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ABT-737 Synergizes with Chemotherapy to Kill Head and Neck Squamous Cell Carcinoma Cells via a Noxa-Mediated Pathway

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Running Title: ABT-737 synergizes with chemotherapy in HNSCC via Noxa

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ABBREVIATIONS: HNSCC, head and neck squamous cell carcinoma; SCLC, small-cell lung cancer; siRNA, small interfering RNA; DMEM, Dulbecco's Modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PARP, poly(ADP-ribose) polymerase; DMSO, dimethyl sulfoxide; MEF, mouse embryo fibroblast.

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ABSTRACT

Overexpression of Bcl-X_L, an anti-apoptotic Bcl-2 family member, occurs in a majority of head and neck squamous cell carcinomas (HNSCCs) and correlates with chemotherapy resistance in this disease. Overexpression of Bcl-2 is also observed in HNSCC, albeit less frequently. We have previously shown that peptides derived from the BH3 domains of pro-apoptotic proteins can be used to target Bcl-X_L and Bcl-2 in HNSCC cells, promoting apoptosis. In this report we examined the impact of ABT-737, a potent small molecule inhibitor of Bcl-X_L and Bcl-2, on HNSCC cells. As a single agent, ABT-737 was largely ineffective at promoting HNSCC cell death. By contrast, ABT-737 strongly synergized with the chemotherapy drugs cisplatin and etoposide to promote HNSCC cell death and loss of clonogenic survival. Synergism between ABT-737 and chemotherapy was associated with synergistic activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase. Treatment with ABT-737 plus chemotherapy resulted in dramatic upregulation of pro-apoptotic Noxa protein, and siRNA-mediated inhibition of Noxa upregulation partially attenuated cell death by the synergistic combination. Treatment with cisplatin or etoposide, alone or in combination with ABT-737, resulted in substantial downregulation of Mcl-1L, a known inhibitor of ABT-737 action. Further downregulation of Mcl-1L using siRNA failed to enhance killing by the cisplatin/ABT-737 synergistic combination, indicating that chemotherapy treatment of HNSCC cells is sufficient to remove this impediment to ABT-737. Together, our results demonstrate potent synergy between ABT-737 and chemotherapy drugs in the killing of HNSCC cells, and reveal an important role for Noxa in mediating synergism by these agents.

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is a common human cancer, and the 5-year survival rates for this disease have remained relatively unchanged for the past few decades (Forastiere et al., 2001; Gibson and Forastiere, 2006; Jemal et al., 2007). The treatment of HNSCC is hampered by frequent resistance to conventional chemotherapeutic agents. Additionally, chemotherapy drugs currently used to treat HNSCC, including cisplatin, are associated with considerable toxicities (Gibson and Forastiere, 2006). Synergistic drug combinations, which would be useful for enhancing treatment efficacy and reducing adverse toxicities, have not been developed for this disease. Thus, there is a need to identify agents that can be used to circumvent chemotherapy resistance and/or synergize with conventional chemotherapy drugs in the killing of HNSCC cells.

The resistance of HNSCC cells to chemotherapy is due, in part, to expression of anti-apoptotic members of the Bcl-2 protein family, including Bcl-X_L and Bcl-2. Bcl-X_L is overexpressed in a majority of primary HNSCC specimens, while overexpression of Bcl-2 occurs somewhat less frequently (Drenning et al., 1998; Trask et al., 2002). Importantly, Bcl-X_L overexpression correlates with chemotherapy resistance in HNSCC patients (Trask et al., 2002). Antisense-mediated downregulation of Bcl-X_L and Bcl-2 has been shown to sensitize HNSCC cell lines to chemotherapy-induced apoptosis (Sharma et al., 2005). Bcl-X_L and Bcl-2 inhibit chemotherapy-induced apoptosis by binding and sequestering pro-apoptotic members of the Bcl-2 protein family (Danial and Korsmeyer, 2004). We have shown that cell-permeable peptides derived from the BH3 domains of pro-apoptotic Bax and Bad localize to mitochondria, the site of Bcl-X_L/Bcl-2 expression, in HNSCC cells (Li et al., 2007). The BH3 peptides promote apoptosis signaling and HNSCC cell death, albeit at relatively high concentrations. The naturally-

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occurring compound (-)-gossypol, an inhibitor of Bcl-X_L and Bcl-2, also has been shown to promote apoptosis of HNSCC cells, both *in vitro* and *in vivo* (Bauer et al., 2005; Wolter et al., 2006). These studies have hinted at the potential therapeutic benefit of targeting Bcl-X_L/Bcl-2 in head and neck cancers.

Recently, a number of small molecule inhibitors of anti-apoptotic Bcl-2 family members have been identified. Among these, the compound with the highest affinity for Bcl-X_L and Bcl-2 is ABT-737 (Oltersdorf et al., 2005; Zhai et al., 2006). ABT-737 binds anti-apoptotic Bcl-X_L, Bcl-2, and Bcl-w, but shows little affinity for anti-apoptotic Mcl-1L and A1/Bfl (Oltersdorf et al., 2005). When used as a single agent, ABT-737 induces apoptosis in the low micromolar range in small-cell lung cancer (SCLC) cells (Oltersdorf et al., 2005), as well as cell lines and primary cells representing a variety of hematologic malignancies, including acute myeloid leukemia, follicular lymphoma, chronic lymphocytic leukemia, and multiple myeloma (Chauhan et al., 2007; Del Gaizo Moore et al., 2007; Konopleva et al., 2006; Kuroda et al., 2006; Oltersdorf et al., 2005; van Delft et al., 2006). Monotherapeutic ABT-737 also exhibits *in vivo* efficacy against SCLC and leukemia xenografts (Konopleva et al., 2006; Oltersdorf et al., 2005). However, cell lines derived from other types of solid tumors have failed to demonstrate sensitivity to single agent ABT-737, and resistance to this compound is associated with aberrant overexpression of Mcl-1L (Chen et al., 2007; Huang and Sinicrope, 2008; Konopleva et al., 2006; Lin et al., 2006; Tahir et al., 2007; van Delft et al., 2006; Wesarg et al., 2007). Despite these limitations, ABT-737 has shown the ability to sensitize cells derived from both solid tumor and hematopoietic malignancies to conventional anti-cancer agents (Oltersdorf et al., 2005; Tahir et al., 2007). ABT-737 sensitizes ovarian cancer cells to carboplatin (Witham et al., 2007) and enhances apoptosis induction by tumor necrosis factor-related apoptosis-inducing ligand

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(TRAIL) in pancreatic, prostate, renal, and lung cancer cells (Huang and Sinicrope, 2008; Song et al., 2008). Additionally, ABT-737 sensitizes cells representing hematopoietic malignancies to agents including bortezomib (Paoluzzi et al., 2008), *N*-(4-hydroxyphenyl)retinamide (Kang et al., 2008), imatinib (Kuroda et al., 2006), vincristine (Kang et al., 2007), dexamethasone (Kang et al., 2007; Trudel et al., 2007), and melphalan (Trudel et al., 2007).

The sensitivity of HNSCC cells to ABT-737 has not been investigated. We report that ABT-737 is ineffective as a monotherapy against HNSCC cells, but potently synergizes with chemotherapy to kill these cells. Upregulation of Noxa was found to play an important role in mediating the synergistic effects of ABT-737 and chemotherapy drugs. Moreover, due to potent downregulation of Mcl-1L by chemotherapy drugs, the impact of ABT-737 was not limited in cells treated with synergistic combinations of ABT-737 plus chemotherapy. These findings suggest that combination of highly selective Bcl-X_L/Bcl-2 targeting agents with conventional chemotherapy drugs may be an effective means for achieving synergistic anti-tumor effects in HNSCC patients.

Materials and Methods

Cell Lines and Reagents. UM-22A, UM-22B, and 1483 are human HNSCC cell lines (Lin et al., 2007) and were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. ABT-737 and A-793844 were provided by Abbott Laboratories (Abbott Park, IL), dissolved in DMSO, and stored at -80^{o}C as 10 mM stocks. Cisplatin was obtained from the University of Pittsburgh Cancer Institute Pharmacy, and etoposide from Sigma (St. Louis, MO). Lipofectamine 2000

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reagent was obtained from Invitrogen (Carlsbad, CA), and Annexin V-FITC apoptosis detection kits were from BD Biosciences (San Jose, CA). Anti-Bcl-2 antibody was from DAKO (Denmark), and antibodies against Bcl-X_L, Bax, Noxa, and Mcl-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against PARP and Bak were obtained from Cell Signaling Technology (Danvers, MA), and anti-caspase-3 was from Assay Designs (Ann Arbor, MI). Anti-β-actin antibody was from Sigma (St. Louis, MO). Horse radish peroxidase-conjugated secondary antibodies were purchased from Promega (Madison, WI).

Cell Viability Assays and Determination of Synergy. Cellular sensitivities to individual agents, or combinations of agents, were determined using trypan blue exclusion assays. Prior to treatment, UM-22A were seeded in triplicate at 10,000 cells/well, UM-22B at 5,000 cells/well, and 1483 at 8,000 cells/well in 48- or 96-well plates. After growth overnight, the cells reached approximately 50% confluence. The cells were then treated for 48 hours at 37^{o}C with varying doses of each agent, or combinations of the agents. Treatment with drug diluent alone (0.1% DMSO) was used as a control in each experiment. Following addition of trypan blue, cell viabilities were determined by counting a minimum of 300 total cells per data point. Data was analyzed using GraphPad PRISM software (San Diego, CA) to determine IC_{50} values. The method of Chou and Talalay (Chou and Talalay, 1984) was used to assess synergy, and combination indexes (CIs) were calculated using CalcuSyn V2 software (BIOSOFT, Cambridge, UK). CIs values below 1.0 were considered evidence of synergism.

In certain experiments, cell viabilities were determined by flow cytometric analysis of Annexin V staining. For these experiments, UM-22A or UM-22B cells were seeded in 6-well plates and treated for 48 hours with ABT-737, cisplatin, etoposide, or combinations of ABT-737 and chemotherapy. Following treatment, adherent cells were detached from plates using trypsin

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and combined with floating cells. Cells were then washed twice with cold PBS and resuspended in 1X Annexin V binding buffer at a concentration of 1 X 10^6 cells/ml. Single cell suspensions (100 μl) were then transferred to 5 ml culture tubes and stained with 5 μl of Annexin V-FITC and 5 μl of propidium iodide for 15 minutes at room temperature in the dark. After staining, 1X Annexin V binding buffer (300 μl) was added to each tube and the samples placed on ice. Two-color flow cytometric analyses were performed using an Epics Coulter XL flow cytometer (Beckman Coulter; Fullerton, CA).

