

# ARC Synergizes with ABT-737 to Induce Apoptosis in Human Cancer Cells

Uppoor G. Bhat<sup>1</sup>, Bulbul Pandit<sup>1</sup>, and Andrei L. Gartel<sup>1,2,3</sup>

## Abstract

Previously, we reported that the nucleoside analogue/transcriptional inhibitor ARC (4-amino-6-hydrazino-7- $\beta$ -D-ribofuranosyl-7H-pyrrolo(2,3-d)-pyrimidine-5-carboxamide) was able to induce p53-independent apoptosis in multiple cancer cell lines of different origins. This occurred, at least in part, by the suppression of short-lived, prosurvival member of the Bcl-2 family, Mcl-1. In contrast, we show here that treatment of human cancer cells with the pan-Bcl-2 inhibitor ABT-737 alone led to upregulation of Mcl-1 protein expression. Combination of subapoptotic concentrations of ABT-737 and ARC induced mitochondrial injury and potent caspase-3/caspase-9-dependent apoptosis in a wide variety of human cancer cell lines. These data suggest that the ABT-737/ARC combination, which simultaneously targets Bcl-2 and Mcl-1, may be efficient against human cancer. *Mol Cancer Ther*; 9(6); 1688–96. ©2010 AACR.

## Introduction

Previously, we identified the nucleoside analogue named ARC (4-amino-6-hydrazino-7- $\beta$ -D-ribofuranosyl-7H-pyrrolo(2,3-d)-pyrimidine-5-carboxamide), which is an inhibitor of P-TEFb kinase (CDK9/CyclinT1 complex) and acts as a transcriptional inhibitor (1, 2). We showed that ARC induced potent apoptosis in transformed and cancer, but not in normal cells, and exhibited potent anti-angiogenic activity *in vitro* (1, 3–5). In addition, we found that ARC targets the labile antiapoptotic protein Mcl-1 (3–5), and overexpression of Mcl-1 protects cells from ARC-induced apoptosis (3, 5).

Abbott Laboratories recently synthesized the pan-Bcl-2 inhibitor ABT-737, a BH3 mimetic developed by structure-based drug design (6). ABT-737 competes with BAD for docking to the hydrophobic groove of Bcl-2 family proteins, thus promoting Bax and Bak activation (6). At the same time, ABT-737 has a low affinity for another member of the Bcl-2 family protein, Mcl-1 (6), which is a critical survival factor for various malignancies (7). Cancer cells with high levels of Mcl-1 expression have been associated with resistance to ABT-737 (8, 9), whereas downregulation of Mcl-1 significantly augmented ABT-737-induced apoptosis

in human cancer cells (8, 10, 11). As a single agent, ABT-737 was efficient against some small-cell lung cancer cell lines (12) and leukemia cells (11), but largely ineffective at promoting cell death in prostate and renal cancer cells (13). We show here that the combination of subapoptotic concentrations of ARC with ABT-737 resulted in synergistic induction of cell death in numerous human cancer cell lines of different origins. Our data suggest that downregulation of Mcl-1 by ARC may contribute to its synergy with ABT-737.

## Materials and Methods

### Cell culture and reagents

The melanoma cell lines DM366 and DM833 were grown in Iscove's modified Dulbecco's medium (Invitrogen) medium. The osteosarcoma cell line U2OS-C3, the colon cancer cells LIM1215 and SW480, and the liver cancer cell lines Huh-7 and HepG2 were all grown in DMEM (Invitrogen). The neuroblastoma cell lines SKNAS and IMR32 were grown in RPMI 1640 (Invitrogen). The HPAC pancreatic cell line was grown in DMEM/F-12 (Invitrogen). All the media were supplemented with 10% fetal bovine serum (Atlanta Biologicals), 2 mmol/L L-glutamine, and 1% penicillin-streptomycin and the cells were grown at 37°C in 5% CO<sub>2</sub>. ARC was obtained from the National Cancer Institute and ABT-737 from Abbott Laboratories. All these drugs were dissolved in DMSO and stored as 10 mmol/L stock solutions. Specific inhibitors to caspase-3 (Z-DEVD-FMK) and caspase-9 (Z-LEHD-FMK) and general/pan-caspase inhibitor (Z-VAD-FMK) were purchased from BD Pharmingen. Specific inhibitor to caspase-8 (granzyme B) was purchased from EMD Biosciences. Solutions for the caspase inhibitors were made according to the manufacturer's instructions.

**Authors' Affiliations:** Departments of <sup>1</sup>Medicine, <sup>2</sup>Biochemistry and Molecular Genetics, and <sup>3</sup>Microbiology and Immunology, University of Illinois at Chicago, Chicago, Illinois

