The Bcl-2/Bcl-X$_L$ Family Inhibitor ABT-737 Sensitizes Ovarian Cancer Cells to Carboplatin

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Abstract

Purpose: The effective treatment of ovarian cancer is hampered by the development of drug resistance, which may be mediated by members of the Bcl-2 family of apoptosis regulators. ABT-737 is a recently described inhibitor of members of this family. We investigated whether this compound could sensitize ovarian cancer cells to chemotherapeutic agents.

Experimental Design: The sensitivity of ovarian cancer cell lines to ABT-737 in combination with either carboplatin or paclitaxel was tested either *in vitro* by assessing cell growth/survival and apoptosis or in xenograft studies.

Results: As a single agent, ABT-737 inhibited the growth of eight ovarian cancer cell lines, although with relatively poor potency. However, ABT-737, but not a less active enantiomer, increased the sensitivity of several cell lines to carboplatin. The increased sensitivity to carboplatin was accompanied by a decrease in time at which apoptosis was observed when assessed according to the number of attached cells, PARP cleavage, and nucleosome formation. ABT-737 was more effective at sensitizing IGROV-1 cells when ABT-737 was administered after carboplatin. In addition, ABT-737 significantly enhanced the activity of carboplatin in one of three primary cultures derived directly from ascitic tumor cells in patients recently treated with chemotherapy. Small interfering RNA directed to Bcl-X$_L$ also increased the sensitivity of ovarian cancer cell lines to carboplatin. ABT-737 was also able to augment the inhibition of IGROV-1 tumor xenograft growth beyond that obtained with carboplatin alone.

Conclusions: These data suggest that ABT-737, in combination with carboplatin, may find utility in the treatment of patients with ovarian cancer.

The currently most frequently used therapy for the treatment of ovarian cancer is a combination of carboplatin and paclitaxel. Although ~80% of patients initially respond well to therapy, the majority of patients suffer recurrent disease (1). In some cases, patients respond well to repeated treatment with the same chemotherapeutic regimen but they will inevitably succumb to the disease following the eventual emergence of drug resistance. As a consequence, the overall 5-year survival is only 30% (1). Thus, there is a pressing need to either identify novel therapies for ovarian cancer or to discover drugs which (re)sensitize tumor cells to existing chemotherapy. Several factors have previously been implicated in drug resistance, including genes which regulate drug influx and efflux, drug metabolism, damage repair, and the apoptotic response to drug-induced damage. Indeed, it is possible that numerous resistance mechanisms could contribute to a drug-resistant phenotype and these mechanisms might be coordinately regulated (2).

One gene that has previously been linked to drug resistance is that encoding the apoptosis inhibitor Bcl-X$_L$. In ovarian cancer, expression of Bcl-X$_L$ is associated with a shorter disease-free interval following therapy, and the expression of Bcl-X$_L$ is increased in patients with recurrent disease following chemotherapy (3). Bcl-X$_L$ expression in clinical samples has also been linked to the resistance of prostate cancer to androgen therapy (4). Preclinical studies have shown that ectopic expression of Bcl-X$_L$ confers resistance to several chemotherapeutic agents including cisplatin, gemcitabine, vincristine, etoposide, doxorubicin, and paclitaxel (5–8). Posttranslational regulation of Bcl-X$_L$ by deamidation has also been implicated in drug resistance to DNA-damaging agents such as cisplatin (9), and the prosurvival function of the oncogenic tyrosine kinase Lck following DNA damage may be mediated in part by inhibition of Bcl-X$_L$ deamidation (10). Inhibition of Bcl-X$_L$ expression with either antisense oligonucleotides (11), with rituximab which inhibits nuclear factor κB–mediated expression of Bcl-X$_L$ (12), or with histone deacetylase inhibitors (13) increases sensitivity to cytotoxic agents. These observations suggest that inhibitors of Bcl-X$_L$, in combination with chemotherapy, may be useful in the treatment of patients with ovarian cancer.

ABT-737 is a recently described Bcl-X$_L$ inhibitor that also inhibits Bcl-2 and Bcl-w. ABT-737 binds to a hydrophobic groove in these proteins and prevents them from sequestering proapoptotic proteins such as BAD and BIM (14). Consequently, ABT-737 promotes cell death following treatment with agents that induce proapoptotic signals (14). ABT-737 also exhibits significant single-agent activity against myeloma, leukemia, and small cell lung cancer cells *in vitro* (14, 15) although only modest activity (IC$_{50}$ > 1 µmol/L) is observed in many other cancer cells types (14). A385358, an analogue of ABT-737 with increased selectivity for Bcl-X$_L$, enhances the potency of chemotherapeutic agents in renal, pancreatic, colon, and non–small cell lung cancer cell lines (15, 16). We have investigated the activity of ABT-737 in ovarian cancer cells. We show that ABT-737 does not potently inhibit the growth/survival of seven ovarian cancer cell lines tested *in vitro*. However, ABT-737 increases the sensitivity of several of these ovarian cancer cells to carboplatin and combined treatment with ABT-737 and carboplatin inhibits the growth of IGROV-1 ovarian tumor xenografts more effectively than when either agent is used alone.

Materials and Methods

Cell culture. All cell lines were grown in RPMI supplemented with 10% FCS with the exception of OVCAR-8 cells, which were grown in RPMI with 5% FCS, and IGROV-1, which were cultured in DMEM with 10% FCS. Ascitic fluid was obtained from patients with ovarian cancer being managed at the Royal Marsden Hospital, Sutton, within a protocol approved by the local ethical committee. Primary cultures were prepared as previously described (17), with the exception that culture flasks were precoated for 2 h at 37°C with a 10 µg/mL solution of rat tail collagen (First Link) in PBS to facilitate adhesion. Cells were used between passages 2 and 8.