Clonogenic Assays. Cells were seeded in 100 mm dishes (3 X 10^6/dish), grown overnight, then treated for 1 hour with ABT-737 or cisplatin, alone or in combination. Following treatment, cells were washed twice with PBS and detached from plates using trypsin. Single cell suspensions were diluted in DMEM containing 10% FBS and an equal number of cells replated into 6-well plates. The cells were grown for 12-15 days, then colonies were stained for 30 minutes in a solution of 6% glutaraldehyde, 0.5% crystal violet in water. The plates were washed in water (5-10 times) until no more dye was detected in the rinse. After air drying, colonies comprised of 50 or more cells were counted.

Immunoblotting. For immunoblotting experiments, treated cells were scraped from plates, centrifuged at 1,300 rpm for 5 minutes at 4°C, washed once in cold PBS, then lysed for 10 minutes on ice in 150 mM NaCl, 50 mM Tris pH 8.0, 0.1% SDS, 1% NP-40, 20 μg/ml aprotinin, 3 μg/ml leupeptin, 1.5 mM PMSF. The lysates were centrifuged at 14,000 rpm and 4^{o}C for 2 minutes and the supernatants were transferred to new tubes. Bio-Rad Protein Assay dye concentrate (Bio-Rad, Hercules, CA) was then used to determine protein concentrations in the lysates. For detection of caspase-3 cleavage products and determination of protein levels of Bcl-2, Bcl-X_L, Mcl-1, Bax, Bak, Noxa, Bik, β-actin, proteins (20 μg/lane) were electrophoresed

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on 12.5% SDS-PAGE gels. For detection of PARP cleavage products or Mcl-1L levels, proteins were electrophoresed on 10% SDS-PAGE gels. Following electrophoresis, proteins were transferred to nitrocellulose membranes for 3 hours at 45 V and 4^{o}C. The membranes were blocked at room temperature for 1 hour in TBST buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.1% Tween 20) containing 5% nonfat milk. The blocked membranes were washed with TBST buffer, probed overnight at 4^{o}C with primary antibodies, washed again in TBST, then probed for 1 hour at room temperature with secondary antibodies. Following four final washes in TBST, the membranes were developed using enhanced chemiluminescence reagent, according to the directions provided by the manufacturer (Perkin Elmer Life Science, Inc., Boston, MA).

When blots were reprobed with anti-β-actin, they were stripped by incubating for 45 minutes at 37^{o}C in 0.1 M glycine pH 2.9. The stripped blots were then washed in TBST buffer before incubating in blocking solution.

siRNA Transfection. Prior to transfection, 2 X 10^6 cells were seeded into 100 mm dishes. Following overnight growth, the cell culture medium was replaced with DMEM/10% FBS without antibiotics. Lipofectamine-2000 (Invitrogen, Carlsbad, CA) was then used to transfect annealed, double-stranded Noxa, Mcl-1, or nonspecific siRNAs (100 nM) into the cells, following the manufacturer's instructions. After 6 hours, the cell culture medium was replaced with fresh DMEM supplemented with 10% FBS and antibiotics, and incubation continued for an additional 18 hours at 37^{o}C. The cells were then either left untreated, or were treated for 24 hours with ABT-737 alone, chemotherapy alone, or ABT-737 plus chemotherapy. Trypan blue exclusion assays were used to assess cell viabilities, and immunoblotting was used to verify inhibition of Noxa or Mcl-1 expression. Nonspecific siRNA was obtained from Ambion (Foster City, CA; Cat. #4635), as were siRNAs targeting Noxa (5'-GUAAUUAUUGACACAUUUCTT-

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3') and Mcl-1 (5'-GGACUUUUAGAUUUAGUGATT-3').

Statistics. Statistical analyses were performed using GraphPad Prism version 4 software (GraphPad Software, Inc., San Diego, CA). Comparisons between groups were carried out by one-way ANOVA followed by Tukey's multiple comparison test. *P* values less than 0.05 were considered significant.

Results

ABT-737 is Ineffective as a Single Agent Against HNSCC Cell Lines. With the exception of small cell lung cancer, cells derived from solid tumor malignancies have failed to demonstrate substantial sensitivity to ABT-737 treatment alone. To determine the impact of ABT-737 on head and neck squamous cell carcinoma (HNSCC) cells we employed three HNSCC cell lines: UM-22A, UM-22B, and 1483 (Lin et al., 2007). UM-22A and UM-22B originated from the same patient, but were derived from primary tumor and a cervical lymph node metastasis, respectively. 1483 originated from the primary tumor of a different patient. As shown in Table 1, cells were treated with varying concentrations of ABT-737, followed by performance of trypan blue exclusion assays and determination of IC_{50} values. As a control, cells were also treated with A-793844, an enantiomeric compound known to possess markedly reduced binding affinity for Bcl-X_L and Bcl-2 (Oltersdorf et al., 2005). In addition, cells were treated with cisplatin, a chemotherapy drug commonly used in the clinical treatment of HNSCC, or with etoposide. Single agent ABT-737 was largely ineffective against the three HNSCC cell lines, exhibiting IC_{50} values ranging from 13.8-53.6 μM. These values were similar to those obtained with either single agent cisplatin (IC_{50}'s from 11.1-23.6 μM) or single agent etoposide

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(IC_{50}'s from 27.6-34.5 μM). Curiously, the metastatic variant UM-22B was somewhat more resistant to cisplatin than were UM-22A cells. Expression profiling of Bcl-2 family members (Supplemental Fig. 1) revealed that UM-22B cells exhibit elevated levels of anti-apoptotic Bcl-2 and reduced levels of pro-apoptotic Bim, which may play a role in the increased drug resistance of these cells.

As a single agent, ABT-737 was unable to overcome the inherent drug resistance of HNSCC cells. However, it should be noted that the modest activity of single agent ABT-737 exceeded that obtained with A-793844, which exhibited IC_{50} values from 42.6 μM to greater than 100 μM. Since ABT-737 and A-793844 differ markedly in their abilities to bind Bcl-X_L and Bcl-2, this suggests that the binding of these proteins by ABT-737 alone provides a weak apoptotic stimulus for HNSCC cells.

ABT-737 Synergizes with Chemotherapy to Kill HNSCC Cells. Bcl-X_L and Bcl-2 are overexpressed in a high percentage of HNSCC tumors, and overexpression of Bcl-X_L has been shown to correlate with chemotherapy resistance in this disease (Trask et al., 2002). Therefore, we predicted that a small molecule inhibitor of Bcl-X_L and Bcl-2 (i.e. ABT-737) might markedly enhance the sensitivity of HNSCC cells to chemotherapy drugs. To investigate this possibility, UM-22A (Fig. 1A), UM-22B (Fig. 1B), and 1483 (Fig. 1C) cells were treated for 48 hours with ABT-737 alone, cisplatin alone, or varying doses of a constant ratio of ABT-737 plus cisplatin. Potential synergism was assessed by calculating combination index (CI) values according to the method of Chou and Talalay (Chou and Talalay, 1984), where CI's less than 1.0 are indicative of synergy. As shown, CI values well below 1.0 were observed in all three cell lines at multiple doses of the ABT-737/cisplatin combination, indicating potent synergy between these two agents. To determine whether ABT-737 would demonstrate synergy with other chemotherapy drugs in

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HNSCC cells, we treated cells with varying doses of a constant ratio of ABT-737 plus etoposide (Fig. 1D-F). Again, CI's well below 1.0 were observed, pointing to potent synergy. To confirm synergy between ABT-737 and either cisplatin or etoposide, treated UM-22A and UM-22B cells were evaluated in Annexin V/propidium iodide flow cytometric assays (Fig. 1G; a representative experiment is shown in Supplemental Fig. 2). Results obtained in these experiments were quantitatively and qualitatively similar to those obtained in trypan blue exclusion assays (Fig. 1A-F) and MTS assays (not shown).

Synergy between ABT-737 and standard chemotherapy drugs in the killing of HNSCC cells was further confirmed in clonogenic survival assays (Fig. 2). UM-22A cells were treated for 1 hour with 0.1% DMSO (drug diluent), ABT-737 alone, cisplatin alone, or ABT-737 plus cisplatin (1:1 fixed ratio). Following treatment, cells were replated in medium lacking drugs, and colonies were counted after 12-15 days (a representative experiment is shown in Supplemental Fig. 3). The loss of clonogenic capacity in cells treated with ABT-737 plus cisplatin exceeded the combined losses in cells treated with ABT-737 alone and cisplatin alone, demonstrating superadditive killing by the ABT-737/cisplatin combination.

ABT-737 and Chemotherapy Induce Synergistic Activation of Apoptosis Signaling. To determine whether combinations of ABT-737 and chemotherapy drugs induce synergistic activation of apoptosis signaling in HNSCC cells, we examined processing/activation of caspase-3 and cleavage of PARP, a caspase-3/-7 substrate (Fig. 3). For these experiments, UM-22A cells were treated for a shorter length of time (24 hours) with 10 μM of each agent alone, or the combination of ABT-737 plus chemotherapy drug (each at 10 μM). Relatively little, if any, processing of procaspase-3 to active caspase-3 was detected in untreated cells, or in cells treated with DMSO, ABT-737 alone, cisplatin alone, or etoposide alone (Fig. 3A). Similarly, only low

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levels of PARP cleavage were detected following treatment with single agents (Fig. 3B). By contrast, the combination of ABT-737 plus cisplatin, or ABT-737 plus etoposide, resulted in marked appearance of active caspase-3 and cleavage of PARP protein. The degree of caspase-3 activation and PARP cleavage in response to the combination treatments greatly exceeded the sum of these events occurring in cells treated with ABT-737 alone or chemotherapy alone. Thus, ABT-737 synergized with chemotherapy drugs to induce key apoptosis signaling events in HNSCC cells.

