Apigenin Sensitizes Colon Cancer Cells to Antitumor Activity of ABT-263

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Abstract

Apigenin is an edible plant-derived flavonoid that shows modest antitumor activities in vitro and in vivo. Apigenin treatment resulted in cell growth arrest and apoptosis in various types of tumors by modulating several signaling pathways. In the present study, we evaluated interactions between apigenin and ABT-263 in colon cancer cells. We observed a synergistic effect between apigenin and ABT-263 on apoptosis of colon cancer cells. ABT-263 alone induced limited cell death while upregulating expression of Mcl-1, a potential mechanism for the acquired resistance to ABT-263. The presence of apigenin antagonized ABT-263-induced Mcl-1 upregulation and dramatically enhanced ABT-263–induced cell death. Meanwhile, apigenin suppressed AKT and ERK activation. Inactivation of either AKT or ERK by lentivirus-transduced shRNA or treatment with specific small-molecule inhibitors of these pathways enhanced ABT-263–induced cell death, mirroring the effect of apigenin. Moreover, the combination response was associated with upregulation of Bim and activation of Bax. Downregulation of Bax eliminated the synergistic effect of apigenin and ABT-263 on cell death. Xenograft studies in SCID mice showed that the combined treatment with apigenin and ABT-263 inhibited tumor growth by up to 70% without obvious adverse effects, while either agent only inhibited around 30%. Our results demonstrate a novel strategy to enhance ABT-263–induced antitumor activity in human colon cancer cells by apigenin via inhibition of the Mcl-1, AKT, and ERK prosurvival regulators. Mol Cancer Ther; 12(12); 2640–50. ©2013 AACR.

Introduction

Colon cancer is one of the leading causes of cancer-related deaths in the United States and worldwide. Since conventional therapeutic approaches are limited in treatment of advanced colon cancer and in prevention of the disease recurrence. Small-molecule inhibitors have been developed under preclinical and clinical studies to treat colon cancer. Apigenin (see chemical structure in Supplementary Fig. S1A) is one of the most common flavonoids widely present in fruits and vegetables. As a plant-derived natural compound, it is a promising and safe antitumor agent (1–5). Studies have shown that apigenin induces cell-cycle arrest and causes apoptosis in different cancer cells including colon cancer through modulation of diverse survival and death effectors, such as PI3K, AKT, ERK, STAT3, JNK, and Mcl-1 (6–10). However, apigenin only showed a modest antitumor activity towards cancer cells (2, 11). New strategies are needed to enhance apigenin's antitumor efficacy.

The PI3K-AKT and Ras-ERK pathways are two of the most important signaling cascades that transduce signals from transmembrane receptors to the nucleus to regulate cell proliferation and survival (12, 13). Gain-of-function mutations in genes of these two signaling pathways, such as Ras, PIK3CA, and Raf are frequently observed in malignant tumors (14–16). Their aberrant activation correlates with poor efficacy of chemotherapies and acquired drug resistance (17–19). Therefore, these genes are promising targets for developing small-molecule inhibitors to treat various cancers. However, inhibition of individual target often results in limited antitumor effects. Instead, a combination approach targeting multiple oncogenic pathways is being investigated to enhance antitumor activities while decreasing their adverse effects. For example, the inhibition of the PI3K-AKT or Ras-ERK pathway upregulates the proapoptotic proteins Bim and Bad and downregulates the prosurvival proteins Bcl-2 and Mcl-1. These effects were significantly enhanced by cotreatment with the BH3 mimetic inhibitor ABT-263 or ABT-737 (20–23).
ABT-263 (Navitoclax, see chemical structure in Supplementary Fig. S1B) is an orally active analog of ABT-737. The BH3 mimetic inhibitors were designed to induce apoptosis by blocking functions of the pro-survival Bcl-2 family proteins such as Bcl-XL, Bcl-2, and Bcl-w but not Mcl-1 or A1 (24). Currently, combination of ABT-263 with other U.S. Food and Drug Administration approved drugs against leukemia or solid tumors is being tested in clinical trials. However, tumor cells expressing high levels of Mcl-1 show resistance to ABT-263 or ABT-737 (25). After long exposure, resistance to ABT-737 is acquired, at least partially resulting from the upregulation of Mcl-1 and/or A1 (26). Therefore, limiting Mcl-1 expression with other agents offers a strategy to conquer acquired resistance and to enhance the antitumor efficacy of ABT-263 or ABT-737 (27, 28).

