

Novel Evidence for Curcumin and Boswellic Acid-Induced Chemoprevention through Regulation of miR-34a and miR-27a in Colorectal Cancer

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Abstract

Colorectal cancer is one of the most common causes of cancer-associated mortality worldwide, but it is truly a preventable disease. Both curcumin and boswellic acids are well-established dietary botanicals with potent antitumorigenic properties that have been shown to modulate multiple oncogenic pathways. Recent data suggest that the chemopreventive effects of these botanicals may, in part, be mediated through regulation of key cancer-related microRNAs (miRNA) and their downstream gene targets. Here, we investigated the antitumorigenic effects of curcumin and 3 acetyl-11-keto- β -boswellic acid (AKBA) on modulation of specific cancer-related miRNAs in colorectal cancer cells and validated their protective effects *in vivo* using a xenograft mouse model. Both curcumin and AKBA inhibited cellular proliferation, induced apoptosis and cell-cycle arrest in colorectal cancer cell lines, and these effects were significantly enhanced with combined treatment. Gene-express-

ion arrays revealed that curcumin and AKBA regulated distinct cancer signaling pathways, including key cell-cycle regulatory genes. Combined bioinformatics and *in silico* analysis identified apoptosis, proliferation, and cell-cycle regulatory signaling pathways as key modulators of curcumin and AKBA-induced anticancer effects. We discovered that curcumin and AKBA induced upregulation of tumor-suppressive miR-34a and downregulation of miR-27a in colorectal cancer cells. Furthermore, we demonstrated in a mouse xenograft model that both curcumin and AKBA treatments suppressed tumor growth, which corresponded with alterations in the expression of miR-34a and miR-27a, consistent with our *in vitro* findings. Herein, we provide novel mechanistic evidence for the chemopreventive effects of curcumin and AKBA through regulation of specific miRNAs in colorectal cancer. *Cancer Prev Res*; 8(5); 431–43. ©2015 AACR.

Introduction

Colorectal cancer is one of the leading causes of cancer-related mortality in the United States (1). There is growing evidence suggesting that dietary and lifestyle modifications can influence risk prevention for colorectal cancer (2). Increasing focus on chemopreventive properties of natural compounds from traditional medicine has led to discovery of potential therapeutic agents targeting colorectal cancer and other cancers. Curcumin,

the principle curcuminoid and a derivative of the spice turmeric *curcuma longa*, is used as a naturally occurring medicine to treat variety of inflammatory disorders, and human cancers (3). In terms of its well-established antitumorigenic properties, curcumin has been shown to interfere with various stages of colorectal tumorigenesis, including tumor initiation, promotion, and progression (4–7). Although curcumin is known to regulate multiple signaling pathways involved in carcinogenesis, the mechanism by which curcumin simultaneously interferes with these pathways remains unclear. One probable explanation is that some botanicals, including curcumin, have the capacity to regulate microRNAs (miRNA) associated with cancer (8). miRNAs are small noncoding RNAs that regulate gene expression via interaction with the 3'-untranslated regions of target mRNAs, thereby regulating up to several hundred genes posttranscriptionally (9). Although miRNAs are known for their involvement in many physiologic processes, dysregulation of miRNA expression in cancer is well established (10). Curcumin has been shown to downregulate putative oncogenic miRNAs, such as miR-21 (11, 12), while it upregulates key tumor-suppressor miRNAs, including miR-200 family, let-7 family, miR-185b, and miR-22 (13–15). Therefore, regulation of miRNAs appears to be a crucial antitumorigenic mechanism of curcumin and warrants further systematic and comprehensive investigation.

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Note: Supplementary data for this article are available at Cancer Prevention Research Online (<http://cancerprevres.aacrjournals.org/>).

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doi: 10.1158/1940-6207.CAPR-14-0354

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Boswellic acid, an organic acid derived from the plant *boswellia serrata*, is another botanical used traditionally for the treatment of various inflammatory diseases, including colitis and arthritis. Although *boswellia serrata* is composed of various derivatives, including acetyl- β -boswellic acid, 11-keto- β -boswellic acid, and 3 acetyl-11-keto- β -boswellic acid (AKBA), AKBA is identified as the most potent anti-inflammatory constituent of boswellic acid (16, 17). Similar to curcumin, AKBA exerts its antitumorogenic effects through regulation of multiple cancer signaling pathways (16, 18–22). Interestingly, we recently demonstrated that AKBA upregulates key putative tumor-suppressive miRNAs in colorectal cancer and the expression of these miRNAs inversely corresponded with tumor size and volume in a xenograft animal model (23).