Noxa is Potently Upregulated by ABT-737/Cisplatin and Mediates HNSCC Killing by this Combination. To investigate the mechanism whereby combinations of ABT-737 and chemotherapy drugs synergistically induce caspase activation and HNSCC cell death, we examined the impact of the agents, alone and in combination, on the expression levels of Bcl-2 family members (Fig. 4A). Treatment with ABT-737 alone did not substantially alter levels of the anti-apoptotic proteins Bcl-2 and Bcl-X_L, or the pro-apoptotic proteins Bax, Bak, and Noxa, but did cause modest induction of anti-apoptotic Mcl-1L. Treatments incorporating cisplatin or etoposide, either alone or in combination with ABT-737, resulted in modest reduction in Bcl-2 and Bcl-X_L, and a very dramatic reduction in Mcl-1L. Overexpression of Mcl-1L in leukemia and solid tumor cell lines has been shown to correlate with resistance to ABT-737 (Chen et al., 2007; Huang and Sinicrope, 2008; Konopleva et al., 2006; Lin et al., 2006; Tahir et al., 2007; van Delft et al., 2006; Wesarg et al., 2007). Thus, the ability of cisplatin or etoposide to promote downregulation of Mcl-1L in HNSCC cells may serve to sensitize these cells to ABT-737.

We also observed that the combination of ABT-737 plus cisplatin, or ABT-737 plus etoposide, resulted in striking upregulation of Noxa, relative to untreated cells or cells treated with single agents (Fig. 4A). Timecourse analysis revealed that Noxa was induced as early as 12

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hours after co-treatment with ABT-737 and cisplatin (Fig. 4B). Noxa is a BH3 domain-only, pro-apoptotic, Bcl-2 family member and is known to potently bind and inhibit Mcl-1L (Chen et al., 2005). To determine the role of Noxa upregulation in cell death mediated by this combination, we utilized siRNA treatment to prevent the upregulation of Noxa. As shown in Figure 5, Noxa was potently upregulated in UM-22A cells transfected with a nonspecific siRNA and treated with ABT-737 plus cisplatin. By contrast, transfection with Noxa siRNA largely attenuated Noxa upregulation by this combination. Importantly, inhibition of Noxa upregulation significantly inhibited cell death induced by ABT-737/cisplatin ($p < 0.01$). Similarly, Noxa siRNA also inhibited cell death resulting from the ABT-737/etoposide combination (Supplemental Fig. 4). These results indicate that upregulation of Noxa is at least partially responsible for mediating induction of HNSCC cell death by synergistic combinations of ABT-737 plus chemotherapy.

We next sought to determine whether siRNA-mediated downregulation of Mcl-1L might serve to enhance ABT-737/cisplatin induction of cell death, since overexpression of this anti-apoptotic protein is known to contribute to resistance to ABT-737. As noted in Figure 4A, treatments incorporating cisplatin already markedly reduced Mcl-1L levels. Transfection of cells with Mcl-1 siRNA served to further reduce the already low levels of Mcl-1L in cells treated with ABT-737 plus cisplatin (Fig. 6A). However, the further reduction of Mcl-1L levels using siRNA failed to enhance cell killing by the ABT-737/cisplatin combination ($p > 0.05$). This suggests that the downregulation of Mcl-1L following treatment of HNSCC cells with chemotherapy may be sufficient to override any inhibitory effects of this protein on ABT-737 action. Alternatively, it is possible that Mcl-1L may not be a significant factor in regulating apoptosis induction by ABT-737 in HNSCC cells. To address this possibility, UM-22A cells stably transfected with an expression construct encoding Mcl-1L were treated for 48 hours with the ABT-737/cisplatin

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combination followed by assessment of cell viability (Fig. 6B). The UM-22A/Mcl-1L cells exhibited enhanced resistance to ABT-737/cisplatin relative to vector-transfected control UM-22A cells ($p < 0.01$). These results supported our contention that endogenous Mcl-1L likely inhibits the action of monotherapeutic ABT-737 in HNSCC cells, but the endogenous protein is effectively removed in treatments incorporating chemotherapy.

Discussion

HNSCC is the sixth most common cancer in the United States (Jemal et al., 2007). An overall 5-year survival rate of roughly 50% places HNSCC among the most deadly of the major types of cancer (Forastiere et al., 2001; Gibson and Forastiere, 2006). Current chemotherapeutic options for HNSCC, including cisplatin, cause considerable adverse toxicities, and recurrent forms of the disease are typically highly resistant to conventional chemotherapy drugs (Gibson and Forastiere, 2006). Recently, cetuximab, an epidermal growth factor receptor blocking antibody, was approved by the FDA for use in HNSCC. This followed demonstration that addition of cetuximab to radiation therapy improved patient survival relative radiation treatment alone (Bonner et al., 2006). Although significant, the impact of cetuximab inclusion was modest in scope. Substantial improvements in therapeutic efficacies in HNSCC and reductions in the toxicities of conventional drug regimens are likely to be achieved with the identification of synergistic drug combinations. In this report we demonstrate that ABT-737, an inhibitor of Bcl-X_L and Bcl-2, potently synergizes with conventional chemotherapy drugs in the killing of HNSCC cells.

Previously, we have shown that the proteasome inhibitor bortezomib synergizes with cisplatin in HNSCC cells (Li et al., 2008). Bortezomib treatment causes multiple changes in the

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cell, including inhibition of NF-κB, a transcription factor that is hyperactivated and contributes to the survival of HNSCC cells (Ondrey et al., 1999; Van Waes et al., 2005). Additionally, bortezomib alters the ratio of pro- and anti-apoptotic Bcl-2 family members in the cell. Treatment of HNSCC cells with bortezomib induces the expression of Bik, Bim, and Noxa, natural cellular antagonists of Bcl-X_L and Bcl-2 (Fribley et al., 2006; Li et al., 2008). Thus, bortezomib treatment provides a means for indirect targeting of Bcl-X_L and Bcl-2 in HNSCC cells. In the current study, we sought to determine whether direct targeting of Bcl-X_L and Bcl-2 using a highly specific small molecule inhibitor would result in synergism with conventional chemotherapy drugs. Indeed, the combination of ABT-737 with cisplatin or etoposide resulted in synergistic induction of HNSCC cell death, as measured by trypan blue exclusion, Annexin V, and clonogenic survival assays (Figs. 1 and 2). Synergism between ABT-737 and chemotherapy was also evident on a molecular level, as assessed by caspase-3 activation and PARP cleavage (Fig. 3), hallmarks of apoptosis.

Our studies reveal an important role for Noxa in the synergism of ABT-737 and chemotherapy drugs against HNSCC cells. Noxa expression was markedly upregulated following treatment with ABT-737 plus chemotherapy, and synergism by this combination was partially dependent on Noxa induction (Figs. 4 and 5). Similar upregulation of Noxa by ABT-737 plus chemotherapy has been observed in H196 SCLC cells that are highly resistant to ABT-737 alone (Tahir et al., 2007). Enforced overexpression of Noxa in ABT-737-resistant H196 SCLC cells (Tahir et al., 2007), NCI-H1299 NSCLC cells (Wesarg et al., 2007), or mouse embryo fibroblasts (MEFs) (van Delft et al., 2006), has been shown to confer sensitivity to ABT-737. Meanwhile, studies employing gene knockout MEFs have revealed that Bax and Bak are essential for ABT-737 activity (Chen et al., 2007; van Delft et al., 2006). The HNSCC cell lines

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employed in our studies are known to harbor mutant p53, as is typical with most HNSCC cell lines and primary patient specimens. This raises the possibility that p73, or an alternative mechanism, may play a role in Noxa induction in HNSCC cells treated with the ABT-737/chemotherapy combination.

Interestingly, Noxa is known to bind with high affinity to Mcl-1L, but not Bcl-X_L or Bcl-2 (Chen et al., 2005). This suggests that Noxa upregulation in response to treatment with ABT-737 plus chemotherapy may serve to functionally inactivate Mcl-1L, causing displacement of pro-apoptotic proteins bound to the Mcl-1L protein. The displaced pro-apoptotic proteins may then bind and directly activate Bax and Bak, as has been suggested by a model of direct Bax/Bak activation (Kuwana et al., 2005; Letai et al., 2002). Alternatively, pro-apoptotic proteins displaced from Mcl-1L may bind to Bcl-X_L and Bcl-2, causing displacement of Bax and Bak, as has been suggested in a model of indirect Bax/Bak activation (Chen et al., 2005; Willis et al., 2007).

Resistance to ABT-737 has been correlated with overexpression of Mcl-1L, which does not bind this compound (Oltersdorf et al., 2005; Tahir et al., 2007). Downregulation of Mcl-1L has been shown to confer sensitivity to ABT-737 (Chen et al., 2007; Huang and Sinicrope, 2008; Konopleva et al., 2006; Lin et al., 2006; Tahir et al., 2007; van Delft et al., 2006; Wesarg et al., 2007). As noted above, the marked upregulation of Noxa in HNSCC cells treated with ABT-737 plus chemotherapy likely serves to functionally inactivate Mcl-1L and promote synergism by this combination. However, we also discovered that chemotherapy alone caused substantial reduction in Mcl-1L levels, independent of Noxa upregulation (Fig. 4). Thus, in HNSCC cells, dual repression of cellular Mcl-1L, via Noxa upregulation and chemotherapy-induced downregulation of the Mcl-1L protein, may explain the highly potent synergism between ABT-

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737 and conventional chemotherapy drugs in promoting cell death. This contention is supported by our findings that further downregulation of endogenous Mcl-1L levels using siRNA failed to enhance killing by the ABT-737/cisplatin combination (Fig. 6A), while enforced overexpression of Mcl-1L inhibited cell death by this combination (Fig. 6B). These findings suggest that overexpression of endogenous Mcl-1L in HNSCC tumors will not prove an insurmountable impediment to therapies combining chemotherapy drugs and ABT-737, or next generation derivatives including ABT-263 (Tse et al., 2008).