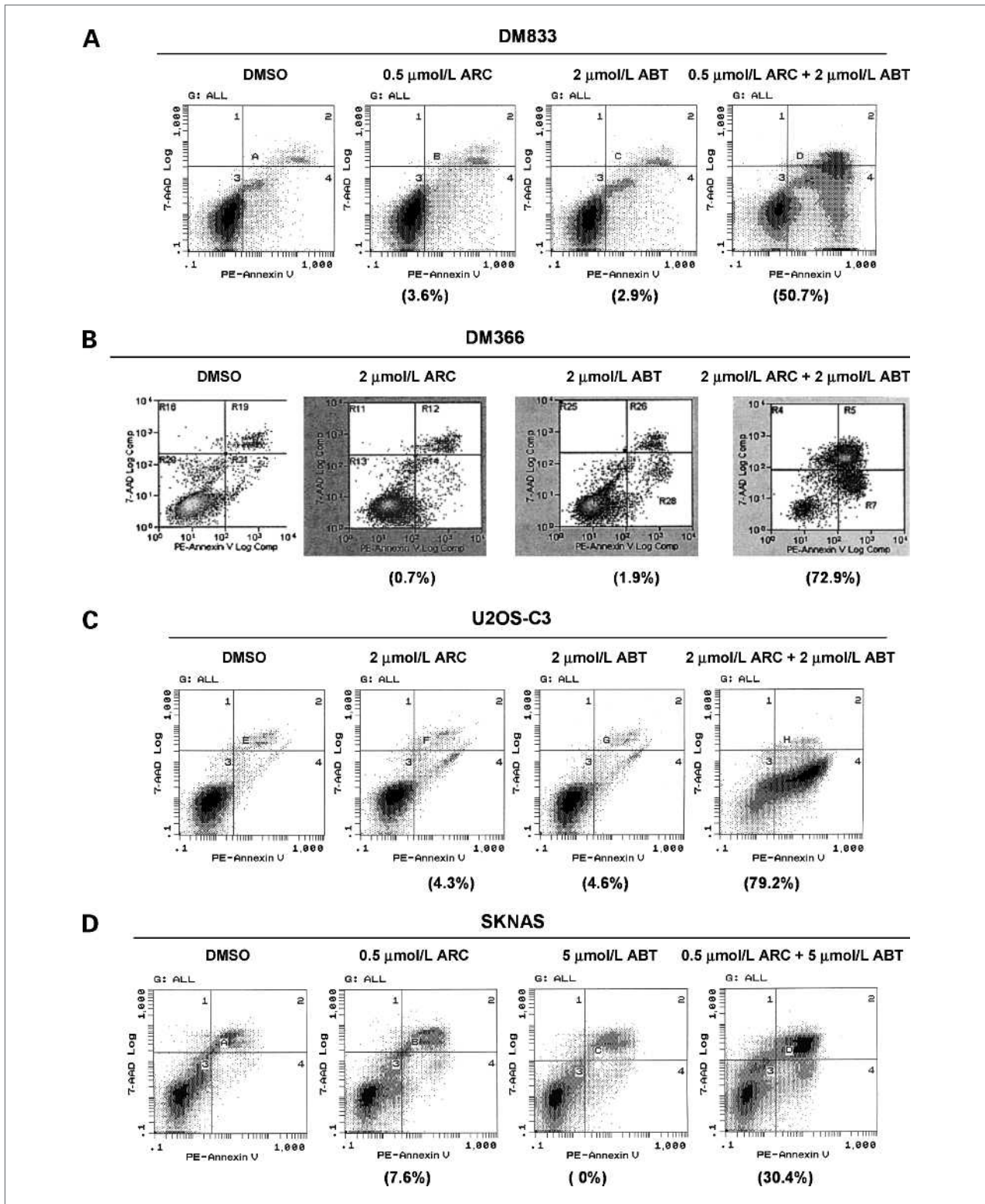
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U.G. Bhat and B. Pandit contributed equally to this work.

**Corresponding Author:** Andrei L. Gartel, University of Illinois at Chicago, Department of Medicine, 840 South Wood Street, Room 1041, Chicago, IL 60612. Phone: 312-996-1855; Fax: 312-996-8697. E-mail: [agartel@uic.edu](mailto:agartel@uic.edu)

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**Figure 1.** Annexin V-PE staining after combination treatment of human tumor cells with ARC and ABT-737. A and B, DM833 and DM366 melanoma cells were treated with subapoptotic concentrations of ARC, ABT-737, or both, as indicated, for 24 h, stained with Annexin V-PE/7-AAD, and analyzed by flow cytometry. C, U2OS-C3 osteosarcoma cells were treated with ARC, ABT-737, or the combination of ARC/ABT-737 for 24 h, stained with Annexin V-PE, and analyzed by flow cytometry. D, SKNAS neuroblastoma cells were treated with ARC or ABT-737 alone or cotreated with ARC and ABT-737, stained with Annexin V-PE and 7-AAD, and analyzed by flow cytometry. The net percentages of apoptotic cells relative to untreated control are shown in parentheses.

### Annexin V-phycoerythrin staining and fluorescence-activated cell sorting analysis

Aliquots of cells were stained using Annexin V-phycoerythrin (PE) apoptosis detection kit (BD Pharmingen) according to the manufacturer's recommendations. Briefly, the cells were trypsinized, washed in PBS, and resuspended in binding buffer ( $1 \times 10^6$ /mL). Five microliters

of Annexin V-PE and 5  $\mu$ L of 7-amino-actinomycin D (7-AAD) were added and incubated for 15 minutes at room temperature in the dark and analyzed by flow cytometry.

### Measurement of combination index value

The synergy between ARC and ABT-737 in human cancer cells was quantitatively evaluated by the median-effect