In the current study, we investigated the role of apigenin, a plant-derived natural compound, in sensitization of colon cancer cells to ABT-263 in vitro and in vivo. Our results indicate that in addition to Mcl-1, apigenin is also linked to inactivation of the AKT and ERK pathways to enhance the antitumor effect of ABT-263.

Materials and Methods

Reagents

FBS was obtained from Atlanta Biological. Culture medium and other related cell culture reagents were from Invitrogen. Apigenin, ABT-263, MK2206, and AZD6244 were purchased from ChemieTek. Cycloheximide was purchased from Sigma-Aldrich. And Z-VAD-FMK was obtained from R&D System. Lentiviral plasmid of pCDH1-HA-Mcl-1 and its control pCDH1-EF1-puro were gifts of Dr. Shengbing Huang.

Cell lines

The human colon cancer cell lines DLD1, HCT116, HCT-8, HT29 and SW48 were obtained from American Type Culture Collection (ATCC). DLD1, HCT-8, and HT29 were cultured in RPMI-1640 medium containing 10% FBS. HCT116 cells were cultured in McCoy’s 5a containing 150 mmol/L NaCl, 50 mmol/L Tris HCl, pH 7.5, and complete mini protease inhibitors (Roche Diagnostics). All the cell lines were expanded at low passages and preserved in liquid N2 after receipt. The cells were used within 10 passages or resuscitated within 1 month. Cell line characterization was conducted by ATCC through short tandem repeat profiling and was not reauthenticated by the authors.

Cell death detection

Cells were seeded into 24-well plates for cell death analysis. After overnight culture, cells were incubated with various compounds for the indicated periods of time. Both floating and adherent cells were harvested and stained with Trypan blue. Cell viabilities were determined with the TC10 Automatic Cell Counter (Bio-Rad). Each experiment was performed in triplicate for at least three times.

Flow cytometry

Cells in 6-well plates were treated with ABT-263, apigenin, or their combination. Floating and adherent cells were harvested and stained with propidium iodide (PI) and Annexin V according to the manufacturer (Invitrogen). The stained cells were analyzed to quantitate Annexin V-positive apoptotic cells by flow cytometry (BD FACSCanto II Analyzer, BD Biosciences).

shRNA

The pGreenPuro shRNA plasmid was obtained from System Biosciences. The shRNA lentivirus vectors were generated by cloning target-specific oligonucleotides into the lentiviral vector according to the manufacturer. The target sequence of shRNA template oligonucleotide used were as follows: sh-Mcl-1: 5'-GGACTTTTATACCTGTAT-3'; sh-ERK1: 5'-GCCATGAGAGATGTCTACA-3'; sh-ERK2: 5'-GAGATTGAAGATGACAG-3'. The details of control shRNA, sh-Bax, sh-AKT1, and sh-AKT2 were described previously (29). The viruses were produced by cotransfection of 293TN cells with a lentiviral vector, packaging and envelope plasmids using lipofectamine 2000 (Invitrogen). The virus containing supernatants were harvested 48 and 72 hours after transfection, used for infection or stored in aliquots at −80°C.

Western blotting

Whole-cell lysates were prepared with cell lysis buffer (Cell Signaling Technologies), separated on SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad). Blots were probed with polyclonal or monoclonal antibodies against Mcl-1, AKT1, AKT, phospho-AKT (S473), phospho-ERK1/2 (T202/Y204), ERK1/2, Bim, XIAP (Cell Signaling Technologies), Bax, Bcl-2, Bcl-XL, Bcl-w, or β-actin (Santa Cruz Biotechnology).

Real-time PCR

Total RNA was extracted from the samples with TRIzol reagent (Invitrogen), and first-strand cDNA was generated using SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer’s instructions. Fluorogenic TaqMan probes were obtained from Applied Biosystems. Mcl-1 mRNA was quantified by real-time PCR (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization.