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Footnotes

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Figure Legends

Fig. 1. ABT-737 synergizes with chemotherapy to kill HNSCC cells. A-C, UM-22A, UM-22B, and 1483 cells were seeded in 96-well trays, grown overnight, then treated for 48 hours with ABT-737 alone, cisplatin (CP) alone, or ABT-737 plus cisplatin. D-F, UM-22A, UM-22B, and 1483 cells were seeded as above, then treated for 48 hours with ABT-737 alone, etoposide (ETO) alone, or ABT-737 plus etoposide. Following treatment, trypan blue exclusion assays were performed to determine % viability. Datapoints represent the average of triplicate wells, and error bars the standard deviations. Combination indexes (CI) were calculated using CalcuSyn V2 software and are shown for each combination of the drugs. G, UM-22A and UM-22B cells were seeded in 6-well plates and treated for 48 hours with 0.1% DMSO, 10 μM ABT-737, 10 μM cisplatin, 10 μM etoposide, or ABT-737 plus chemotherapy drug (10 μM each). Following treatment, adherent cells were detached with trypsin and combined with floating cells. The percent of Annexin V-positive cells was determined by flow cytometric analysis. *, $P < 0.01$.

Fig. 2. ABT-737 and cisplatin synergize in clonogenic survival assays. UM-22A cells were treated for 1 hour with 0.1% DMSO, ABT-737 alone (7.5, 10, or 12.5 μM), cisplatin alone (7.5, 10, or 12.5 μM), or ABT-737 plus cisplatin (equal concentrations of each). The treated cells were washed twice in PBS, detached from plates, diluted in DMEM containing 10% FBS, then replated in 6-well plates. Colonies were stained with crystal violet solution and colonies comprised of 50 cells or greater were counted. Data were plotted as the % inhibition of colony formation relative to DMSO-treated cells. The graphed data represent the average of 3 independent experiments, and error bars represent standard deviations. *P* values were calculated

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using one-way ANOVA followed by Tukey's multiple comparison test. *, $P < 0.01$; **, $P < 0.001$.

Fig. 3. Synergistic activation of caspase signaling by the combination of ABT-737 plus chemotherapy. UM-22A cells were left untreated, or were treated for 24 hours with 0.1% DMSO, 10 μM ABT-737 alone, 10 μM cisplatin alone, 10 μM etoposide alone, the combination of ABT-737 plus cisplatin (10 μM each), or the combination of ABT-737 plus etoposide (10 μM each). Following treatment, whole cell lysates were prepared and proteins (20 μg/lane) were electrophoresed on SDS/PAGE gels, transferred to nitrocellulose, then probed with antibodies against caspase-3 (A) or PARP (B). Blots were stripped and reprobed with anti-β-actin to demonstrate equal protein loading. Similar results were seen in three independent experiments.

Fig. 4. Synergistic upregulation of pro-apoptotic Noxa by the combination of ABT-737 plus chemotherapy. A, UM-22A cells were left untreated, or were treated for 24 hours with 0.1 % DMSO, 10 μM ABT-737 alone, 10 μM cisplatin alone, 10 μM etoposide alone, ABT-737 plus cisplatin (10 μM each), or ABT-737 plus etoposide (10 μM each). Following treatment, whole cell lysates were prepared and subjected to immunoblotting for the indicated proteins. Similar results were seen in three independent experiments, and representative blots are shown. B, UM-22B cells exposed to ABT-737, cisplatin, or ABT-737 plus cisplatin, for the indicated number of hours were subjected to immunoblotting for Noxa. The depicted results are representative of three independent experiments.

Fig. 5. Inhibition of Noxa upregulation attenuates killing by the ABT-737/cisplatin combination.

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UM-22A cells were seeded in 100 mm dishes, grown overnight, then transfected for 24 hours with nonspecific siRNA or Noxa siRNA, as described in Materials and Methods. The transfected cells were then either left untreated, or were treated for an additional 24 hours with 0.1% DMSO, 10 μM ABT-737 alone, 10 μM cisplatin alone, or ABT-737 plus cisplatin (10 μM each). Following treatment, cells were harvested and analyzed by immunoblotting to determine Noxa protein levels. In addition, trypan blue exclusion assays were used to determine percent cell survival. The plotted data represent the average of means from three independent experiments, and error bars represent the standard error of the means. *P* values were determined using one-way ANOVA followed by Tukey's multiple comparison test. *, $P < 0.01$ when comparing ABT-737/cisplatin-treated cells transfected with Noxa siRNA versus nonspecific siRNA.

Fig. 6. Impact of Mcl-1 downregulation or overexpression on killing by the ABT-737/cisplatin combination. A, UM-22A cells were seeded in 100 mm plates, grown overnight, then transfected for 24 hours with Mcl-1 siRNA or nonspecific siRNA. The cells were then left untreated, or were treated for an additional 24 hours with 0.1% DMSO, 5 μM ABT-737 alone, 5 μM cisplatin alone, or ABT-737 plus cisplatin (5 μM each). Immunoblotting was used to demonstrate inhibition of Mcl-1L expression, and trypan blue exclusion assays were used to determine percent cell survival. Columns represent the average of means from three independent experiments and error bars the standard errors of the means. $P>0.05$ when comparing ABT-737/cisplatin-treated cells transfected with Mcl-1 siRNA versus nonspecific siRNA. B, UM-22A cells stably transfected with Mcl-1L expression construct, or empty vector, were seeded in 48-well plates and treated for 48 hours with DMSO, ABT-737, cisplatin, or ABT-737 plus cisplatin. Following treatment, cells were detached with trypsin and combined with floating

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cells. Trypan blue exclusion assays were used to determine cell viabilities. *, $P < 0.01$. The experiment was performed 3 times with similar results each time.

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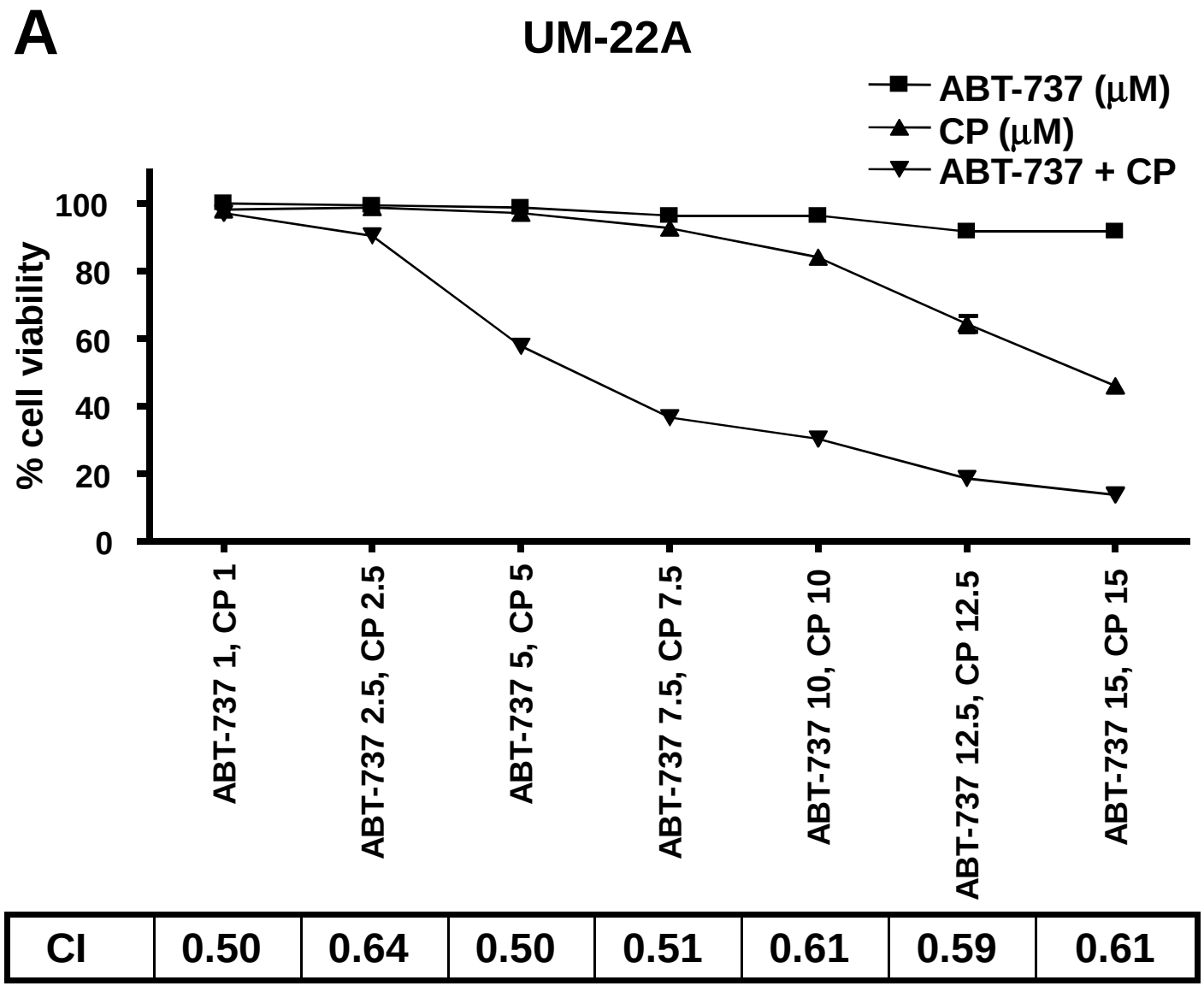
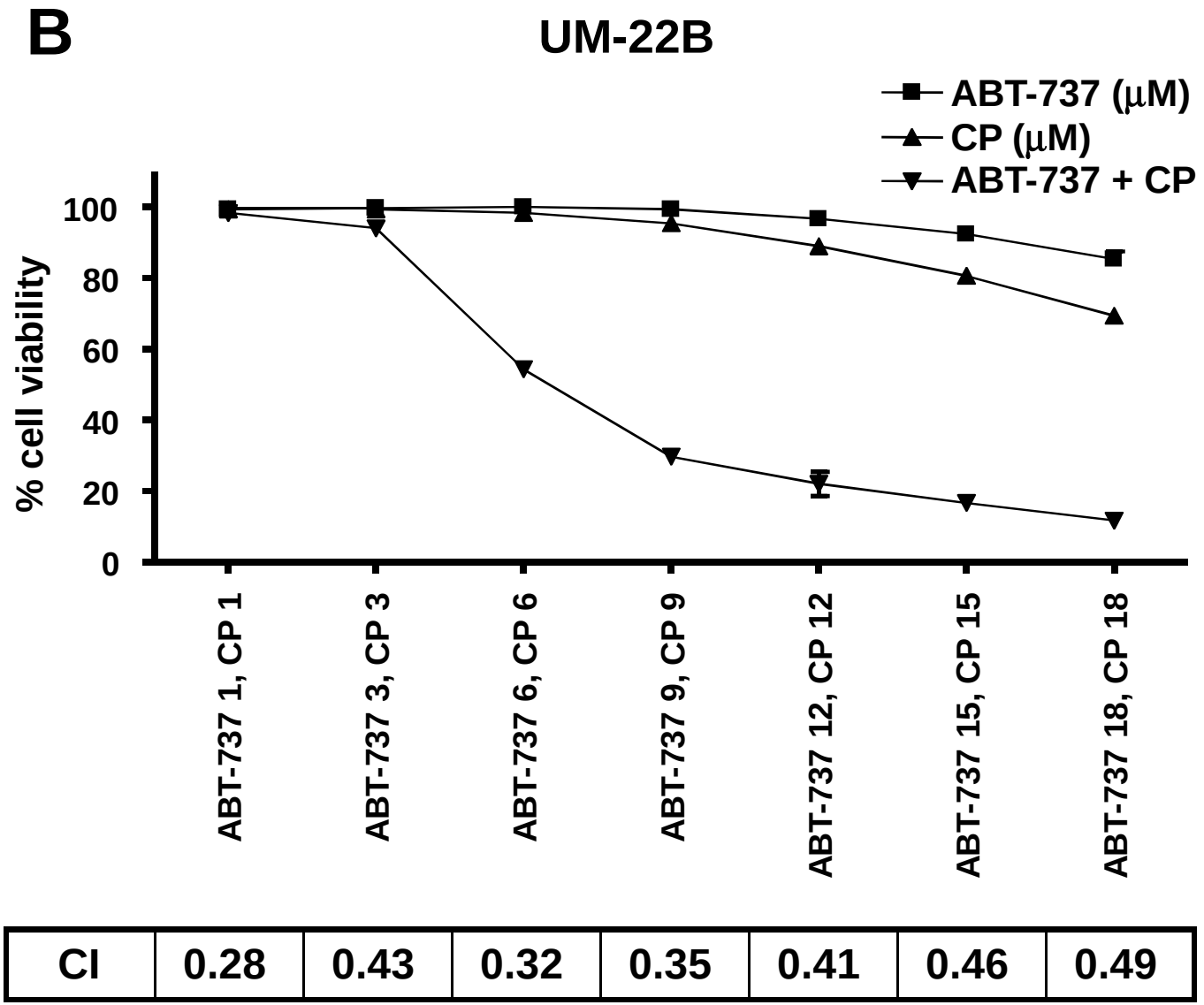
TABLE 1. IC_{50} values (μM) of single agent ABT-737, cisplatin, etoposide, or A-793844 against HNSCC cell lines. UM-22A, UM-22B and 1483 cells were seeded in 48-well plates, grown overnight, then treated for 48 hours with varying doses of the indicated agents. Cell viabilities were assessed in triplicate via trypan blue exclusion assays and IC_{50} values were calculated.