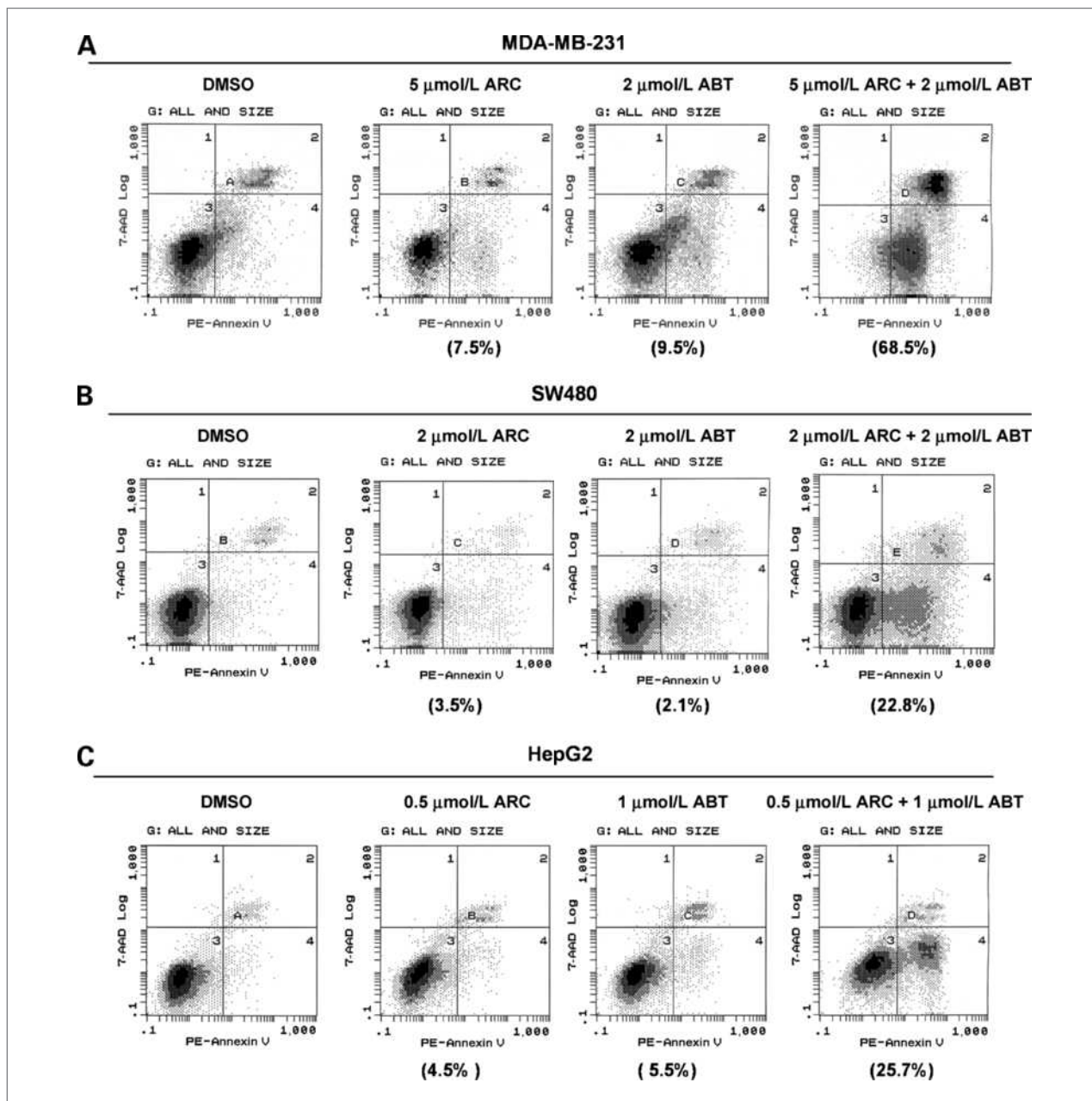
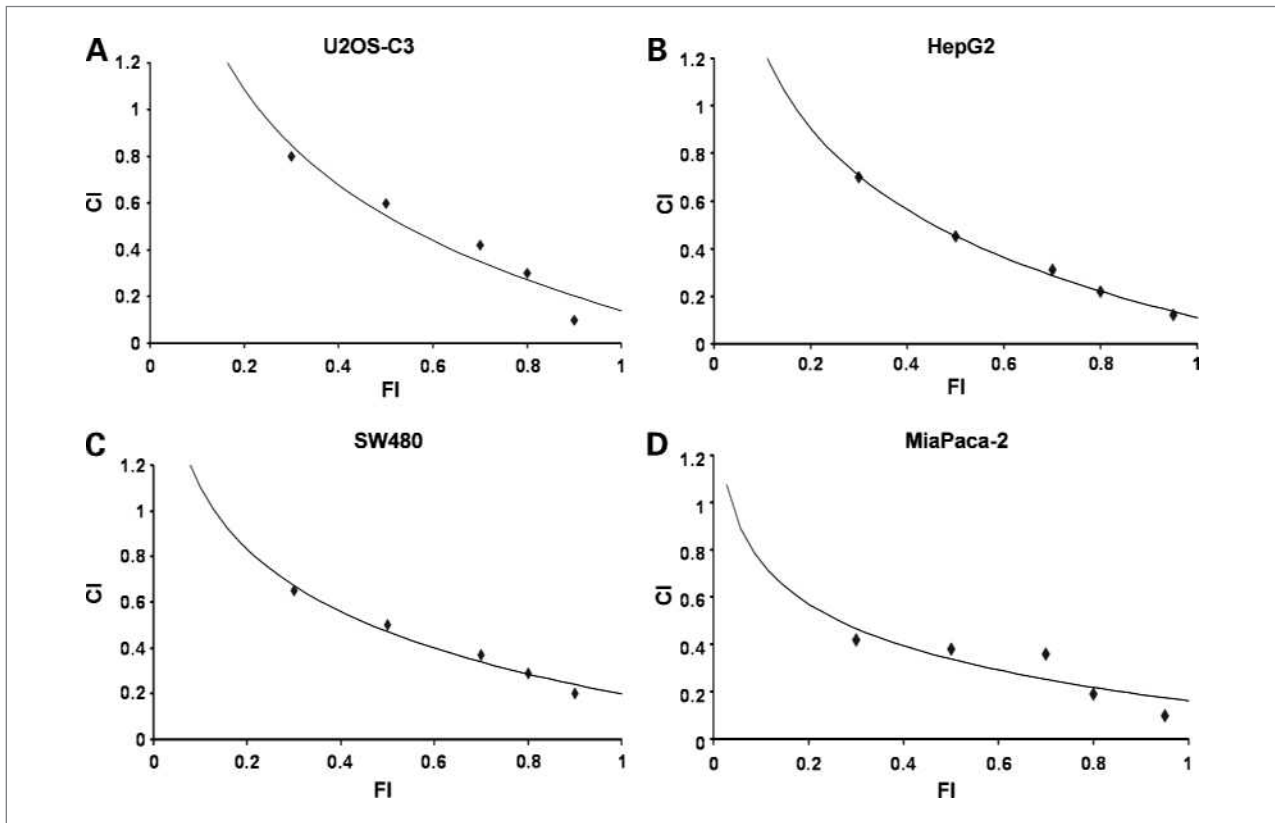