Immunocytochemistry. To analyze the cells isolated from ascites, cells were plated on collagen-coated coverslips and allowed to adhere overnight. Cells were fixed in methanol/acetone (50%/50% v/v) at -20°C and stained to identify cells of epithelial origin. Cytokeratin was detected with 1 µg/mL of FITC-conjugated antibody MNF116 (DAKO) in PBS, whereas epithelial antigen was detected with either 10 µg/mL of FITC-conjugated antibody BerEP4 (DAKO) or 1 µg/mL of antibody E29 (DAKO). In all cases, samples were compared with samples stained with a FITC-conjugated isotype-matched control rabbit IgG. The cells were costained with 5 µg/mL of propidium iodide to indicate total cell number. The proportion of the cells stained with the antibodies was determined by counting between 50 and 100 cells per ascites sample.

Cell growth/survival and apoptosis assays. ABT-737 (Steven Elmore, Abbot Laboratories, Abott Park, IL) or its inactive enantiomer ABT-737E was prepared as a 10 mmol/L solution in DMSO. Stock solutions of carboplatin (13.5 mmol/L) were prepared in PBS and solutions of paclitaxel (8.3 mmol/L) were prepared in ethanol. Cells (5,000 cells/well except for OVCAR-8, for which 2,500 cells/well were used) were plated in 96-well plates in 80 µL of 10% FCS/RPMI. The following day, the cells were treated with drug or solvent and the volume adjusted to 200 µL with 10% FCS/RPMI. After 96 h, the culture medium was removed and the cells fixed in 10% TCA on ice for 30 min. After washing in water, the cells were stained in 0.4% sulforhodamine B. After washing in 1% acetic acid and drying, the dye was solubilized and A$_{540}$ was determined. Data were analyzed using GRAPHPAD Prism to fit a four-parameter Hill equation. Combination indices ("CI values") were calculated as previously described (18).

For experiments in which cell number was determined by counting, detached cell debris was removed by washing twice with PBS and the

attached cells were collected in 50 µL of trypsin (0.1%) and quenched with 50 µL of RPMI/10% FCS before counting with a hemocytometer.

For experiments in which apoptosis was quantified, a "Cell Death ELISA" (Roche) kit was used. Cells (5,000 cells/well) were plated in 96-well plates in 80 µL of 10% FCS/RPMI. The following day, the cells were treated with drug or solvent and the volume adjusted to 200 µL with 10% FCS/RPMI. After 16 h, nucleosomes were quantified according to the manufacturer's instructions.

Western blotting. To measure PARP cleavage, 100,000 cells were plated in a 12-well plate and treated with drug(s) for the indicated times. Detached cells were collected and the adherent cells collected by trypsinization. Both populations of cells were recombined, washed with PBS, and the cells lysed as described (19). Protein concentration was estimated with a bicinchoninic acid assay (Sigma). Proteins were separated on a 3% to 12% Novex gel (Invitrogen) and transferred to nitrocellulose. PARP was detected by blotting with a polyclonal antibody (Cell Signaling Technology) and visualized with Supersignal West Dura reagent (Pierce) using a Fluorchem8900 imager (AlphaInnotech). For detection of Bcl-2 family members, a prosurvival Bcl-2 family sampler kit was used (Cell Signaling Technology) according to the manufacturer's instructions.

Small interfering RNA experiments. Cells (5,000 cells/well except for OVCAR-8, 2,500 cells/well) were seeded in 96-well plates in 80 µL of antibiotic-free RPMI containing 10% FCS. After 16 h, the cells were transfected by the addition of 20 µL of Optimem containing the indicated concentration of small interfering RNA (siRNA) and in the presence of 0.1% Dharmafect-1. The siRNA (Dharmacon) used were

Bcl-X$_L$ no. 2, ACAAGGAGAUGCAGGUAUUUU
Bcl-X$_L$ no. 4, GGUAGUGAAUGAACUCUUCUU

and the control siRNA were nontargeting siRNA no. 1 and RISC-free siRNA (both from Dharmacon). Previous experiments have shown that these conditions offer close to 100% transfection efficiency with minimal toxicity. After 24 h, the medium was replaced with 10% FCS/RPMI. After a further 24 h, the cells were treated for 48 h with the indicated concentration of carboplatin and cell number estimated using a sulforhodamine B assay as described above.

Tumor xenograft experiments. All work was done in accordance with U.K. Home Office regulations under the Animals (Scientific Procedures) Act 1986 and the U.K. Coordinating Committee on Cancer Research guidelines for animal experimentation (20). Tumors were established from tissue-cultured cells released from plastic flasks by a short exposure to trypsin, washed, and resuspended in PBS. Two million IGROV-1 cells (American Type Culture Collection) were s.c. injected bilaterally in the flanks of 6- to 8-week-old female NCr athymic mice. Dosing commenced when tumors were well established (~ 5-6 mm diameter, 19 days following implantation). Animals received either vehicle alone [30% propylene glycol, 5% Tween 80, <1% DMSO, 65% D5W (pH 4-5) i.p. daily plus saline i.p. once weekly], ABT-737 at 100 mg/kg in the first vehicle daily i.p for 28 days, carboplatin at 30 mg/kg in saline once weekly i.p., or a combination of both compounds. Animals received the dose required in 0.1 mL of solution per 10 g of body weight. They were observed daily, weighed, and tumors measured thrice weekly. Tumors were measured across two perpendicular diameters and volumes calculated from the following formula:

$$V = 4/3\pi[(d1 + d2)/4]^3$$

as previously described (21).

Results

Single-agent activity of ABT-737 in ovarian cancer cell lines. To investigate whether inhibition of Bcl-X$_L$ sensitizes ovarian cancer cells to chemotherapeutic agents, the activity of ABT-737 as a single agent was first investigated using eight

ovarian cancer cell lines. These include the ovarian cancer cell lines in the "NCI-60" panel and possess a range of sensitivities to carboplatin (Table 1). These were supplemented with A2870 cells to allow comparison with its derivative A2780cis. These cells were rendered resistant to cisplatin by prolonged exposure of A2780 cells to cisplatin *in vitro* (22). ABT-737 inhibited the growth/survival of each of the cell lines with comparable potencies (IC_{50} = 8-14 μmol/L; Table 1, *column 1*) between the cell lines. To provide evidence suggesting that the activity of ABT-737 results from the inhibition of Bcl-X_L family members, an enantiomer of ABT-737 (here designated ABT-737E), which is a 40-fold less potent inhibitor of Bcl-X_L (14), was tested. ABT-737E also inhibited cell growth/survival, but 2- to 3-fold less potently than ABT-737 (IC_{50}, ∼23-28 μmol/L; Table 1, *column 2*). This suggested that at concentrations above ∼20 μmol/L, ABT-737E inhibited cell growth/survival independently of Bcl-X_L and the same might be anticipated of ABT-737.

