. (C) 95-D and 5637 cells treated with ABT-737 for 12 and 18 h 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 h.
Fig. 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 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 μM gemcitabine, either alone or in combination with 2 μM ABT-737 for 18 h were examined.

Fig. 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. Relative tumor volume are expressed as mean ± SD (n=10 per group). (B) Expression of the apoptosis related proteins extracted from tumor tissues in the animal experiment were detected by western blot.
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Mol Cancer Ther Published OnlineFirst May 12, 2011.

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Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-10-1091

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