Figure 2. Combination treatment of ARC and ABT-737 induces apoptosis in human tumor cell lines. A, MDA-MB-231 breast cancer cells were treated with subapoptotic concentrations of ARC, ABT-737, and the ARC/ABT-737 combination for 24 h, stained with Annexin V-PE and 7-AAD, and analyzed by flow cytometry. B, SW480 colon cancer cells were treated with subapoptotic concentrations of ARC, ABT-737, or the combination of ARC and ABT-737, stained with Annexin V-PE/7-AAD, and analyzed by flow cytometry. C, HepG2 liver cancer cells were treated with sublethal concentration of ARC alone, ABT-737 alone, and the combination of ARC and ABT-737, as indicated, and analyzed after Annexin V-PE/7-AAD staining by flow cytometry. The net percentages of apoptotic cells relative to untreated control are shown in parentheses.



**Figure 3.** ARC and ABT-737 display synergistic CI values in human cancer cells. CI chart for the ARC/ABT-737 drug combination was plotted with CI (y axis) and fractional effect (FI; x-axis). Percentage inhibition of proliferation was determined following treatment with a single agent or the ARC/ABT-737 combination. CI values of the two agents plotted for U2OS-C3 (A), HepG2 (B), SW480 (C), and MiaPaca-2 (D) cells.

plot method formulated by Chou-Talalay (14). Cells were treated with varied doses of ARC alone, ABT-737 alone, or ARC and ABT-737 in combination. In our experiments, the  $IC_{30}$ ,  $IC_{50}$ ,  $IC_{70}$ ,  $IC_{80}$ , and  $IC_{90}$  values (i.e., the drug concentration needed to cause 30%, 50%, 70%, 80%, and 90% reductions in cell viability) were chosen for comparison. Percent viability of cells was determined using standard MTT assay. Combination index (CI) values were calculated using the following formula:

$$CI = \frac{CA, x}{IC_{x, A}} + \frac{CB, x}{IC_{x, B}}$$

where  $CA, x$  and  $CB, x$  are the concentrations of drug A and drug B used in combination to achieve  $x\%$  drug effect;  $IC_{x, A}$  and  $IC_{x, B}$  are the concentrations for single agents to achieve the same effect (14). A CI value of  $<1$  indicates synergy, a value of 1 indicates additive effects, and a value of  $>1$  indicates antagonism.

#### Immunoblot analysis

The cells were harvested and lysed in immunoprecipitation buffer [20 mmol/L HEPES (pH 7.4), 1% Triton X-100, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 100 mmol/L NaF, 10 mmol/L  $Na_4P_2O_7$ ,

1 mmol/L sodium orthovanadate, 0.2 mmol/L phenylmethylsulfonyl fluoride] supplemented with protease inhibitors, and the protein concentrations were measured by the Bio-Rad method. Fifty micrograms of the cell lysates were separated by electrophoresis on a 12% SDS-polyacrylamide mini gel and transferred onto a polyvinylidene difluoride membrane. Immunoblotting was done as described (15) with specific antibodies for Mcl-1 (MS-683, Lab Vision), Bax (2774, Cell Signaling), Bcl-2 (7382 Santa Cruz), cleaved caspase-3 (9664, Cell Signaling), and  $\beta$ -actin (AS441, Sigma).

#### Nuclear-ID green chromatin condensation detection

Cells were stained using *in vitro* apoptosis detection kit (Enzo Life Sciences) according to the manufacturer's recommendations. Briefly,  $3 \times 10^4$  to  $4 \times 10^4$  cells were plated in 96-well black-wall clear-bottom plate and allowed to grow overnight. Cells were pretreated with apoptosis inhibitors for 2 hours, following which they were treated with either DMSO or the ARC/ABT-737 combination and incubated for 24 hours. Cells were washed with assay buffer and stained with 1  $\mu$ mol/L nuclear-ID green dye. The plate was read in a fluorescence microplate reader with excitation wavelength 488 nm and emission wavelength 520 nm.

**Clonogenic assay**

HepG2 and SW480 cells were grown in RPMI 1640 to 50% to 70% confluence and treated with various combinations of ARC and ABT-737 for 24 hours. The cells were then trypsinized, resuspended in the media, and counted. The cells were reseeded (2,000 per dish) into 100-mm new tissue culture dishes and incubated for 10 days. Fresh medium was added on the 5th day. On the 10th day, medium was removed from the dishes and washed once with ice-cold PBS. The colonies were stained with 2 mL of 0.25% 1,9-dimethyl-methylene blue

in 50% ethanol for 45 minutes on a rocking platform. The dishes were rinsed three times with PBS and air-dried, and the colonies were counted.

**Mitochondrial injury**

Cells ( $10^6$ ) were resuspended in fresh RPMI I640, treated with tetramethylrhodamine ethyl ester (TMRE) to a final concentration of 25 nmol/L, and incubated at 37°C for 20 minutes. The cells were centrifuged and resuspended in 25 nmol/L TMRE in PBS. The mitochondrial membrane potential was measured by flow cytometry.