Detection of Bax conformational change

Cells were lysed in a lysis buffer containing 2% CHAPS, 150 mmol/L NaCl, 50 mmol/L Tris HCl, pH 7.5, and complete mini protease inhibitors (Roche Diagnostics). Approximately 600 µg of proteins were used for immunoprecipitation with the anti-Bax antibody 6A7 (Sigma-Aldrich). The 6A7 antibody recognizes only active form of Bax that has undergone a conformational change. The immunoprecipitates were then subjected to Western
blotting with an anti-Bax polyclonal antibody (Santa Cruz Biotechnology).

**Tumorigenesis assay in SCID mice**

All animal procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. The C.B.-17 SCID mice (female, 7-week-old, Jackson Laboratory) were subcutaneously implanted into the right flank with 5 × 10^5 HCT116 cells in 0.2 mL 50% Matrigel (BD Biosciences). Tumor growth was monitored by periodic visual inspection initially followed by measuring the dimensions of xenografts 3 times a week. Tumor volumes were calculated using the following formula: volume = longest tumor diameter × (shortest tumor diameter)^2/2.

Control and treatment groups were subdivided once the average tumor size reached 60 mm^3 so that the average tumor burden in each group was similar before onset of drug treatment. ABT-263 and apigenin were formulated in 10% ethanol, 30% polyethylene glycol 400, and 60% Phosal 50 PG and administered by oral gavage. The control group received the vehicle only, and the treatment groups received apigenin, ABT-263, or both. ABT-263 was used at 100 mg/kg daily and apigenin at 25 mg/kg daily. For combination treatment, the agents were formulated at a higher concentration so that the total volume of the two agents together was equivalent to the volume of each agent alone. At the endpoint, mice were sacrificed and tumors were harvested for Western blotting analysis.

**Statistical analysis**

Data from studies were expressed as the mean ± SD. The statistical significances of differences between groups were determined using student t test. Difference was considered statistically significant if P < 0.05.

**Results**

**Apigenin enhanced ABT-263–induced apoptosis in colon cancer cells**

Previous studies have shown that apigenin inhibits cell growth and induces apoptosis in cancer cells by modulating various signaling pathways, including downregulation of the Bcl-2 family proteins Mcl-1 (9). We thus investigated whether apigenin sensitized colon cancer cells to ABT-263–triggered cell death. As shown in Fig. 1A (left), HCT116 cells were exposed to varying doses of apigenin in the presence or absence of a fixed concentration of ABT-263 for 1 day. While a minimal cell death rate was detected when the cells were exposed to apigenin or ABT-263 alone (approximately 15% cell death with either 20–30 μm/L of apigenin or 1 μm/L ABT-263), there was a significant increase in death rate when the cells were cotreated with apigenin and ABT-263 (up to 80%). In the presence of 1 μm/L ABT-263, cell death was induced by apigenin in a dose-dependent manner. On the other hand, 20 μm/L apigenin increased cytotoxicity in an ABT-263 dose-dependent manner (Fig. 1A, right). Furthermore, HCT116 cells were exposed to a fixed concentration of ABT-263 (0.5 μmol/L) and apigenin (20 μmol/L) alone or in combination for 3 days. Either compound alone demonstrated limited cytotoxicity. The combination, however, led to robust cell death (Fig. 1B). The combination index (CI) was analyzed on the basis of a fixed ratio of apigenin and ABT-263 using the CalcuSyn program (30). A value less than 1.0 indicated a synergistic interaction between apigenin and ABT-263 (Fig. 1C).

To determine whether the synergistic interaction between ABT-263 and apigenin also applies to other colon cancer cell lines, we tested HCT116, DLD1, SW48, HT29, and HCT-8 cells. As shown in Fig. 1D, apigenin significantly enhanced ABT-263–induced cell death in all these colon cancer cell lines. Supplementary Fig. S2A–S2C showed details of the time- and dose-dependent synergistic interaction between ABT-263 and apigenin in DLD1 cells. Taken together, our results suggest that apigenin sensitizes colon cancer cells to ABT-263–induced antitumor efficacy.