Despite lack of preclinical studies on combined treatment with curcumin and AKBA together, curcumin has been used in combination approaches with other dietary components. Treatment with curcumin and green tea catechins attenuated aberrant crypt formation in a carcinogen-induced colorectal cancer mouse model (24), while a combination of curcumin and resveratrol synergistically suppressed tumor proliferation in a mouse xenograft model (25). Although further investigations are required to fully understand the antitumorogenic properties of these compounds individually and in combination, these studies highlight the enormous therapeutic potential of using these safe and cost-effective botanicals together to help prevent and possibly treat colorectal cancer. Here, we identified key molecular mechanisms by which curcumin and AKBA both individually and in combination affect specific miRNAs and their downstream target genes involved in the cell-cycle regulation of colorectal cancer cell lines. Furthermore, we confirmed these antitumorogenic properties of curcumin and AKBA, both alone and together, in a mouse xenograft model.

Materials and Methods

Materials and cell lines

Human colorectal cancer cell lines, HCT116, RKO, SW480, SW620, HT29, and Caco2 were purchased from the American Type Culture Collection. All cell lines were routinely authenticated by analyzing a panel of specific genetic and epigenetic biomarkers. The HCT116p53^{-/-} cell line was a generous gift from Bert Vogelstein (Johns Hopkins Medical Institute, Baltimore, MD). All cells were grown in Iscove Modified Dulbecco's medium (IMDM; Invitrogen) with 10% fetal bovine serum and 1% penicillin and streptomycin and maintained at 37°C in a humidified incubator (5% CO₂). Both curcumin (BCM-95) and AKBA (Bospure) were provided by Dolcas Biotech. These botanicals were dissolved in DMSO and diluted to appropriate experimental concentrations with tissue culture medium. In addition, aspirin (Acetylsalicylic acid) was purchased from Sigma-Aldrich and dissolved in DMSO and diluted to appropriate experimental concentration with tissue culture medium.

Cellular cytotoxicity, cell cycle, apoptosis, and clonogenic assays

Cellular cytotoxicity was determined by the 3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; MTT) assay as described previously (23). In brief, approximately 4,000 cells were seeded in each well and treated with various concentrations of curcumin and/or AKBA for 72 hours. Optical density was determined using Tecan Infinite 200 Pro multireader and i-control 1.10 software (Tecan Group Ltd.). The Chou–Talalay

equation (26) was used to calculate the combination index (CI) to determine the interaction between curcumin and AKBA treatments. Cell-cycle analysis was conducted using a Cell-cycle assay kit (MCH100106; Millipore) and apoptotic cell fraction was measured using Annexin V and Dead Cell Assay Kit (Millipore) according to the manufacturer's instructions using the Muse Cell Analyzer (Millipore). For clonogenic assays, approximately 500 cells were seeded in each well of a 6-well plate, and were treated with various concentrations of curcumin and/or AKBA. After 8 days, colonies were stained with crystal violet (Sigma-Aldrich) and dried overnight at room temperature. The number of colonies with >50 cells were counted using GeneTools (Syngene). All experiments were conducted in replicates and at least three independent experiments.

Gene-expression microarray analysis

Microarray gene-expression analysis was performed in HCT116 and SW480 cell lines treated with curcumin and/or AKBA- and DMSO-treated controls according to the manufacturer's instructions and a method described previously (27). Detailed methodologies are supplied in Supplementary Materials and Methods.