ABT-737 sensitizes ovarian cancer cells to carboplatin. The potential activity of ABT-737 at high concentrations and independent from inhibition of Bcl-X_L family members suggested that combination experiments should be done using a fixed low concentration of ABT-737 and a range of concentrations of the cytotoxic agent. For example, interpretation of an experiment using a fixed ratio of carboplatin and ABT-737 would be confused at high concentrations of ABT-737 (i.e., >20 μmol/L) by effects that are independent of inhibition of Bcl-X_L family proteins.

For initial combination experiments, ovarian cancer cell lines were treated simultaneously with solvent or ABT-737 at a concentration selected to inhibit growth/survival by <10% (shown in Table 1, *column 3*), after which the potency of either paclitaxel or carboplatin were then determined (Table 1, *columns 4-6*). There was no increase in sensitivity to paclitaxel in any of the cell lines treated with ABT-737 (data not shown). Previous results have shown that A385358, a compound related to ABT-737 but with increased selectivity for Bcl-X_L, more efficiently sensitizes cells to paclitaxel if the Bcl-X_L inhibitor was added 24 h after paclitaxel. However, in all the cell lines tested here, scheduling ABT-737 24 h after paclitaxel did not increase

sensitivity to paclitaxel (data not shown). In contrast, in most of the cell lines, ABT-737 increased the sensitivity to carboplatin, and most prominently in IGROV-1, OVCAR-8, and OVCAR-3 cells in which an ∼2-fold increase in sensitivity was observed (Fig. 1; Table 1). Treatment of IGROV-1 cells with ABT-737E did not increase the sensitivity to carboplatin, suggesting that the effect of ABT-737 was mediated by the inhibition of a Bcl-X_L family member. A2780cis cells, which are resistant to cisplatin (22), were also more resistant to carboplatin than their parental A2780 line (Table 1). However, little increase in the sensitivity of either of these two cell lines to carboplatin was observed following treatment with ABT-737 (Table 1).

ABT-737 decreases the time to cell death. Visual examination of the cells treated with the drug combination suggested that ABT-737 reduced the time at which cell death was observed. To confirm this, and in many of the subsequent experiments, IGROV-1 cells were used because they showed the greatest increase in sensitivity to carboplatin when treated with ABT-737 (Table 1). IGROV-1 cells were treated with a high concentration of carboplatin (to ensure synchronous cell death) and examined by photomicroscopy. Inclusion of ABT-737 decreased the time at which cell death became evident; after 24 h of treatment with ABT-737 and carboplatin, almost all of the cells were detached or displayed plasma membrane blebbing, whereas cells treated with carboplatin appeared to be morphologically unchanged (Fig. 1A). However, after 48 h, almost all the cells treated with carboplatin alone were also detached. To quantify this, cells were treated with carboplatin and the attached cells were collected by trypsinization and counted. Inclusion of ABT-737 significantly decreased the number of attached cells measured at both 24 and 48 h (Fig. 1B), consistent with the increase in drug sensitivity previously observed. The decrease in the number of detached cells caused by the inclusion of ABT-737 was most prominent at 24 h. However, by 48 h, <20% of the cells treated with 100 μmol/L of carboplatin alone remained attached. These data are consistent with ABT-737 both increasing the sensitivity to carboplatin as well as decreasing the time at which cell death is observed.

Table 1. The potency of ABT-737 and carboplatin in cell proliferation/survival assays

	Single agent		Combination with carboplatin			
	ABT-737, IC_{50} (μmol/L)	**ABT-737E, IC_{50} (μmol/L)**	**ABT-737 (μmol/L)**	**IC_{50} (μmol/L), no ABT-737**	**IC_{50} (μmol/L), with ABT-737**	**Combination index**
A2780	14 ± 1	26 ± 2	3.0	11 ± 1	10 ± 1	1.2 ± 0.1
cisA2780	11 ± 1	28 ± 1	3.0	110 ± 20	89 ± 14	1.2 ± 01
IGROV-1	8.2 ± 1.1	23 ± 1	0.6	21 ± 4	10 ± 3	0.54 ± 0.04*
OVCAR-3	14 ± 3	27 ± 1	1.0	4.6 ± 0.9	2.7 ± 0.7	0.65 ± 0.05*
OVCAR-4	10 ± 2	27 ± 2	1.0	25 ± 12	21 ± 9	0.93 ± 0.06
OVCAR-5	8.8 ± 0.9	25 ± 1	0.6	64 ± 10	47 ± 9	0.80 ± 0.04 [†]
OVCAR-8	13 ± 1	27 ± 1	1.0	70 ± 11	37 ± 7	0.61 ± 0.06*
SK-OV-3	13 ± 3	28 ± 1	1.0	30 ± 4	25 ± 4	0.90 ± 0.04