Cell line	ABT-737	Cisplatin	Etoposide	A-793844
	IC_{50}	IC_{50}	IC_{50}	IC_{50}
UM-22A	53.6	14.5	34.5	>100
UM-22B	36.2	23.6	34.3	>100
1483	13.8	11.1	27.6	42.6



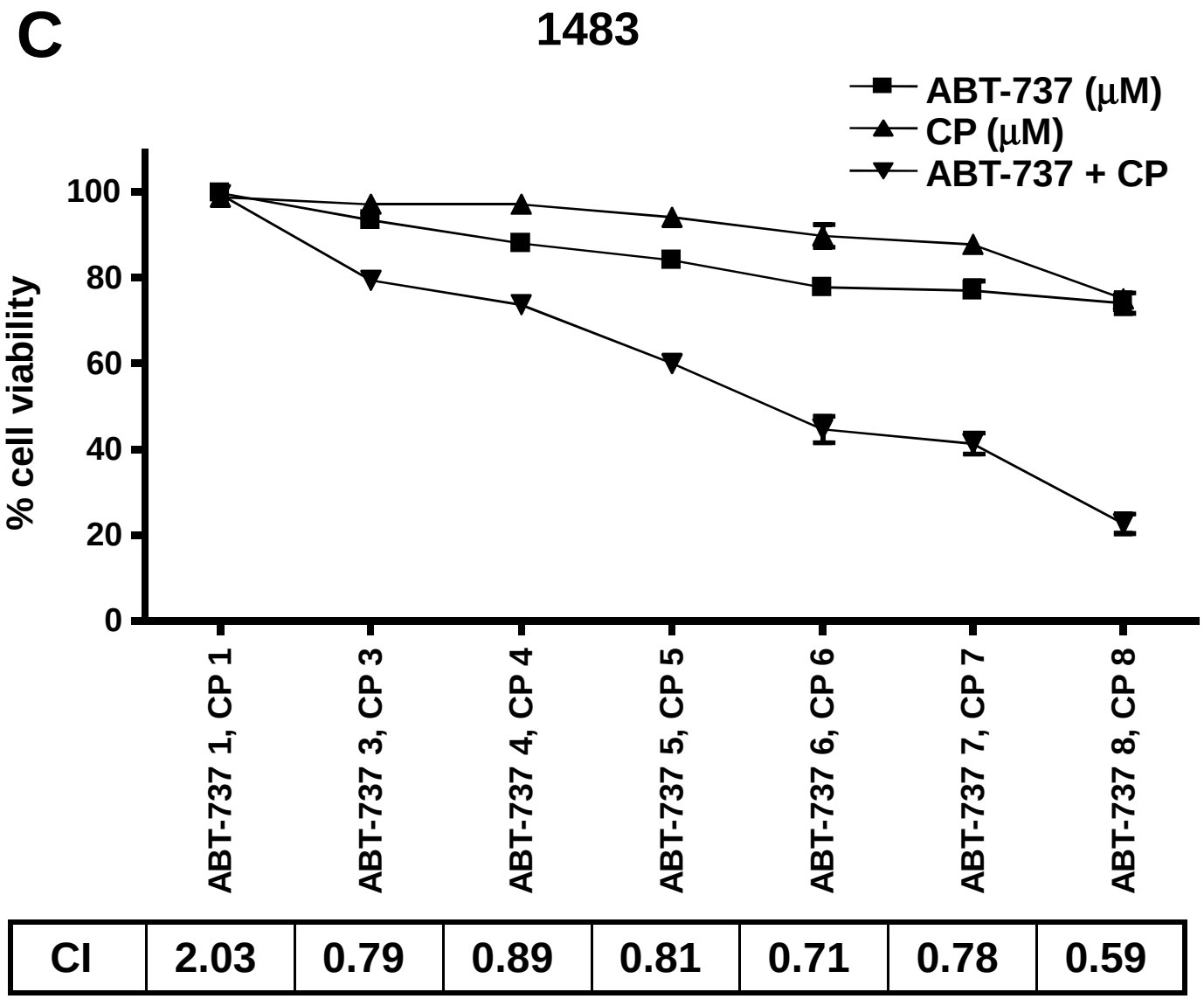
CI	0.50	0.64	0.50	0.51	0.61	0.59	0.61

Figure 1



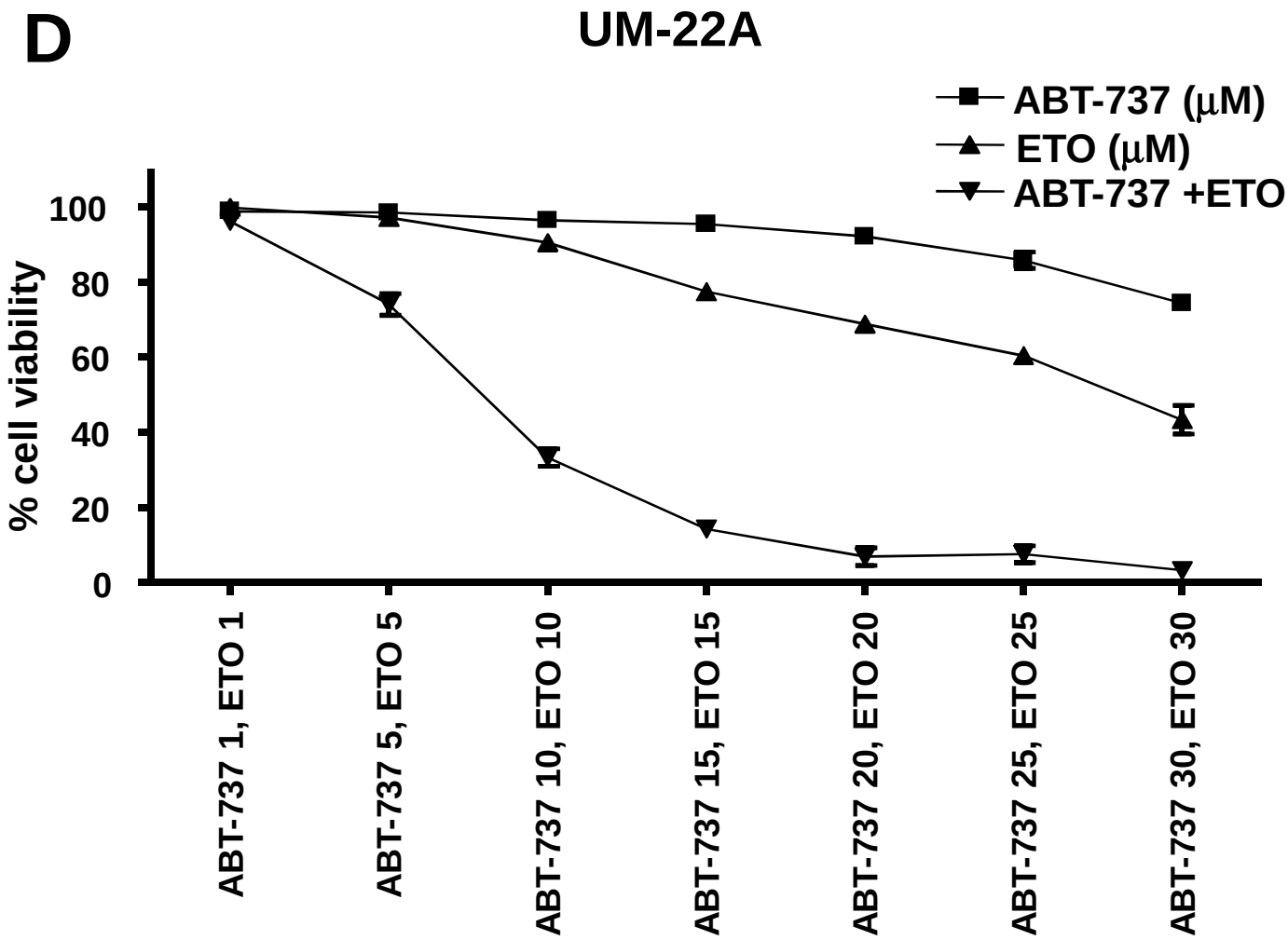
CI	0.28	0.43	0.32	0.35	0.41	0.46	0.49

Figure 1