Quantitative real-time PCR analysis

Total RNA was extracted from curcumin and AKBA-treated colorectal cancer cell lines (10 μ mol/L curcumin, 30 μ mol/L AKBA, or the combination of both botanicals for 24 hours) and from the xenograft tumor tissue samples using the miRNeasy Mini Kit (Qiagen) following the manufacturer's instructions. For analysis of the mRNA expression, 1 μ g of total RNA was reverse transcribed to complementary DNA using the Advantage RT PCR kit (Clontech Laboratories Inc.). Power SYBR Green (Applied Biosystems) real-time PCR was performed using StepOnePlus system (Applied Biosystems). For specific primer sequences, refer to Supplementary Figures (Supplementary Table S1). All qRT-PCR target genes expression was normalized to the expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and analyzed using the $\Delta\Delta C_t$ method. The expression of miRNAs was analyzed using TaqMan real-time PCR assay kit (Applied Biosystems). All miRNA primers used in this study were purchased from Ambion. Ten nanograms of RNA from each sample was reverse transcribed using TaqMan microRNA Reverse Transcription kit (Applied Biosystems) and 6 ng of complementary DNA was used for real-time qRT-PCR. All data were analyzed using the $\Delta\Delta C_t$ method and normalized to RNU6B.

Transfection of miRNA

A total of 1×10^5 cells were seeded and transfected with pre-miR-34a, anti-miR-27a, pre-miR-negative-control 1, or anti-miR-negative-control 1 (Ambion) at a final concentration of 50 nmol/L using siPort NeoFX (Applied Biosystems) and Opti-MEM (Gibco) according to the manufacturer's instructions. The relative levels of miRNAs were measured to confirm the efficiency of miRNA transfection and functional analyses were conducted as described above.

Western blotting

Western immunoblotting experiments were performed as described previously (28). In brief, cells were treated with curcumin and/or AKBA for 24 hours, thereafter lysed using 100 μ L of 1 \times SDS sample buffer containing β -mercaptoethanol. All antibodies are listed in Supplementary Table S2. 1. All samples were compared against β -actin as a reference (Sigma-Aldrich). The bands

were visualized using GeneTools (Syngene) and images were captured using Syngene GBox.

DAPI staining of apoptotic cells

Apoptotic cells were evaluated using 4',6-diamidino-2-phenylindole (DAPI) staining as described previously (29). In brief, cells were treated with different concentrations of curcumin and/or AKBA for 24 hours. The cells were then fixed with methanol for 30 minutes at 4°C and incubated in DAPI solution for 1 hour in the dark. The number of apoptotic cells was evaluated under fluorescence microscope (Leica).

3D cultures

Three-dimensional (3D) cultures were generated as described previously (30). In brief, approximately 1×10^6 cells were seeded on a cellulose filter on top of a steel mesh bridge. Cells were then treated with curcumin (10 $\mu\text{mol/L}$), AKBA (30 $\mu\text{mol/L}$), or the combination of both for 1, 3, 7, or 10 days. The growth of 3D cultures was assessed by determining the diameter of the 3D cultures using light microscopy.

Mouse xenograft model

The 5-week-old male athymic nude mice were purchased from Harlan Laboratories and kept under controlled conditions of light (12-hour cycles), fed *ad libitum* diet, and had free access to water. Xenograft tumors were generated by subcutaneous injection of 5×10^6 HCT116 cells. Tumor growth was determined by measuring the length (*L*) and width (*W*) of the tumor every other day using calipers and the tumor volume was calculated according to the following formula: $1/2LW^2$. Once average tumor size reached 50 mm^3 , animals were randomly divided into four groups with 10 animals in each group: (i) control vehicle (DMSO), (ii) curcumin (25 mg/kg), (iii) AKBA (75 mg/kg), or (iv) curcumin and AKBA. Treatments or vehicle were injected intraperitoneally daily for 3 weeks. The tumor samples were fixed in RNAlater (Sigma-Aldrich) and then stored in a -80°C freezer for subsequent analyses. For the xenograft tumor growth experiment, the interaction between curcumin and AKBA was evaluated as described previously (31). The fraction of tumor volume (FTV) affected by curcumin and/or AKBA was calculated individually and together as a ratio to control. The ratio of expected FTV (Exp FTV) and observed FTV (Obs FTV) was calculated for the combined treatment (A ratio >1 indicates synergistic effect, <1 indicates an additive effect). The animal protocol was approved by Institutional Animal Care and Use Committee at the Baylor Research Institute (Dallas, TX).