NOTE: The potency of ABT-737 or ABT-737E as single agents was measured in cell proliferation/survival assays (columns 1 and 2; mean ± SD, n = 4-11). To measure the sensitization of ovarian cancer cells to carboplatin, cells were simultaneously treated with carboplatin and the indicated concentration of ABT-737 (column 3) and the potency of carboplatin determined (columns 4-5; mean ± SD, n = 4-7). Combination index values (mean ± SD) are quoted at a fraction unaffected of 0.5, and where shown, are significantly different from unity.
*$P < 0.001$, paired t test.
[†]$P < 0.01$, paired t test.
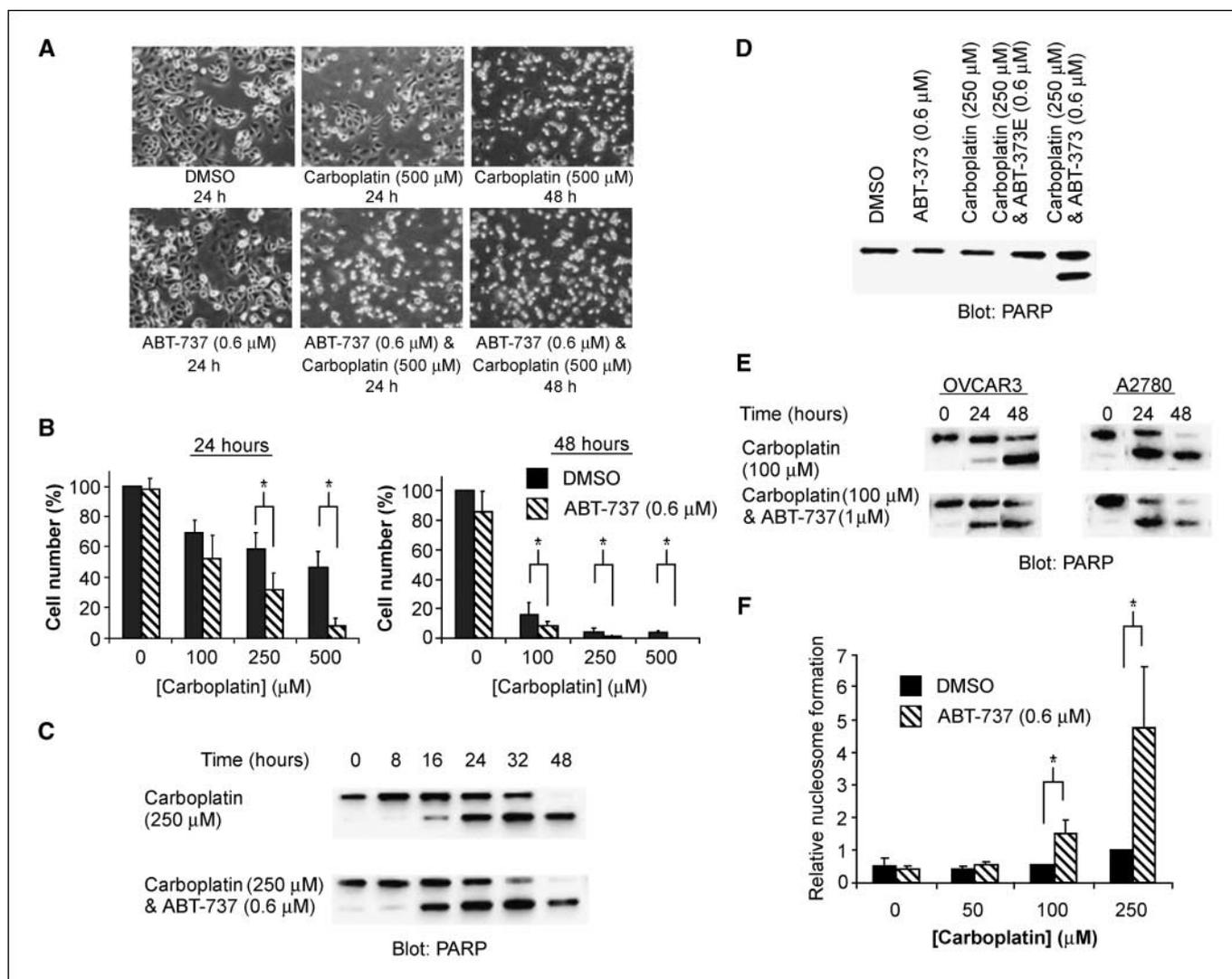
Fig. 1. Sensitization of IGROV-1 cells to carboplatin by treatment with ABT-737. *A,* IGROV-1 cells were treated with either solvent (DMSO), 500 μmol/L of carboplatin, and/or 0.6 μmol/L of ABT-737. The cells were assessed by phase contrast microscopy after either 24 or 48 h. The results are representative of three experiments. *B,* IGROV-1 cells were treated with the indicated concentration of carboplatin in the absence (*solid columns*) or presence (*striped columns*) of 0.6 μmol/L of ABT-737. After either 24 or 48 h, the cells which remained attached were collected by trypsinization and counted. Columns, mean percentage of cell numbers measured in samples treated with DMSO alone; bars, SD (*n* = 4). *C,* IGROV-1 cells were treated with either 250 μmol/L of carboplatin or carboplatin and 0.6 μmol/L of ABT-737. After the indicated time, the cells were lysed and PARP cleavage assessed by Western blotting. *D,* IGROV-1 cells were treated with DMSO, ABT-737 (0.6 μmol/L), or carboplatin combined with either ABT-737 or ABT-737E. After 16 h, the cells were lysed and PARP cleavage was assessed. The results are representative of three experiments. *E,* OVCAR-3 or A2780 cells were treated with carboplatin (100 μmol/L) and either DMSO or ABT-737 (1 μmol/L). After the indicated times, the cells were lysed and PARP cleavage was assessed by Western blotting. The results are representative of three experiments. *F,* IGROV-1 cells were treated with the indicated concentrations of carboplatin and either DMSO (*solid columns*) or 0.6 μmol/L of ABT-737 (*striped columns*). After 16 h, the adherent and detached cells were combined and DNA fragmentation assessed using an ELISA to measure nucleosome formation. Columns, mean fraction of the results obtained from samples treated with 250 μmol/L of carboplatin alone; bars, SD (*n* = 3). *, *P* < 0.05, paired *t* test.