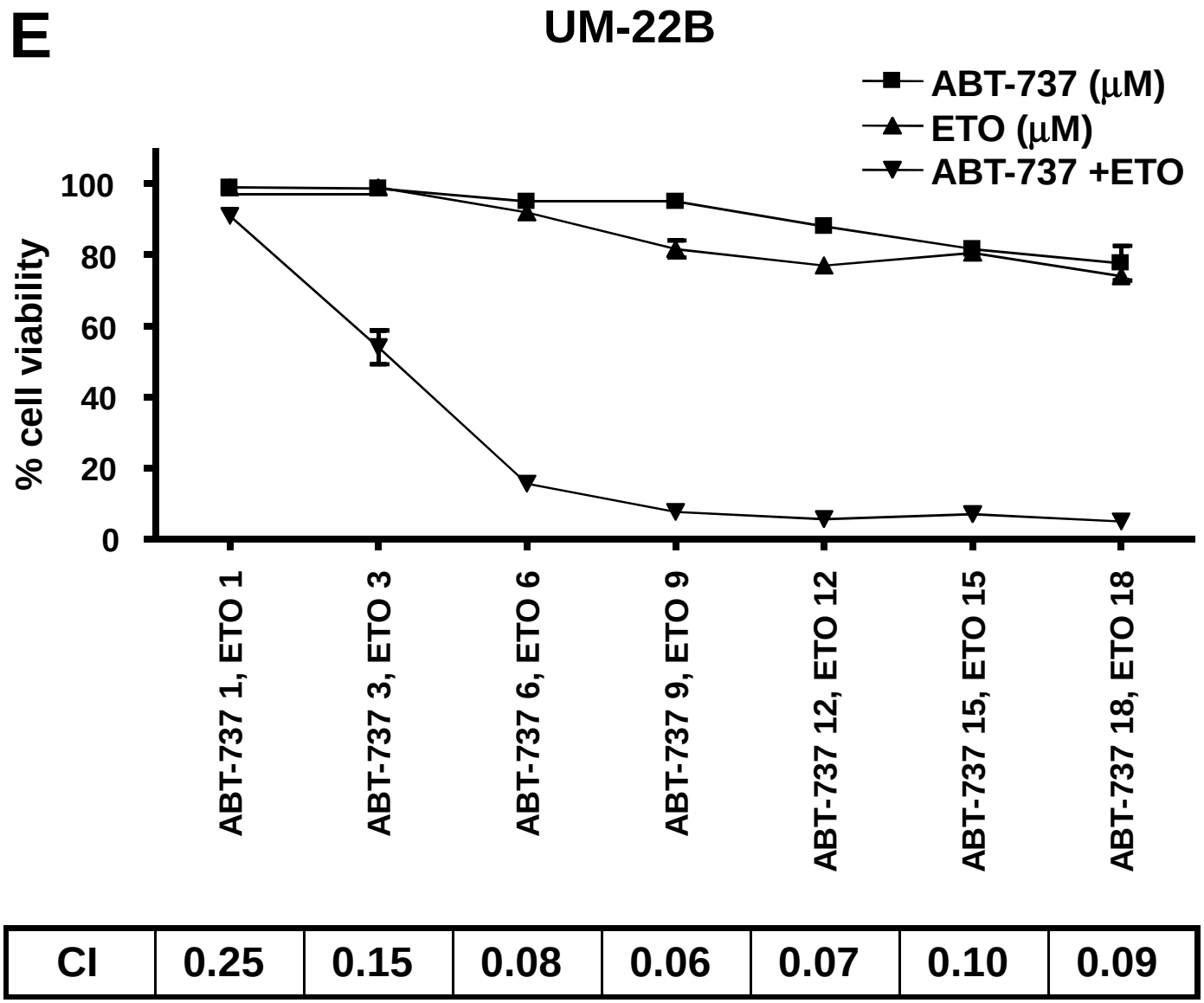
CI	2.03	0.79	0.89	0.81	0.71	0.78	0.59

Figure 1



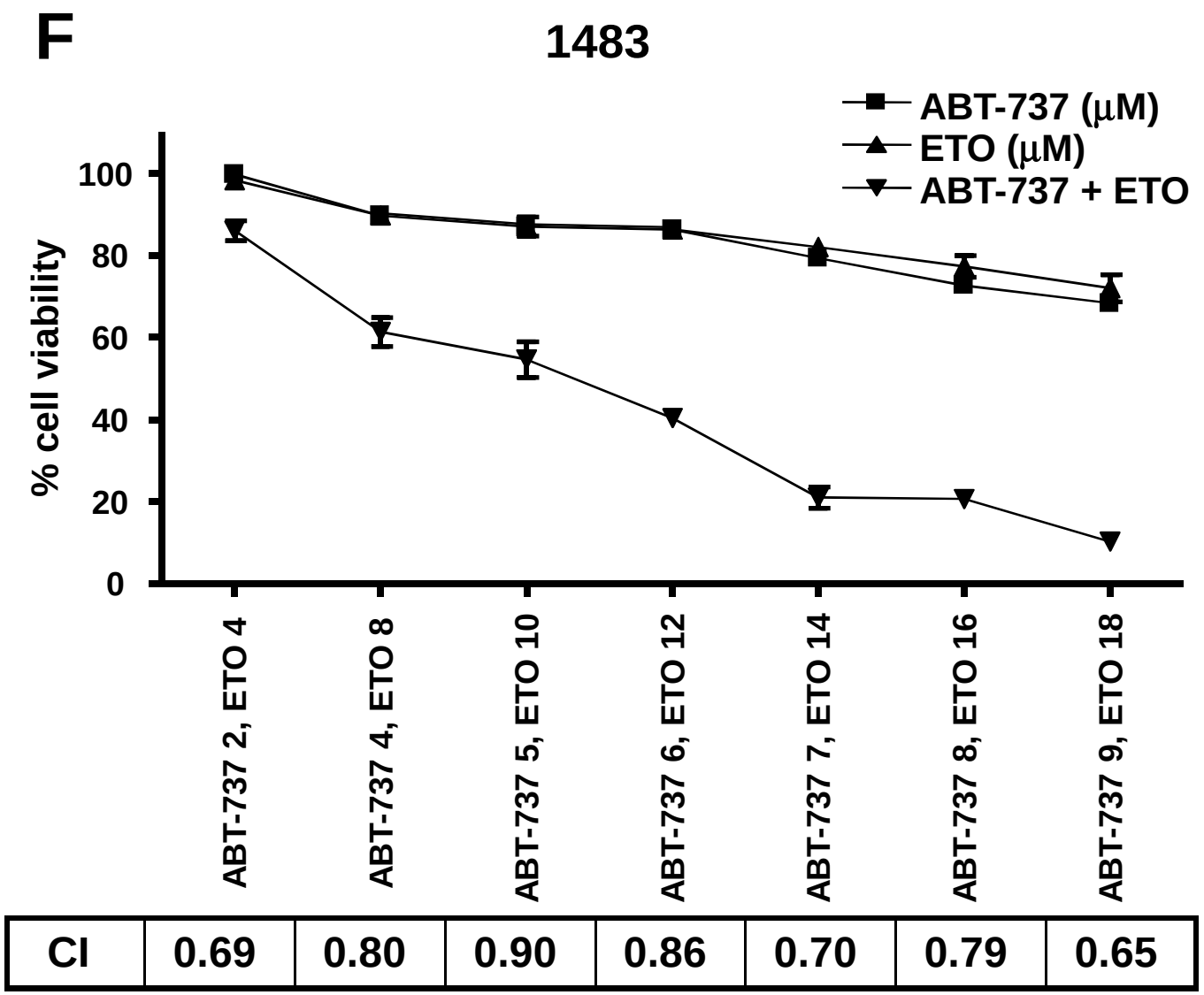
CI	0.37	0.38	0.24	0.18	0.15	0.12	0.14

Figure 1



CI	0.25	0.15	0.08	0.06	0.07	0.10	0.09

Figure 1



CI	0.69	0.80	0.90	0.86	0.70	0.79	0.65

Figure 1

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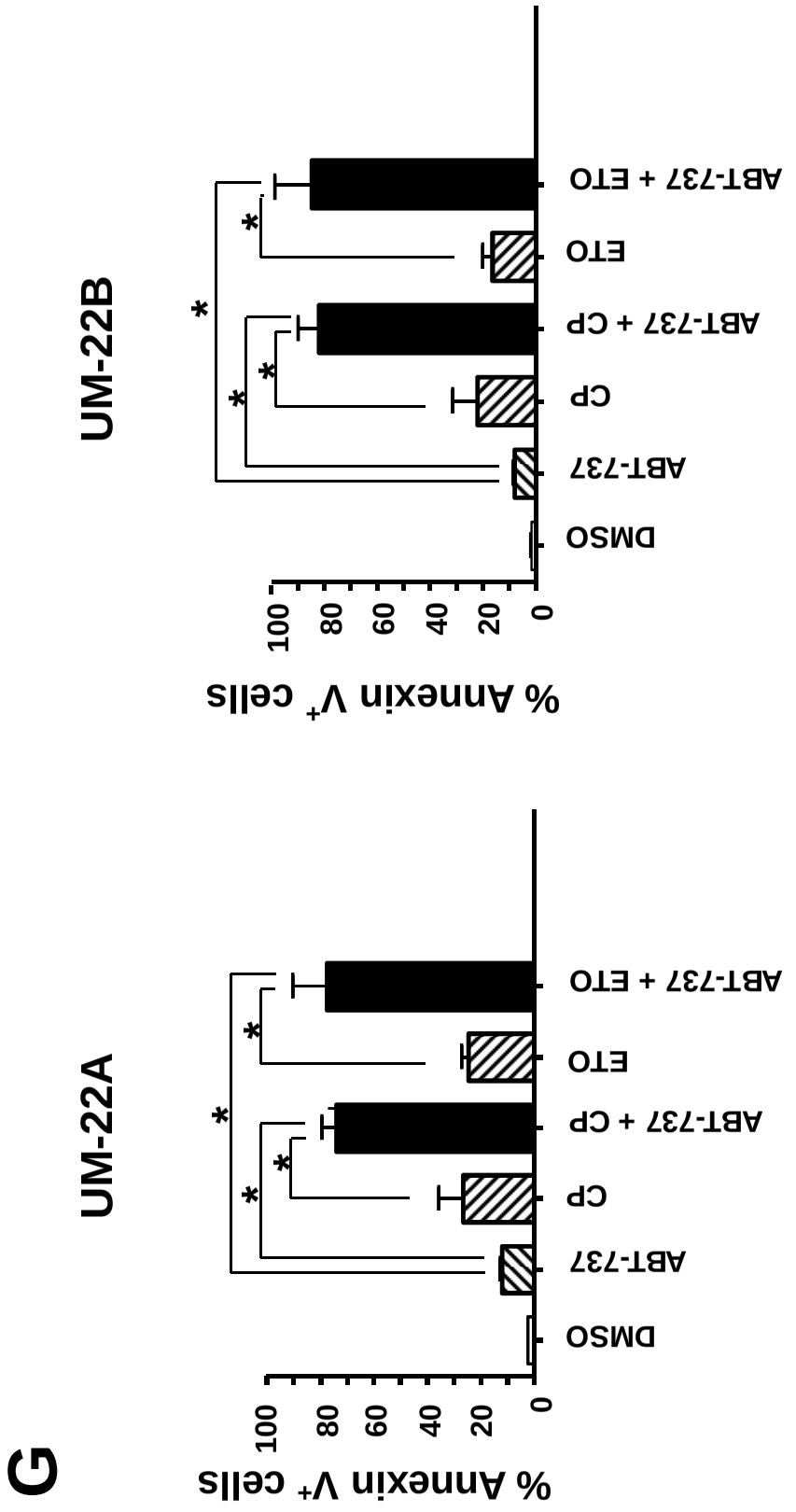