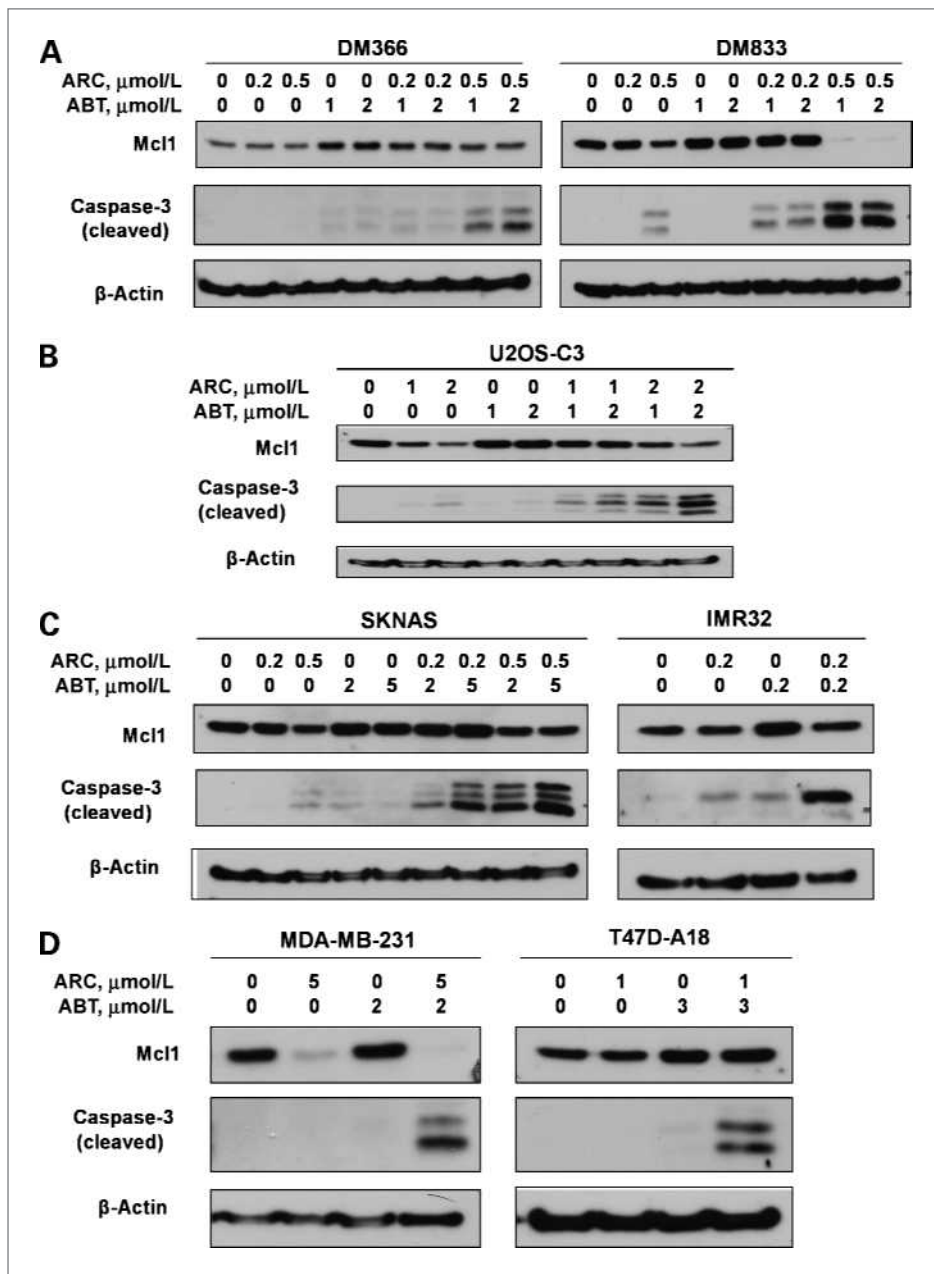
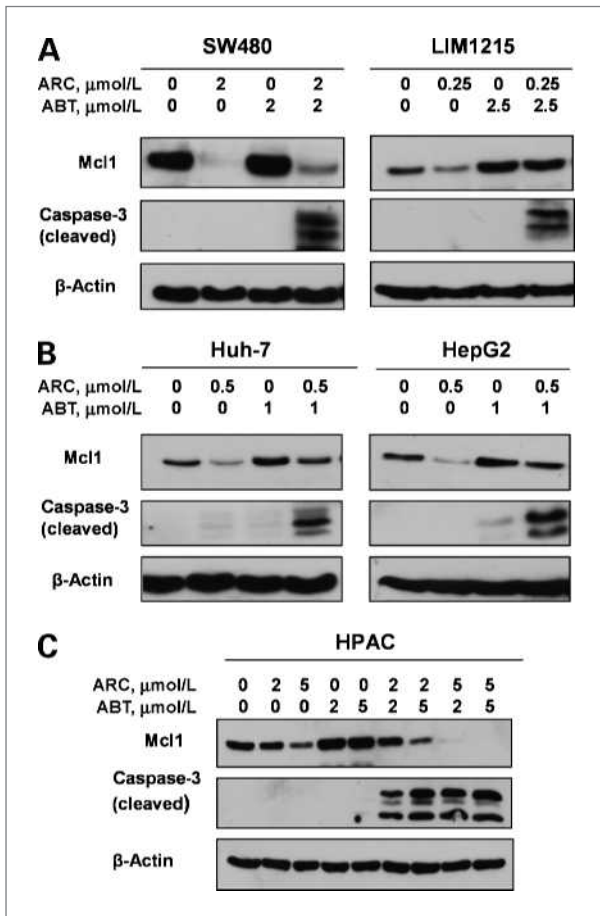