To determine whether ABT-737 decreased the time to onset of apoptosis caused by carboplatin, PARP cleavage was measured in IGROV-1 cells. PARP cleavage was observed in cells treated with either carboplatin or the combination of carboplatin and ABT-737. However, the inclusion of ABT-737 decreased the time at which PARP cleavage was first observed and the time at which the cleavage was essentially complete (Fig. 1C). The ability of ABT-737 to potentiate the PARP cleavage was dependent on the ABT-737 enantiomer used. Following 16 h of treatment with either carboplatin alone, ABT-737 alone, or carboplatin combined with the less active enantiomer ABT-737E, very modest PARP cleavage was observed. In contrast, carboplatin and ABT-737 again led to a

substantial increase in PARP processing (Fig. 1D). This is consistent with the earlier onset of apoptosis being mediated by inhibition of a Bcl-X_L family member. In OVCAR-3 cells, ABT-737 also promoted earlier cleavage of PARP (Fig. 1E), consistent with the increase in sensitivity of this cell line to carboplatin when cells were treated with ABT-737 (Table 1). In contrast, ABT-737 did not increase the sensitivity of A2780 cells to carboplatin (Table 1) and ABT-737 did not accelerate the cleavage of PARP (Fig. 1E). To substantiate these observations using an alternative measure of apoptosis, DNA fragmentation was measured using an ELISA which detects nucleosome formation. After treatment of IGROV-1 cells for 16 h with carboplatin alone or ABT-737 alone, little increase in

Table 2. The potency of carboplatin in cells treated with the siRNA

siRNA	Carboplatin IC$_{50}$ (μmol/L)		
	IGROV-1	OVCAR-3	OVCAR-4
NT	49 ± 15	4.6 ± 0.3	30 ± 1
RISC-free	55 ± 13		
SCNN1A no. 1	52 ± 10	5.3 ± 0.6	27 ± 7
SCNNIA no. 2	57 ± 12		
Bcl-X$_L$ no. 2	22 ± 8*	3.1 ± 0.9 †	28 ± 3
Bcl-X$_L$ no. 4	30 ± 13*	3.1 ± 1.4 ‡	23 ± 2 †

NOTE: IC$_{50}$ values are expressed as mean ± SD, n = 3-4.
*$P < 0.001$, paired t test.
†$P < 0.05$, paired t test.
‡$P < 0.01$, paired t test.

nucleosome number was detected. However, the combination of ABT-737 with carboplatin led to an ~5-fold increase in nucleosome formation (Fig. 1F).

Bcl-X$_L$ siRNA also sensitizes ovarian cancer cells to carboplatin. To confirm that inhibition of Bcl-X$_L$ increases the sensitivity of ovarian cancer cells to carboplatin, RNA interference was used as an alternative method to inhibit Bcl-X$_L$. IGROV-1, OVCAR-3, and OVCAR-4 cells were transfected with two separate siRNA directed to Bcl-X$_L$ and the sensitivity to carboplatin was compared with cells transfected with a "nontargeting" siRNA, which is not expected to interfere with the expression of another gene (A2780 cells were not used in these experiments because we were unable to transfect them at sufficiently high efficiency). In all three cell lines, both siRNA reduced the expression of Bcl-X$_L$ by >80%. In IGROV-1 and OVCAR-3 cells, in which ABT-737 had previously been shown to cause sensitization to carboplatin, both Bcl-X$_L$ siRNAs caused a significant sensitization to carboplatin (Fig. 2). In OVCAR-4 cells, in which ABT-737 caused very modest sensitization to carboplatin, both siRNA had a modest effect on sensitivity to carboplatin (Table 2). Thus, the cells in which ABT-737 caused the greatest increase in sensitivity to carboplatin were also the cells in which Bcl-X$_L$ siRNA had the most significant effect. To confirm the specificity of the Bcl-X$_L$ siRNA, two siRNA directed to a SCNN1A, which has not been reported to be involved in regulating drug sensitivity, or an siRNA which does not enter the RISC complex, were also tested. These siRNA had no measurable effect on sensitivity to carboplatin. These data suggest that inhibition of Bcl-X$_L$ expression by RNA interference also sensitizes cells to carboplatin.
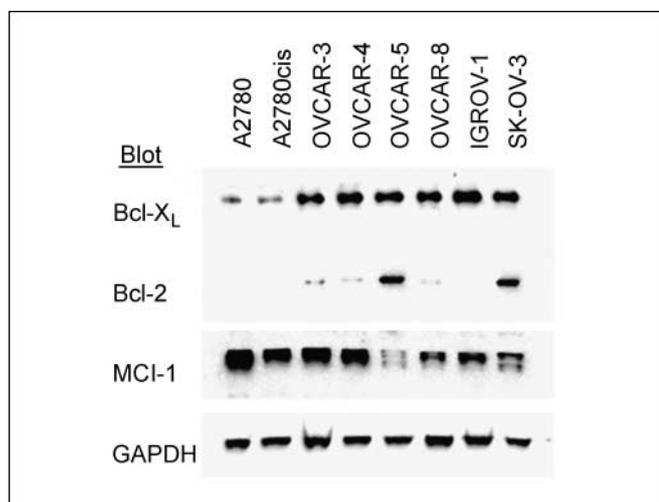
Expression of Bcl-X$_L$ family members in ovarian cancer cells. When tested as a single agent, ABT-737 was previously shown to potently inhibit the survival of certain cell types, whereas other cells are less sensitive. It has been proposed that differences in sensitivity may result from the expression of different members of the Bcl-X$_L$ family. In particular, it has been reported that expression of MCL-1, which is not potently inhibited by ABT-737, could antagonize cell death induced by ABT-737 (23, 24). To assess this, the expression of Bcl-X$_L$ family members was measured in of each of the ovarian cancer cell lines by immunoblotting (Fig. 3). Bcl-X$_L$ was expressed in all eight cell lines, although expression was noticeably lower in A2780 and A2780cis, the lines in which ABT-737 caused the

least sensitization to carboplatin. This is also consistent with the effects of ABT-737 being mediated by inhibition of Bcl-X$_L$. However, there was no clear correlation between Mcl-1 expression and the sensitization induced by ABT-737. For example, Mcl-1 expression was evident in OVCAR-3 cells, although ABT-737 caused significant sensitization of these cells. Thus, other than Bcl-X$_L$ expression, the gene expression profile that renders cells sensitive to ABT-737/carboplatin remains to be determined.