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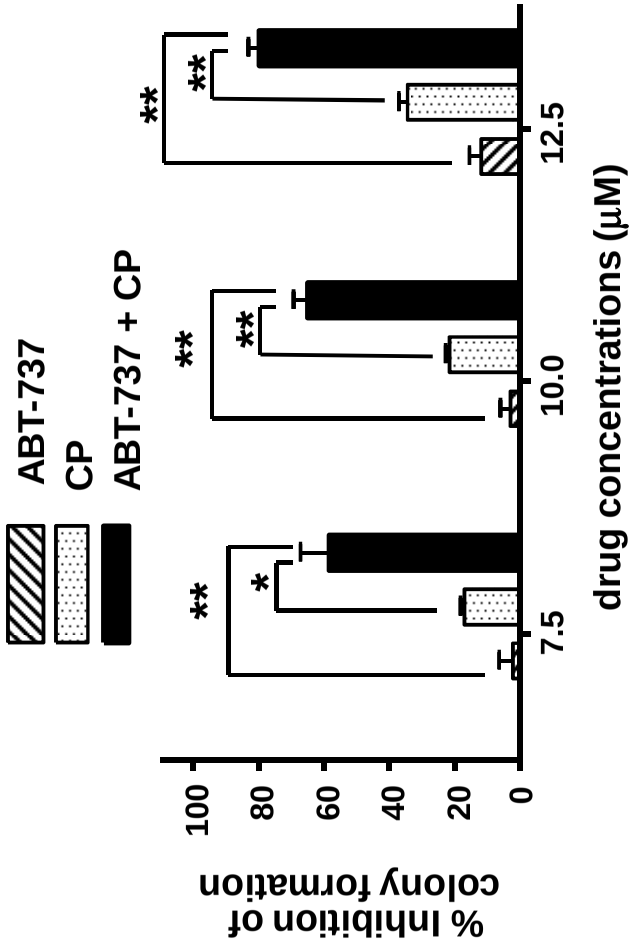

Figure 2

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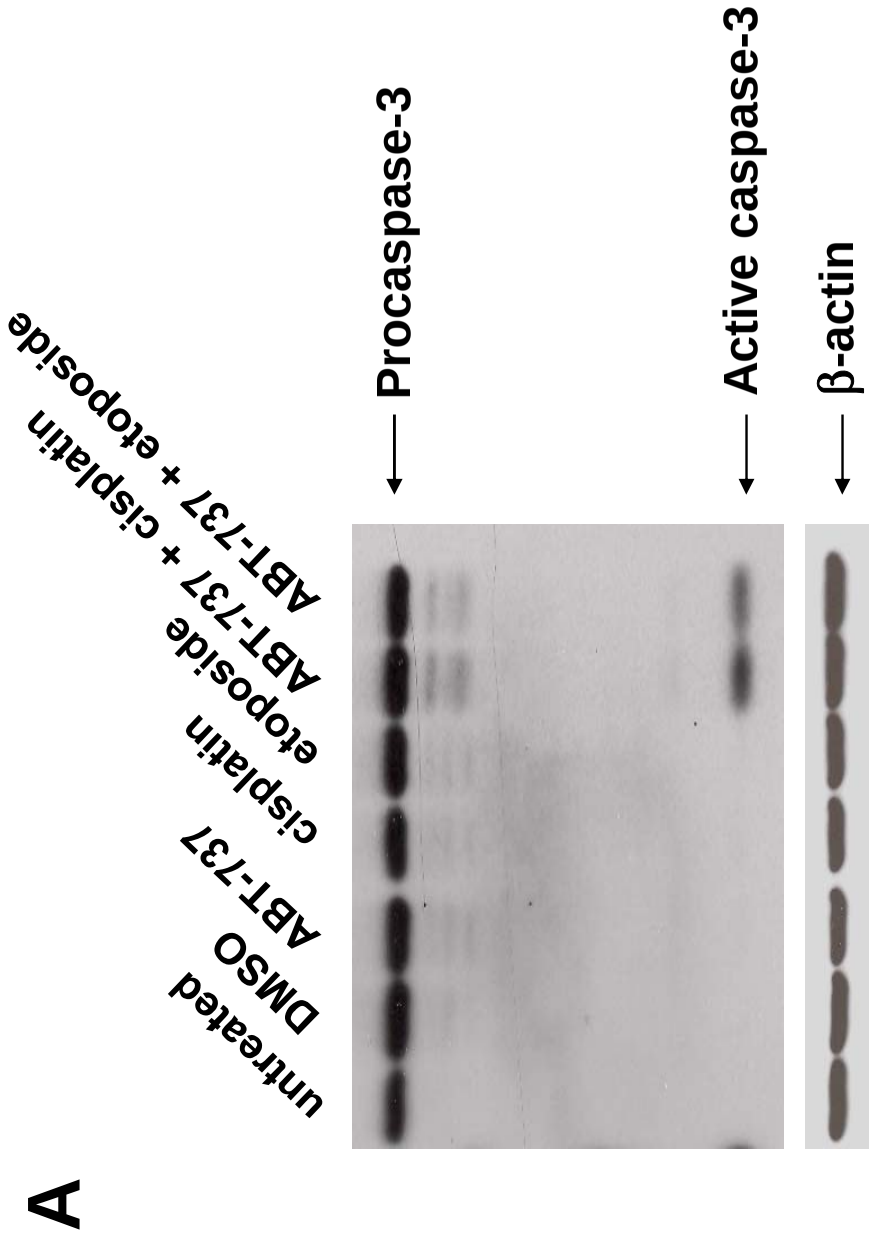

Figure 3

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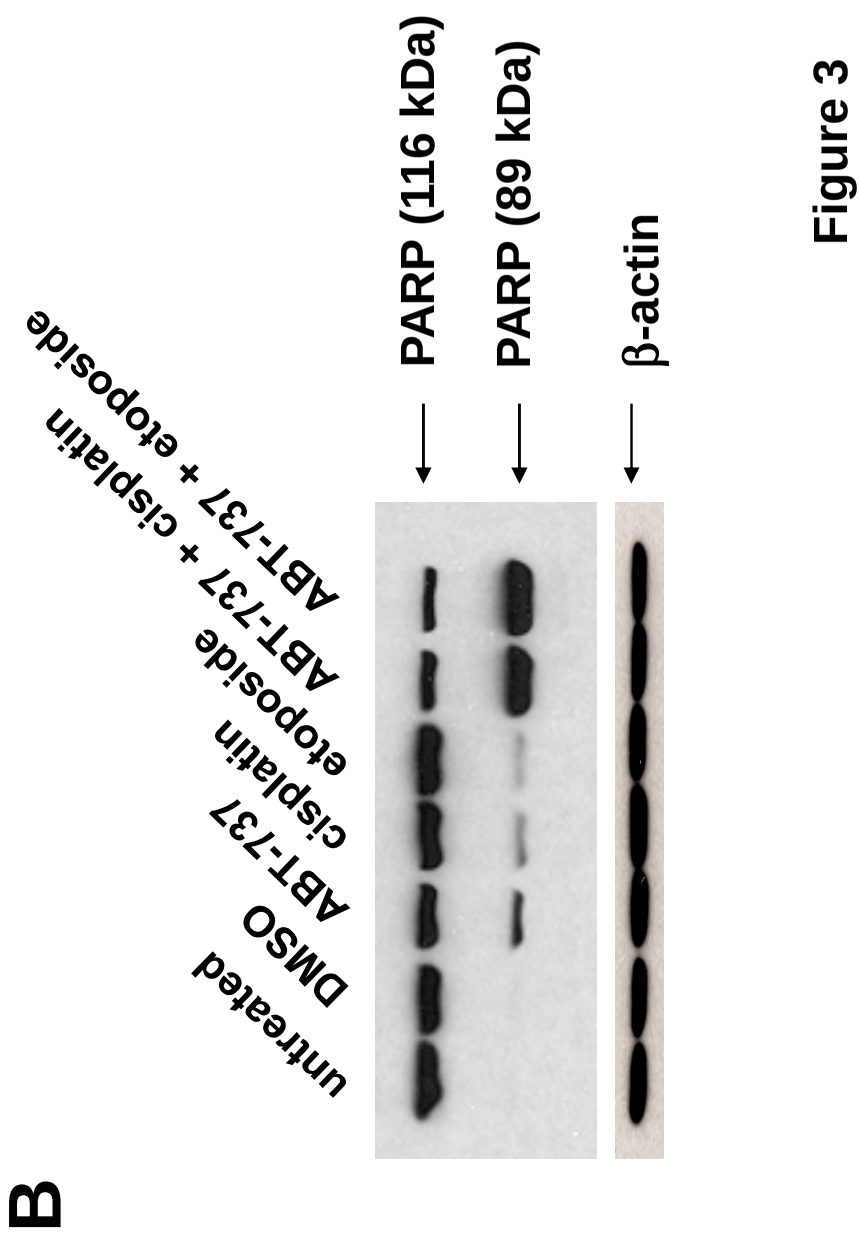