Figure 4. ARC and ABT-737 induce caspase-3 cleavage in human melanoma, osteosarcoma, neuroblastoma, and breast cancer cells. A, melanoma DM366 and DM 833 cells were treated with ARC, ABT-737, or both, as indicated, for 24 h; total cell lysates were extracted and immunoblotted with antibodies for Mcl-1, cleaved caspase-3, and  $\beta$ -actin. B, U2OS-C3 osteosarcoma cells were treated with the drugs as indicated for 24 h and the cell lysates were immunoblotted for Mcl-1, cleaved caspase-3, and  $\beta$ -actin. C, neuroblastoma SKNAS and IMR32 cells were treated with ARC, ABT-737, or both, as indicated, for 24 h and immunoblotted with specific antibodies for Mcl-1, cleaved caspase-3, and  $\beta$ -actin. D, breast cancer cell lines MDA-MB-231 and T47D-A18 were treated with ARC, ABT-737, or both for 24 h and immunoblotted for Mcl-1, cleaved caspase-3, and  $\beta$ -actin.



**Figure 5.** ARC synergizes with ABT-737 in inducing caspase-3 cleavage in human colon, liver, and pancreatic cancer cells. **A**, SW480 and LIM1215 colon cancer cells were treated as indicated for 24 h and analyzed by immunoblotting. **B**, Huh-7 and HepG2 liver cancer cells were treated with ARC, ABT-737, and the combination of ARC/ABT-737 and immunoblotted for the proteins, as indicated. **C**, HPAC pancreatic cancer cells were treated with ARC, ABT-737, or both and analyzed for the expression of Mcl-1, cleaved caspase-3, and  $\beta$ -actin by immunoblotting.

## Results and Discussion

We showed earlier that ARC inhibited the growth of and induced apoptosis in melanoma, neuroblastoma, liver, breast, and colon cancer cell lines (1, 3–5). To determine whether ARC may synergize with ABT-737 against human cancer cell lines of different origins, we treated melanoma, osteosarcoma, neuroblastoma, breast, pancreatic, liver, and colon cancer cells either with subapoptotic concentrations of ARC or ABT-737 alone or with combinations of the two for 24 hours and used Annexin V-PE/7-AAD staining and flow cytometry to determine the percent of apoptotic cells (Figs. 1 and 2). As shown in Fig. 1A, treatment of DM833 cells with 0.5  $\mu\text{mol/L}$  ARC or 2  $\mu\text{mol/L}$  ABT-737 induced apoptosis of only 3.6% and 2.9% of cells, respectively, over the control, whereas treatment with both drugs at the same doses caused 50.7% of

cells to undergo apoptosis (Fig. 1A). Similarly, in osteosarcoma cells, treatment with 2  $\mu\text{mol/L}$  ARC or 2  $\mu\text{mol/L}$  ABT-737 induced only 4.3% and 4.6% apoptosis over the control, whereas combined treatment with both drugs resulted in 79.2% cell death (Fig. 1C). In addition, enhanced apoptotic effects of ARC/ABT-737 combinations were also seen in other cell types such as neuroblastoma (Fig. 1D), breast cancer (Fig. 2A), colon cancer (Fig. 2B), and liver cancer (Fig. 2C). All these data suggest that the combination of ARC with ABT-737 resulted in synergistic programmed cell death in human cancer cell lines of different origins.

To quantitatively validate the synergistic nature of the interaction between ARC and ABT-737, we examined cell viability after single and combination drug treatments by using the Chou-Talalay median-effect equation method (14). CI values below 1 indicate a synergistic antiproliferative effect, and the CI range values for the combined treatments with ARC/ABT-737 in four different human cancer cell lines were 0.1 to 0.8 (Fig. 3) for fractional effect corresponding to 0.3 to 0.9, suggesting a strong synergistic effect.

To further confirm that combination treatment of ARC and ABT-737 induces synergistic apoptosis, we used caspase-3 cleavage in drug-treated and control cells to serve as an indicator of apoptotic cell death. We treated DM366 and DM833 melanoma cells with ARC, ABT-737, or both, as indicated, for 24 hours and performed immunoblotting for cleaved caspase-3 (Fig. 4A). Whereas treatment with ARC or ABT-737 alone induced little or no caspase-3 cleavage in these cells, treatment with several different combinations of these drugs showed potent caspase-3 cleavage. Similar synergistic effects of ARC/ABT-737 combinations on caspase-3 cleavage were seen in osteosarcoma (U2OS-C3, Fig. 4B), neuroblastoma (SKNAS and IMR32; Fig. 4C), breast (MDA-MB-231 and T47D-A18; Fig. 4D), colon (SW480 and LIM1215; Fig. 5A), liver (Huh-7 and HepG2; Fig. 5B), and pancreatic (HPAC; Fig. 5C) cancer cells.