Sensitization of primary cultures of cells derived from patients with ovarian cancer. To confirm that the increase in sensitivity of cells to chemotherapy after treatment with ABT-737 was not restricted to established cell lines, primary cultures were
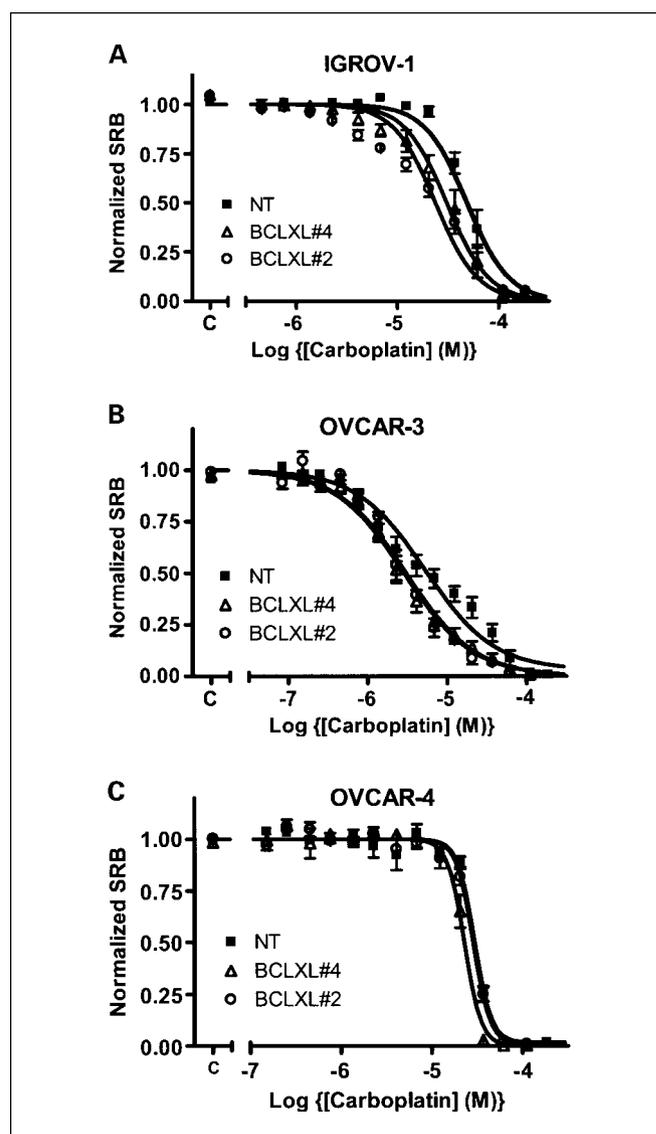
Fig. 2. Inhibition of Bcl-X$_L$ expression by siRNA increases the sensitivity to carboplatin. IGROV-1 (*A*), OVCAR-3 (*B*), or OVCAR-4 (*C*) cells were transfected with either two separate siRNA directed to Bcl-X$_L$ (○, Bcl-X$_L$ no. 2 or △, Bcl-X$_L$ no. 4), a nontargeting siRNA, an siRNA that does not enter the RISC complex ("RISC-free"), or two siRNA (SCNN1A no. 1 and no. 2) directed to a gene not previously implicated in drug resistance. Quantitative PCR studies indicated that in all three cell lines, the Bcl-X$_L$ siRNAs afforded >80% reduction in Bcl-X$_L$ mRNA (data not shown). The potency of carboplatin was subsequently measured in a sulforhodamine B assay (*SRB*). *C*, cells not treated with carboplatin (*X*-axis).

Fig. 3. Expression of Bcl-X$_L$ family members in ovarian cancer cells. Lysates were prepared from cultures of the indicated cell lines and protein expression assessed by Western blotting. The results are representative of three separate experiments.

established from cells collected from ascites fluid that had been drained from patients diagnosed with ovarian cancer. More than 80% of the cells expressed cytokeratin or epithelial antigen and adopted a cobblestone morphology in culture, suggesting that the cells were of epithelial origin. Similar to the established cell lines, these cells were only modestly sensitive to ABT-737 (Table 3). However, the sensitivity of one of these cultures to carboplatin was increased almost 2-fold by cotreatment with carboplatin. No significant sensitization was seen in the other two cultures. Bcl-X$_L$ was expressed in all three cultures (Fig. 4). This suggests that ABT-737 could, in principle, increase the sensitivity of primary cultures of cancer cells to carboplatin, but a larger study is necessary to quantify the proportion of such cultures that respond to ABT-737.

Scheduling of ABT-737. The foregoing observations suggested that inhibition of Bcl-X$_L$ family members could sensitize ovarian cancer cells *in vitro* to carboplatin. Prior to determining whether these observations could be extended to a xenograft model *in vivo*, we investigated how to schedule the administration of ABT-737 with carboplatin. IGROV-1 cells were treated with carboplatin for 24 h, and after washing, cell numbers were measured after a subsequent 48 h. The cells were either pretreated with ABT-737, treated simultaneously

with ABT-737 or treated with ABT-737 after the carboplatin (Fig. 5). Pretreatment of the cells or simultaneous treatment of the cells with ABT-737 (schedules 4, 5, 6; Fig. 5) did not significantly increase sensitivity to carboplatin. This is unlikely to be due to the degradation of ABT-737 because in schedule no. 6, the cells were treated with fresh ABT-737 after 24 h. However, we cannot unequivocally rule out the degradation of ABT-737 reducing the extent of sensitization to carboplatin. If the cells were treated with ABT-737 after treatment with carboplatin, an increase in sensitivity was observed (schedules 2 and 3). These data are consistent with a model in which carboplatin induces DNA damage, and subsequently, the cells undergo apoptosis in a manner which is dependent on Bcl-X$_L$ family members.