To investigate whether the cell death caused by cotreatment with apigenin and ABT-263 was apoptotic, HCT116 and DLD1 cells were treated with apigenin and ABT-263 alone or in combination for 24 hours before harvesting and staining with PI and Annexin V for flow cytometry analysis. As shown in Fig. 1E, cotreatment with ABT-263 and apigenin significantly increased percentages of Annexin V-positive cells compared with single drug treatment. A significant percentages of Annexin V-positive cells were also PI-positive, suggesting that they could be late-stage apoptotic cells or alternatively, necrotic cells. To distinguish the possibilities, we examined cleavage of caspase-3, a hallmark of cellular apoptosis. As shown in Fig. 1F, cotreatment with ABT-263 and apigenin markedly increased cleavage of caspase-3, consistent with promotion of apoptosis instead of necrotic cell death by the combination. In further support of this, a caspase inhibitor, Z-VAD-fmk which prevented cleavage of caspase-3, protected HCT116 cells from cell death (Supplementary Fig. S2D), confirming that cotreatment with ABT-263 and apigenin caused apoptotic cell death via a caspase-dependent mechanism.

**Downregulation of Mcl-1 by apigenin sensitized colon cancer cells to ABT-263–triggered cell death**

As previously reported, expression or induction of Mcl-1 is a major obstacle to proapoptotic effect of ABT-263 (26, 27). Mcl-1 was detectable in multiple colon cancer cell lines including HCT116, DLD1, SW48, HT29, and HCT-8 (Supplementary Fig. S3). In HCT116 and DLD1 cells, apigenin downregulated Mcl-1 expression in a dose- and time-dependent manner (Fig. 2A). In contrast to apigenin, ABT-263 treatment upregulated Mcl-1 expression in HCT116 and DLD1 cells. As shown in Supplementary Fig. S4, ABT-263 induced a time- and dose-dependent upregulation of Mcl-1 in HCT116 cells. However, when these cells were cotreated with apigenin and ABT-263, apigenin significantly attenuated stimulatory effect of
ABT-263 on Mcl-1 (Fig. 2B), suggesting that apigenin downregulation of Mcl-1 is implicated in the potentiation of the antitumor activity of ABT-263. To confirm the importance of Mcl-1 modulation in apigenin and ABT-263–induced cell death, we knocked down Mcl-1 expression by shRNA in HCT116 cells to mimic the impact of apigenin. As expected, knockdown of Mcl-1 enhanced ABT-263–induced cell death by an average of 1.8-fold (Fig. 2C), indicating that downregulation of Mcl-1 by apigenin plays a role in sensitization of the cells to ABT-263–triggered cytotoxicity. However, Mcl-1 knockdown also moderately increased the combined effect of ABT-263 and apigenin on cell death by 0.6-fold. The remaining potentiation activity is likely due to Mcl-1–independent input.
cells from apigenin and ABT-263–induced apoptosis, as reflected by reduction in cleavage of caspase-3 and PARP.

To investigate the mechanism for apigenin downregulation of Mcl-1, we treated HCT116 cells with cycloheximide in the presence or absence of apigenin for up to 6 hours to monitor Mcl-1 protein turnover. Western blotting analysis showed that cellular Mcl-1 protein displayed similar pattern of turnover in the presence and absence of apigenin (Supplementary Fig. S6A). Consistent with this, the exogenous HA-Mcl-1 was not affected by exposure to apigenin (Supplementary Fig. S5). These results suggest that apigenin does not alter Mcl-1 protein stability. We next examined Mcl-1 mRNA in apigenin-treated cells by real-time PCR. Exposure to apigenin induced decreases in Mcl-1 mRNA levels in HCT116 cells (Supplementary Fig. S6B), suggesting that apigenin downregulated Mcl-1 expression at mRNA level.