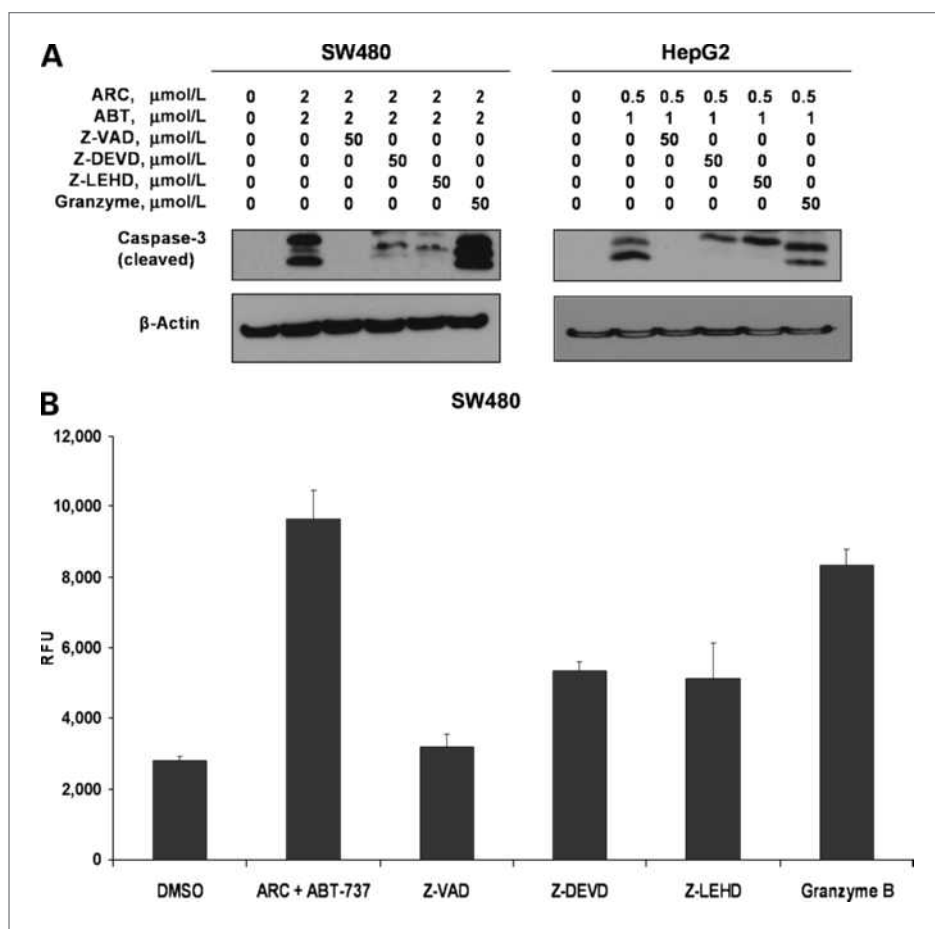
To determine how downregulation of Mcl-1 by ARC/ABT-737 treatment correlates with cell death, we measured Mcl-1 protein levels in cells treated with either ARC or ABT-737 or their combination by immunoblotting with specific Mcl-1 antibody (Figs. 4 and 5). In accordance with our previous results (3–5), treatment with ARC alone attenuated Mcl-1 protein levels in all the cell lines in a dose-dependent manner. In contrast, treatment with ABT-737 upregulated Mcl-1 in all the cell lines, although to varying degrees. However, the effect of treatment with the drug combinations on Mcl-1 expression was variable in the different cell lines, but in all cases, these levels were lower than after treatment by ABT-737 alone. These data suggest that the proapoptotic synergy between ABT-737 and ARC is partially based on the suppression of Mcl-1 protein by ARC.

To determine the roles of different caspases in ARC/ABT-737-induced apoptosis, we treated the SW480 colon cancer and the HepG2 liver cancer cell lines with this

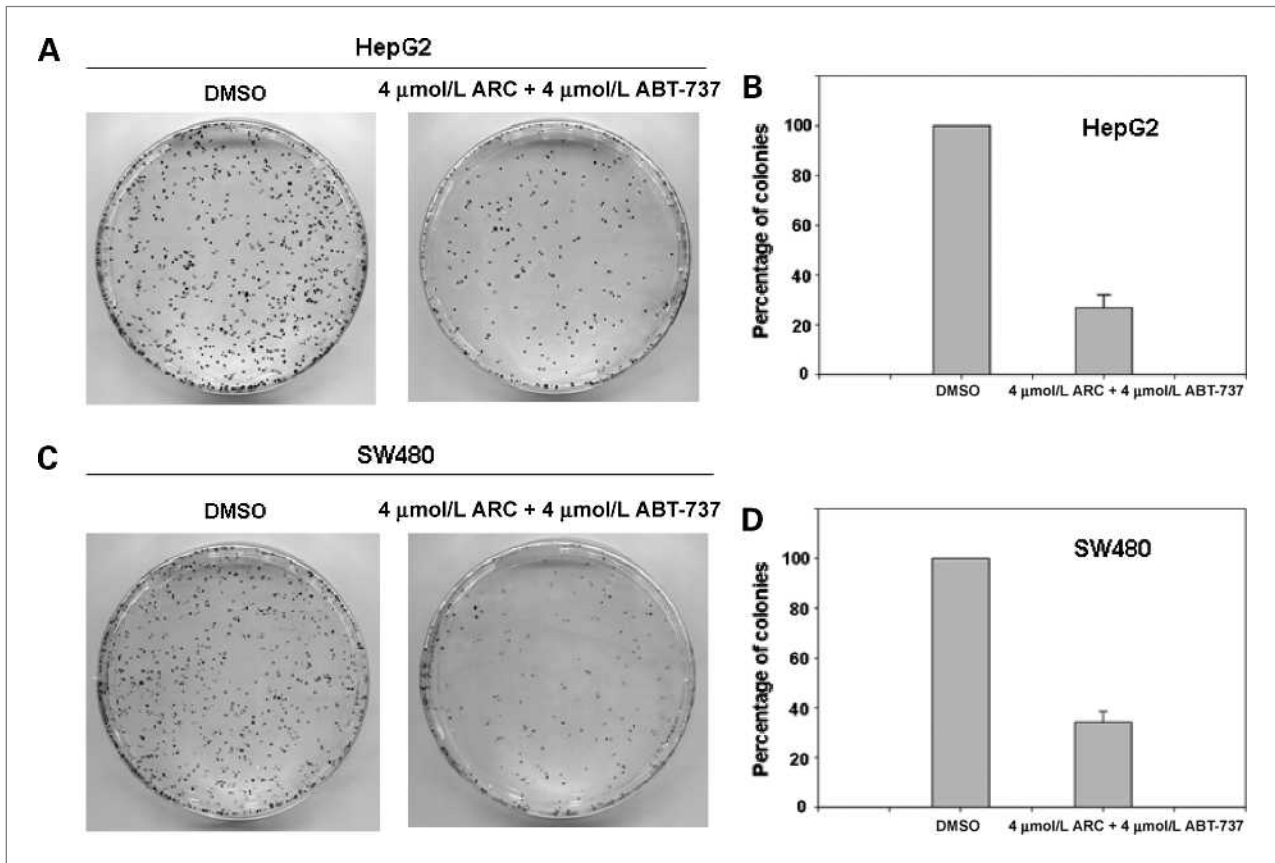
drug combination in the presence of caspase inhibitors and evaluated apoptosis by Western blot with antibodies specific for cleaved caspase-3 or by using *in vitro* chromatin condensation detection assay (Fig. 6). Using Western blot, we found that caspase-3 and caspase-9 inhibitors, but not caspase-8 inhibitor, inhibit caspase-3 cleavage after treatment with ARC/ABT-737 (Fig. 6A). Similarly, using a fluorescent green probe (chromatin condensation detection kit from Enzo Life Biosciences; see Materials and Methods), we measured the DNA condensation in cells induced by ARC/ABT-737. Cells pretreated with specific inhibitors to caspase-3 and caspase-9 lowered the fluorescence induced by the ARC/ABT-737 combination (Fig. 6B). In contrast, preincubation with specific inhibitor to caspase-8 did not affect ARC/ABT-737-induced DNA condensation and apoptosis. Therefore, apoptosis induced by the combination of ARC/ABT-737 in human cancer cells is dependent on caspase-3 and caspase-9, but not on caspase-8, which is required for extrinsic apoptosis (Fig. 6). Our findings contradict the data of Keuling et al. that caspase-8 is required for combination treatment of ABT-737 and Mcl-1 inhibitors in melanoma cells (16). Additional experiments are needed to resolve these differences.