Figure 3

Figure 4

B

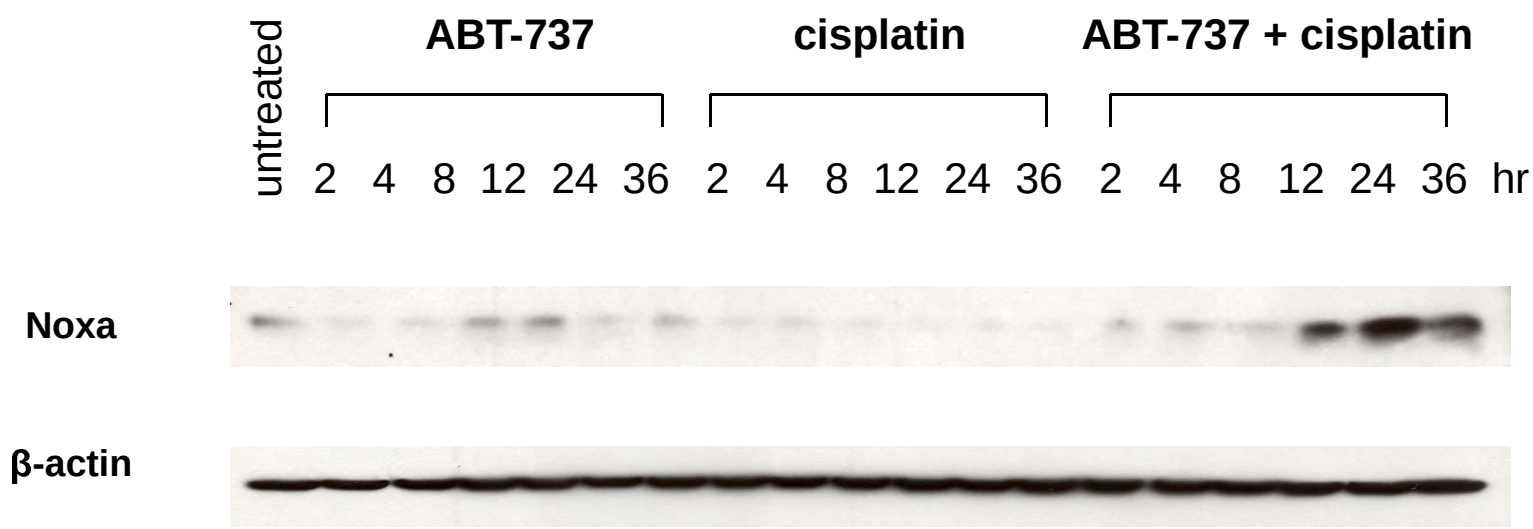

Figure 4

Figure 5

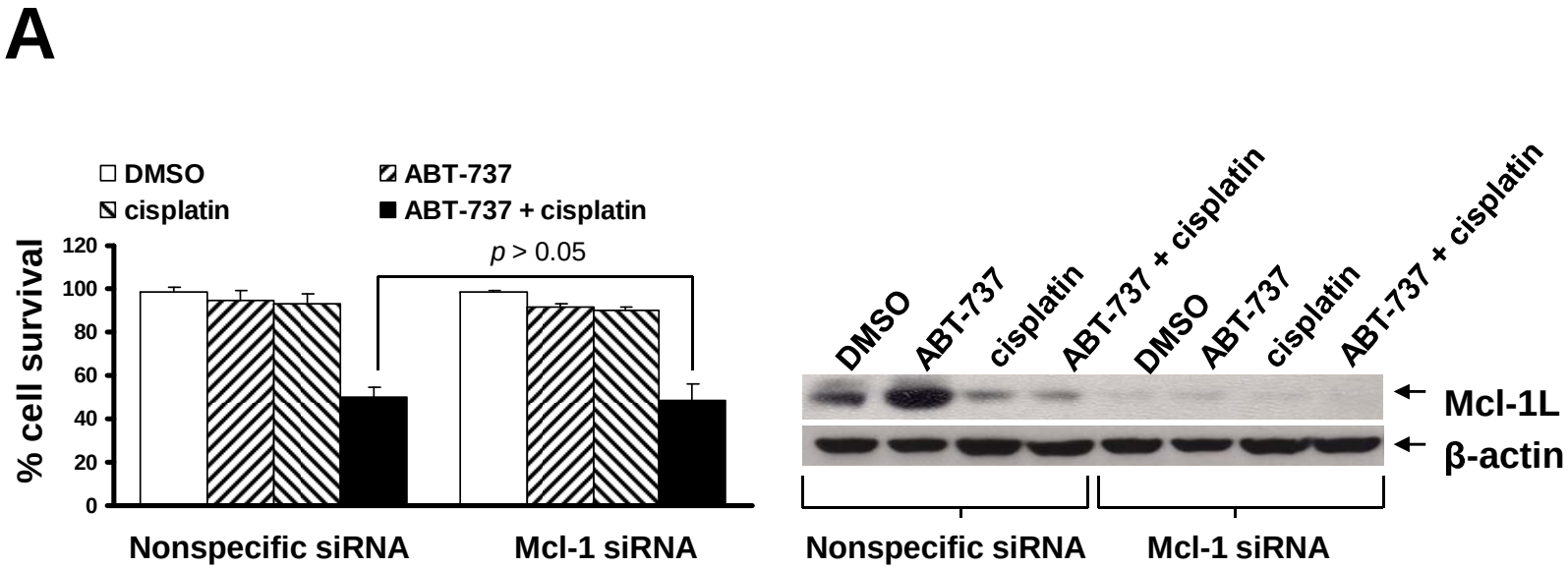

Figure 6

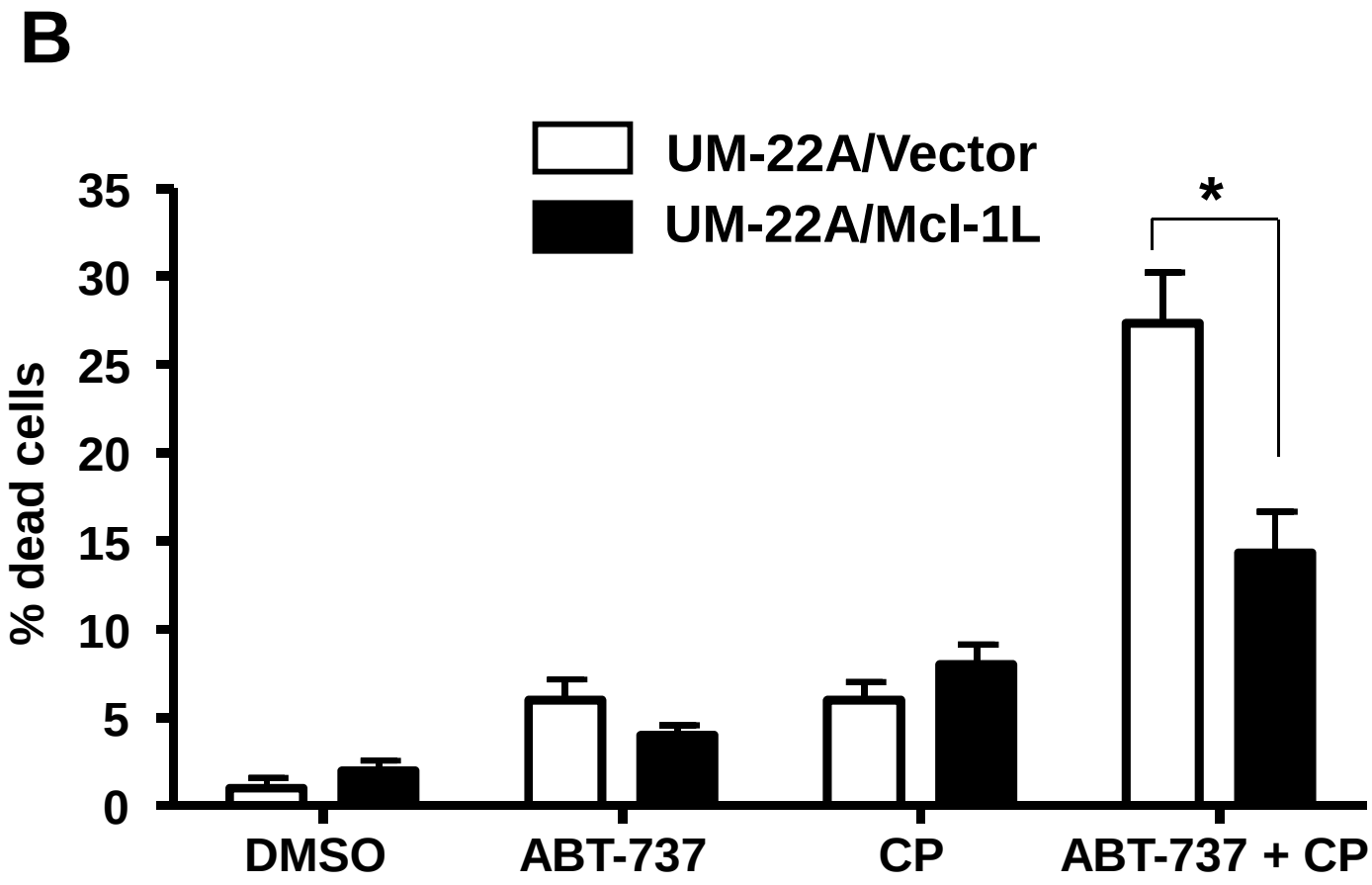

Figure 6