Inhibition of AKT by apigenin is another modulator of synergistic interaction with ABT-263

As reported previously, the PI3K-AKT pathway was inhibited by apigenin in leukemia cells (9). To figure out whether the AKT pathway was affected by apigenin and played a role in ABT-263 and apigenin-induced cell death in colon cancer cells, HCT116 and DLD1 cells were treated with increasing doses of apigenin for 24 hours. As shown in Fig. 3A, activation of AKT was dose dependently inhibited by apigenin. HCT116 and DLD1 cells were then exposed to ABT-263 and apigenin alone or in combination for 24 hours. While ABT-263 had no effect on AKT activation, apigenin inhibited AKT in the absence and presence of ABT-263 (Fig. 3B). To determine the significance of AKT inhibition in apigenin-ABT-263 synergistic interaction, we knocked down expression of AKT1, AKT2 or both with AKT isoform-specific shRNA in HCT116 cells. Knockdown of each AKT isoform enhanced ABT-263–induced cell death in HCT116 cells. Downregulation of both AKT isoforms further enhance ABT-263–induced cell death (Fig. 3C), suggesting that both AKT1 and AKT2 are involved in counteracting ABT-263–induced cell death. Furthermore, when HCT116 and DLD1 cells were treated with ABT-263 in the presence of MK2206, a selective AKT inhibitor (31), MK2206 enhanced ABT-263-induced cell death dramatically in both cell lines (Fig. 3D). Enhanced cleavage of caspase-3 was also observed in the presence of MK2206 (Fig. 3E). Together, these results suggest that inactivation of AKT contributes to ABT-263 and apigenin-induced antitumor responses in colon cancer cells.

Apigenin inhibition of ERK contributes to synergistic interaction with ABT-263

In an effort to explore other potential mechanisms contributing to the synergistic role of apigenin in ABT-263–induced cell killing, we examined whether apigenin targets ERK, a prototype prosurvival regulator. As shown in Fig. 4A, apigenin itself decreased ERK phosphorylation levels in HCT116 and DLD1 cells. This effect of apigenin remained significant when HCT116 cells were cotreated

from other additional apigenin-activated prosurvival regulators.

To determine whether overexpression of Mcl-1 could prevent the antitumor effect of apigenin, HA-tagged Mcl-1 was transduced into HCT116 cells. As shown in Supplementary Fig. S5, overexpression of Mcl-1 protected
with ABT-263. In DLD1 cells, ABT-263 also reduced ERK phosphorylation. Combination of apigenin and ABT-263 led to further inhibition of ERK (Fig. 4B). Consistent with inhibition of ERK contributing to the enhancement of ABT-263-induced cytotoxicity by apigenin, silencing of either ERK1 or ERK2 by shRNA also sensitized HCT116 and DLD1 cells to ABT-263-induced cell death. Down-regulation of both ERK1 and ERK2 further enhanced ABT-263 synergy compared to ERK2 knockdown cells (Fig. 4C). Similarly, AZD6244, a MEK inhibitor (32), blocked ERK1/2 activation and augmented the proapoptotic activity of ABT-263 (Fig. 4D and E). These results support a role of ERK suppression in ABT-263 and apigenin-induced cell death.

**Cotreatment with apigenin and ABT-263 upregulated Bim and activated Bax**

The Bcl-2 family members are important mediators of cell death and survival. It has been shown that the AKT and ERK pathways are linked to regulation of certain Bcl-2
family members including Bim (33, 34). Upon treatment with apigenin, Bim-L expression was robustly upregulated in HCT116 cells (Fig. 5A, left; Supplementary Fig. S7A, left). Cotreatment with ABT-263 and apigenin further enhanced Bim-L expression (Fig. 5A, right; Supplementary Fig. S7A, right). When the cells were treated with a small-molecule inhibitor of AKT or MEK, although expression of both Bim-EL and Bim-L was upregulated significantly (Fig. 5B; Supplementary Fig. 7B), Bim-L expression was increased more than Bim-EL (2.5 vs 1.7-fold, respectively, upregulation upon treatment with MK2206; 3.2 vs. 2.2-fold upregulation upon treatment with AZD6244). Thus Bim-L is a common effector between apigenin and AKT or MEK inhibition. Since these cells express high levels of endogenous Bim-EL, the moderate increase in Bim-EL by AKT or MEK inhibitor may not be as important as Bim-L induction. This is also consistent with a previous observation that Bim-L is more potent than EL in inducing apoptosis (35).