Inhibition of IGROV-1 xenograft growth by carboplatin and ABT-737. To determine whether ABT-737 could sensitize tumor cells *in vivo* to carboplatin, IGROV-1 xenografts were established in nude mice. Once tumors were well established, treatment commenced with either carboplatin (30 mg/kg, i.p., once weekly), ABT-737 (100 mg/kg, i.p., daily), or both carboplatin and ABT-737. Daily i.p. administration of ABT-737 at this dose has been successfully used in other xenograft models (14). All these treatments seemed to be well tolerated with <5% decrease in mean body weights over the duration of the experiment. When used as single agents, both ABT-737 and carboplatin had a modest effect (Fig. 6). However, the tumor volume following treatment with both ABT-737 and carboplatin was substantially reduced, with little increase in volume from that observed immediately prior to treatment. To confirm these data, after 28 days of treatment, six representative tumors were excised and their mass determined. Both ABT-737 and carboplatin reduced tumor mass compared with that measured in animals treated with vehicle alone (70 ± 32% and 73 ± 26%, respectively). An at least additive effect was observed in tumors collected from animals which were treated with the drug combination (tumor mass, 41 ± 13% of control). The lack of significant tumor regression may reflect the submaximal dose of carboplatin used; this dose was selected to allow the detection of any increase in tumor growth inhibition resulting from the inclusion of ABT-737.

Discussion

We have shown that treatment of several ovarian cancer cell lines with ABT-737 increased their sensitivity to carboplatin.

Table 3. The potency of ABT-737 and carboplatin in primary cultures of ovarian cancer

| Patient | ABT-737 | IC$_{50}$ (μmol/L) | | Combination index |
		Carboplatin	Carboplatin and ABT-737	
A	26 ± 3	22 ± 3	11 ± 4	0.66 ± 0.12*
B	19 ± 7	18 ± 7	15 ± 3	11 ± 0.3
C	12 ± 6	13 ± 6	10 ± 4	11 ± 0.3

NOTE: The IC$_{50}$ (mean ± SD, n = 3-6) of ABT-737, carboplatin or the combination of ABT-737 (1 μmol/L) and carboplatin was measured using a sulforhodamine B assay.
*$P < 0.005$, paired t test.
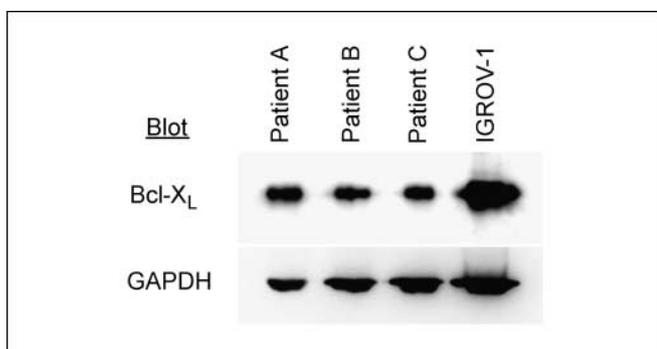
Fig. 4. Drug sensitivity of primary cultures of epithelial cells derived from patients with ovarian cancer. Primary cultures were established from cells isolated from ascitic fluid collected from three patients diagnosed with ovarian cancer (*Patient A:* age 51, stage IV, poorly differentiated adenocarcinoma. *Patient B:* age 62, stage III papillary serous carcinoma. *Patient C:* age 49, stage III, serous carcinoma). The expression of Bcl-X_L family members was measured in lysates prepared from cultures and protein expression assessed by Western blotting. Results representative of three separate experiments.

This was reflected both as an increase in the measured potency of carboplatin but also as a reduction in the time at which cell death is observed either morphologically by counting cell number, by cleavage of PARP, or by nucleosome formation. The activity of ABT-737 probably reflects the inhibition of Bcl-X_L because siRNA oligonucleotides directed to Bcl-X_L were also able to sensitize cells to carboplatin. ABT-737 was most effective when present after cells were treated with carboplatin, consistent with ABT-737 potentiating the induction of apoptosis triggered by DNA damage and ABT-737 could be combined with carboplatin to enhance the inhibition of tumor xenograft growth.

When tested as a single agent in ovarian cancer cells, ABT-737 displayed modest cytotoxic/cytostatic activity (IC_{50}, ~10 μmol/L). This contrasts with the potent activity (IC_{50}, ~100 nmol/L) observed in small cell lung cancer and leukemia cells (14). Why ovarian cancer cells are markedly less sensitive to ABT-737 is not clear but it might reflect the expression of regulators of the intrinsic apoptosis pathway in different cell types. Mcl-1 confers resistance to ABT-737 in several cancer cell types (23, 24), and Mcl-1 was expressed in all the ovarian cancer cells tested in this study, although lower levels were detected in SK-OV-3 and OVCAR-5 cells. Despite this, these latter cells were not markedly more sensitive to ABT-737, suggesting that additional factors may also contribute to determining the sensitivity to ABT-737. One possibility is that cells which are sensitive to ABT-737 are "primed" for cell death (25). In this state, a constitutive proapoptotic signal is suppressed by members of the Bcl-X_L family. The ovarian cancer cells we have tested with ABT-737 might not be primed and so might not require Bcl-X_L for survival without an additional proapoptotic stimulus. Together with the expression of Mcl-1, this may render these cells relatively resistant to ABT-737 when used as a single agent.

In several cell lines, treatment with a relatively low concentration of ABT-737, which on its own had a minimal cytotoxic/cytostatic effect, rendered the cells more sensitive to carboplatin. This may also be consistent with the concept of "priming" if carboplatin induces DNA damage, which results in a proapoptotic signal. In this scenario, Bcl-X_L may sequester

apoptotic mediators until the apoptotic signal becomes overwhelming and cell death ensues. ABT-737 may prevent Bcl-X_L from sequestering the proapoptotic mediators and consequently lowers the threshold at which apoptosis occurs. This model is consistent with the observations that ABT-737 increases sensitivity to carboplatin, reduces the time to apoptosis, as well as with ABT-737 being most effective if present after treatment with carboplatin. This contrasts with scheduling experiments done with the epidermal growth factor receptor inhibitor gefitinib in which pretreatment with gefitinib causes sensitization to paclitaxel (26).