To confirm that the combination of these drugs may induce intrinsic apoptosis, we investigated whether the drugs cause mitochondrial injury, one of the hallmarks of the intrinsic pathway. We stained treated and control cells with the mitochondria staining dye TMRE and analyzed the mitochondrial membrane potential by flow cytometry. The results show that combined treatment of ARC and ABT-737 caused depolarization of the mitochondrial membrane of melanoma (Supplementary Fig. S1A) and osteosarcoma (Supplementary Fig. S1B) cells, whereas treatment with either drug alone had little effect. These data suggest that mitochondrial injury induced by ARC/ABT-737 in human cancer cells correlated with cell death (Fig. 1A and C) after combination treatment.

We also tested the levels of other proteins that play important role in apoptosis, such as Bcl-2 and Bax, after treatment with ARC alone, ABT-737 alone, or the ARC/ABT-737 combination in a few human cancer cell lines (Supplementary Fig. S2), and we found that in contrast to Mcl-1, these treatments did not change the expression of Bax or Bcl-2. The long-term effects of combination treatment with ARC and ABT-737 were assessed by clonogenic assay. We found that colony formation in colon and liver



**Figure 6.** ARC/ABT-737-induced apoptosis in human cancer cells is dependent on caspase-3 and caspase-9, but not caspase-8, activity. **A**, specific inhibitors for caspase-3 and caspase-9 and pan-caspase inhibitor reduce ARC/ABT-737-induced apoptosis, whereas specific inhibitor for caspase-8 shows no reduction in apoptosis detected by immunoblotting for cleaved caspase-3 in SW480 and HepG2 cells. **B**, quantitation of apoptotic cells by using *in vitro* chromatin condensation detection kit. The combination of ARC/ABT-737 enhances fluorescence intensity in SW480 cells; pan-caspase inhibitor and caspase-3 and caspase-9 inhibitors, but not caspase-8 inhibitor, lower the fluorescence intensity of SW480 cells (columns, mean of three separate determinations; bars, SD).



**Figure 7.** Clonogenic assay shows the long-term effects of combination treatment of human cancer cells with ARC and ABT-737. A, clonogenic assay of HepG2 cells treated with DMSO or the ARC/ABT-737 combination for 24 h as detailed; photographs of petri dishes in a representative experiment are shown. B, percentage inhibition of colony formation by treatment with the drug combination (columns, mean of three separate experiments; bars, SD). C, clonogenic assay of SW480 cells treated with DMSO or the ARC/ABT-737 combination for 24 h. D, percentage inhibition of colony formation by treatment with the drug combinations (columns, mean of three separate determinations; bars, SD).

cancer cells treated with these drugs for 24 hours was suppressed 3- to 4-fold (Fig. 7).

It has been shown in preclinical studies that ABT-737 synergizes with Mcl-1 inhibitors against leukemia (8, 17–19), melanoma (16), multiple myeloma (20), lymphoma (10), prostate (21), and small-cell lung cancer (12). In this article, we showed the same effect for osteosarcoma, neuroblastoma, breast, colon, pancreatic, and liver cancer cells, suggesting that the synergy of ABT-737 used in combination with Mcl-1 inhibitors to induce cell death in human cancer cells is a very general phenomenon. Our results suggest that it will be important to investigate the efficacy of ABT-737 in combination with ARC or with other transcriptional inhibitors against human solid tumors.

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## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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