To determine whether there are any other potential mitochondrial Bcl-2 family members were involved in apigenin enhancement of ABT-263-induced cell death in colon cancer cells. A, HCT116 and DLD1 cells were treated for 1 day with the indicated concentrations of apigenin and analyzed by Western blotting for phospho-ERK1/2 (T202/Y204) and total ERK1/2. B, HCT116 and DLD1 cells were treated for 1 day with ABT-263 (0.5 μmol/L) apigenin (20 μmol/L), or their combination before Western blotting analysis phospho-ERK1/2 (T202/Y204) and ERK1/2 as in A. C, ERK1 and ERK2 in HCT116 and DLD1 cells were knocked down individually or simultaneously by shRNA and the knockdown efficiencies were confirmed by Western blotting (left). The control cells and ERK knockdown cells were treated for 1 day with ABT-263 and analyzed for cell death rates (right). D, HCT116 and DLD1 cells were treated with ABT-263, AZD6244 (AZD), or their combination (comb) at the indicated concentrations for 1 day before determination of cell death as in C. E, the cells were treated as described in D and subjected to Western blotting analysis of phospho-ERK1/2 and cleaved caspase-3. *P < 0.05; **P < 0.01.
colon cancer cells, HCT116 and DLD1 cells were treated with increasing doses of apigenin for 24 hours. Western blotting analysis did not detect any significant changes in the Bcl-2 family members including Bcl-2, Bcl-XL, Bcl-w, and Bax (Supplementary Fig. S8).

However, Bim has been shown to directly activate Bax, leading to translocation of Bax from cytosol to the mitochondria where it triggers mitochondrial outer membrane permeabilization (MOMP) to induce apoptosis. Associated with this, Bax undergoes transformational change with exposure of its N-terminus, which could be recognized by the 6A7 antibody (36). As shown in Fig. 5C, cotreatment with apigenin and ABT-263 led to marked increase in Bax activation as detected by immunoprecipitation with the 6A7 antibody despite the constant levels of total Bax. This effect on Bax activation could be detected as early as 4 hours of exposure to apigenin and ABT-263. In addition, apoptosis induced by apigenin, ABT-263 or their combination was dependent on Bax, as Bax depletion with shRNA abrogated the death responses to these agents (Fig. 5D and E).

Apigenin enhanced antitumor effect of ABT-263 in vivo

The antitumor efficacies of ABT-263 and apigenin were further analyzed in SCID mice. The SCID mice bearing HCT116 xenografts were treated daily with ABT-263, apigenin or both of them for 21 days. The growth of the xenografts and body weights were monitored 3 times per week. As shown in Fig. 6A, either apigenin or ABT-263 alone resulted in approximately 30% inhibition of tumor growth as compared to the vehicle control group. Cotreatment with ABT-263 and apigenin caused more than 70% inhibition, significantly higher than single drug treatment groups. The mice from all groups maintained comparable body weights during the course of the drug treatment, indicating minimal detrimental effects of single or combinatorial treatment (Fig. 6B). When the tumor tissues were examined by immunoblotting, decreases in Mcl-1 and phosphorylation of AKT and ERK were observed in the groups treated with apigenin or apigenin and ABT-263 (Fig. 6C), indicating that apigenin also targets these pro-survival modulators in vivo.

Discussion

In this study, we showed the synergistic interaction in colon cancer cells between ABT-263, a BH3 mimetic inhibitor of the Bcl-2 family members currently under clinical trials, and apigenin, an edible plant-derived flavonoid. Although apigenin has been tested as an antitumor agent against various human tumors, its antitumor activity is generally limited. Its potential application in combination with other anticancer drugs has not been thoroughly explored. Our results herein provided such an example that apigenin enhanced ABT-263–induced cell killing in colon cancer cells. Detailed studies demonstrated that apigenin downregulated Mcl-1, and inhibited...
AKT and ERK prosurvival mediators. Targeting each of these pathways independently aided in ABT-263-induced cell killing. Furthermore, the synergistic interaction between apigenin and ABT-263 in colon cancer cells was supported by the xenograft experiment in SCID mice wherein coadministration of apigenin and ABT-263 suppressed tumor growth more potently than apigenin or ABT-263 alone.