Although in several cell lines, ABT-737 caused an evident increase in the sensitivity to carboplatin, in some cell lines, this was modest or absent. For example, no sensitization of A2780 cells was observed. The A2780cis cell line, which is relatively resistant to both cisplatin and carboplatin, was also not sensitized by treatment with ABT-737. This cell line was originally derived by prolonged exposure of A2780 cells to cisplatin. A single cell line may not provide a model for all potential drug resistance pathways and it seems likely that mechanisms of resistance other than Bcl-X_L may predominate in A2780cis cells. It is noticeable that of the eight lines tested, A2780 and A2780cis expressed the lowest amount of Bcl-X_L. When tested on primary cultures, only one of three samples was more sensitive to the combination of carboplatin and ABT-737 compared with carboplatin alone, even though all three cultures expressed Bcl-X_L. This suggests that expression of Bcl-X_L family members is necessary but not sufficient to predict sensitivity to a combination of ABT-737 and carboplatin. We found no evident correlation between
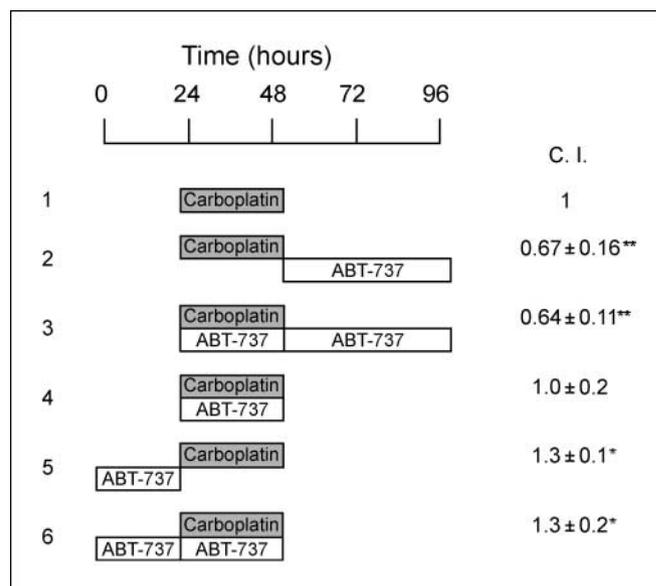
Fig. 5. Schedule-dependent sensitization with ABT-737. IGROV-1 cells were treated with a range of concentrations of carboplatin and either pretreated, cotreated, or subsequently treated with 0.6 μmol/L of ABT-737. After 72 h, the potency of carboplatin was determined using a sulforhodamine B assay. Rectangular boxes represent a new addition of drugs (and removal of any drug previously added). For example, in schedule no. 3, cells were treated for 24 h with carboplatin and ABT-737, then washed, and retreated with fresh ABT-737. In schedule no. 6, cells were treated with ABT-737 for 24 h, washed and the cells retreated with fresh ABT-737 together with carboplatin for a further 24 h. Combination indices (mean ± SD) are quoted at a fraction unaffected of 0.5, and where shown, are significantly different from unity (paired *t* test; **, $P < 0.005$; *, $P < 0.05$).
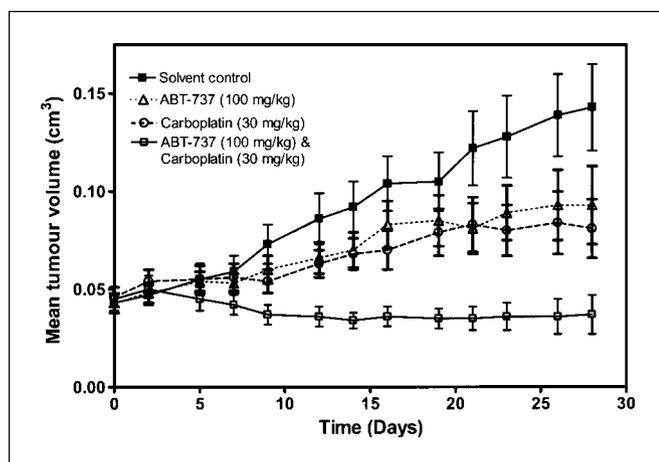
Fig. 6. Inhibition of IGROV-1 xenograft growth. Mice were s.c. inoculated with IGROV-1 cells. Once tumors became measurable, the animals were treated weekly with vehicle (■), ABT-737 (△, 100 mg/kg, i.p., q.d.), carboplatin (○, 30 mg/kg, i.p., q7d) or a combination of carboplatin and ABT-737 (□). Tumor volume was monitored for 28 d after which drug dosing was ceased. None of the drug treatments reduced body weight by >5%.

sensitivity to ABT-737 and carboplatin as a combination and the expression of Bcl-X_L family members, including Mcl-1. Further work to understand the molecular basis of this sensitivity is required and this will be important to predict which patients will likely benefit from treatment with ABT-737 and carboplatin.

When tested as a single agent, ABT-737 exhibited a noticeable inhibition of tumor growth *in vivo*. ABT-737 potently inhibits cell growth *in vitro* in the presence of a proapoptotic stimulus. It is possible that in the xenograft studies, hypoxia or deprivation of contact with extracellular matrix in the tumors stimulates apoptosis, but this is countered by Bcl-X_L. Consequently, treatment with ABT-737, on its own, noticeably inhibits tumor growth. However, a substantially larger inhibition of tumor growth was achieved by treatment with both carboplatin and ABT-737 compared with either agent alone. Taken together, the data presented here suggest that clinical trials of compounds such as ABT-737 in patients with ovarian cancer are warranted and should evaluate the activity of ABT-737 (or analogues) in combination with carboplatin in patients whose tumors express Bcl-X_L.

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