Previous studies of ABT-263 showed that cells expressing high levels of Mcl-1 are more resistant to ABT-263. In addition, prolonged exposure to ABT-263 resulted in upregulation of Mcl-1, one of the major mechanisms for acquired resistance of cancer cells to ABT-263 (37, 38). Our initial idea that apigenin might synergize with ABT-263 to promote cell death stemmed from the observation that apigenin exhibited an opposite effect on Mcl-1 expression in colon cancer cells (Fig. 2A). We assume that if Mcl-1 upregulation is indeed the major mechanism for resistance to ABT-263, inclusion of apigenin might be able to overcome this resistance to optimize the antitumor activity of ABT-263. Our data showed that knockdown of Mcl-1 via shRNA enhanced ABT-263-induced cell killing, while overexpression of exogenous Mcl-1 protected cells from combination-induced cell death (Supplementary Fig. S5), suggesting an important role of Mcl-1 modulation in apigenin promotion of ABT-263-induced cell death.

Mcl-1 mRNA and protein have been reported to have short half-lives (39). In addition to the predominant transcriptional regulation, Mcl-1 expression can be regulated at translational levels by a wide range of survival or apoptotic signals. The stability and function of Mcl-1 protein may be affected by phosphorylation on specific sites. As reported previously, ERK activation induces Mcl-1 phosphorylation at Threonine 163 which stabilizes Mcl-1 protein and lowers its turnover (40). Activation of glycogen synthase kinase-3 (GSK-3) by inhibition of AKT causes phosphorylation of Mcl-1 at Serine 159, leading to its degradation (41). Although these previous studies suggest that AKT and ERK activities regulate Mcl-1 expression or turnover (42–44), our data showed that apigenin regulation of Mcl-1 occurs at mRNA levels (Supplementary Fig S6). Furthermore, Mcl-1 protein expression was not affected in our hands by administration of specific AKT or ERK inhibitors, indicating that apigenin-mediated downregulation of Mcl-1 is independent of inactivation of AKT or ERK in colon cancer cells.

It should be pointed out that apigenin did not completely block the stimulatory effect of ABT-263 on Mcl-1. Downregulation of Mcl-1 by shRNA only showed partial enhancement of ABT-263-induced cell death in colon cancer cells. The observation is consistent with involvement of AKT and ERK in the observed synergistic interaction in addition to Mcl-1 downregulation. When we knocked down or pharmaceutically inhibited AKT or ERK to mimic the actions of apigenin, we found that inhibition of AKT or ERK also synergized with ABT-263 to promote cell death. These results indicate that multiple prosurvival cascades are targeted by apigenin to confer the sensitivity to ABT-263.

A limitation of our study is that we have not succeeded in identifying a central mechanism that accounts for the entire synergistic activity of apigenin and ABT-263. Although inhibition of Mcl-1, AKT or ERK each could impair the effect of apigenin, it remains unclear whether these pathways contribute to the action of apigenin independently or act through any common death and survival modulators that have not been recognized in the present work. Nevertheless, our results open a new avenue to explore the direct molecular mediator(s) of apigenin and ABT-263 interactions.

It is worth noting that, similar to other studies (7–9), the concentrations of apigenin we used in our study were lower than those used in other studies. Nevertheless, the concentrations of apigenin we used in our study were...
high micromolar concentrations. This less ideal efficacy may be related to its limited permeability across cell membranes and its stability in culture and in vivo. Before the molecular targets of apigenin are fully resolved, apigenin may be synthetically developed into more stable and more membrane permeable compounds to enhance its antitumor efficacy. In summary, our data showed that apigenin enhanced ABT-263-induced antitumor activities in colon cancer cells by modulating Mcl-1 expression along with other prosurvival effectors in colon cancer cells. Further exploration of this synergistic interaction and the underlying mechanisms could lead to the development of a new therapeutic approach to treating colon cancer and other malignant diseases.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: H. Shao, E. Mahmoud, H. Huang, X. Fang, C. Yu
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Shao, K. Jing, E. Mahmoud, C. Yu
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