Statistical analysis

All analyses were performed using GraphPad Prism Ver. 6.0 (GraphPad Software Inc.). All data were expressed as mean \pm SEM with statistical significance indicated when $P < 0.05$. Statistical comparisons between control and treatment groups were determined using an unpaired *t* test or one-way ANOVA with the Tukey post-hoc tests.

Results

Combination of curcumin and AKBA enhances cytotoxicity and inhibits proliferation of colorectal cancer

The average IC_{50} of four cell lines (HCT116, RKO, SW480, and SW620; Supplementary Fig. S1A) was used to determine the

potency of both botanicals and the ratio between these botanicals was calculated (Curcumin:AKBA, 1:2.76; Supplementary Fig. S1B). On the basis of these data, we selected the concentration of AKBA as three times the dose of curcumin for this study. We then examined the cytotoxicity of curcumin and/or AKBA on two primary colorectal cancer cell lines (HCT116 and SW480). Both curcumin and AKBA treatment increased the cytotoxicity in a dose-dependent manner (Fig. 1A). The combined treatment further enhanced cytotoxicity in both cell lines. Next, we determined whether this enhanced cytotoxicity by the combined treatment was synergistic using the Chou-Talalay CI (26). Interestingly, while the cytotoxicity of the both treatments together was additive in HCT116 cell line (CI = 1.09), combined treatment of curcumin and AKBA in the SW480 cell line resulted in synergism (CI = 0.768; Fig. 1A, insets). Considering tumor-suppressor p53 is mutated in the SW480 cell line, but not in the HCT116 cell line, we next investigated whether p53 dysregulation contributed to the discrepancy of AKBA and curcumin-induced cytotoxicity between these cell lines using p53^{-/-} HCT116 cells (HCT116p53^{-/-}). Although treatment of curcumin or AKBA resulted in a dose-dependent increase in cytotoxicity in HCT116p53^{-/-} cell line, the combination of curcumin and AKBA showed synergistic enhancement in cytotoxicity (CI < 1 ; Fig. 1A, inset), suggesting that AKBA complements cytotoxicity of curcumin in cell lines lacking active p53. In addition, to ensure that the combined botanicals exert synergistic enhancement in cytotoxicity in p53-mutated cell lines, we determined synergism between curcumin and AKBA in RKO (p53 WT), Caco3, and HT29 (both p53-mutant cell lines; Supplementary Fig. S2A). Although RKO cells showed no synergistic cytotoxicity between curcumin and AKBA (CI = 1.21), the combined botanicals exerted synergistic cytotoxicity in both HT29 and Caco2 cell lines (CI = 0.989 and 0.971, respectively). Next, we evaluated the effects of these botanicals on cell survival and proliferation using the clonogenic assays (Fig. 1B and Supplementary Fig. S2B). To our surprise, even at a low dose of 2.5 $\mu\text{mol/L}$, curcumin significantly suppressed colony formation, while over a 10-fold higher concentration of AKBA was required to exert a similar effect (Fig. 1B), indicating that curcumin has superior inhibition of cell survival and proliferation compared with AKBA.

Combination of curcumin and AKBA regulates cell death, proliferation, and cell cycle

To identify cancer signaling pathways modulated by curcumin and AKBA in colorectal cancer, we used microarray analysis to examine gene-expression alterations caused by curcumin and AKBA individually, and in combination in the two colorectal cancer cell lines. Hierarchical clustering analysis showed that a markedly different cluster of genes was regulated by curcumin and AKBA in HCT116 and SW480 cell lines (Fig. 2A). Furthermore, gene-expression profiles of the combined treatments revealed that a large cluster of genes unaffected by curcumin treatment alone was regulated by the combined treatment with curcumin and AKBA (Fig. 2B), suggesting that these botanicals in combination modulate additional cluster of genes compared with individual treatments. We then assessed all genes regulated by the dual treatments in both colorectal cancer cell lines and identified 355 genes with overlapping expression alterations (Fig. 2C). Next, we used the Ingenuity Pathway Analysis (IPA) to determine potential molecular signaling pathways regulated by the combined curcumin and AKBA treatment (Fig. 2D). Top cancer-related molecular functions common to both cell lines were cell death

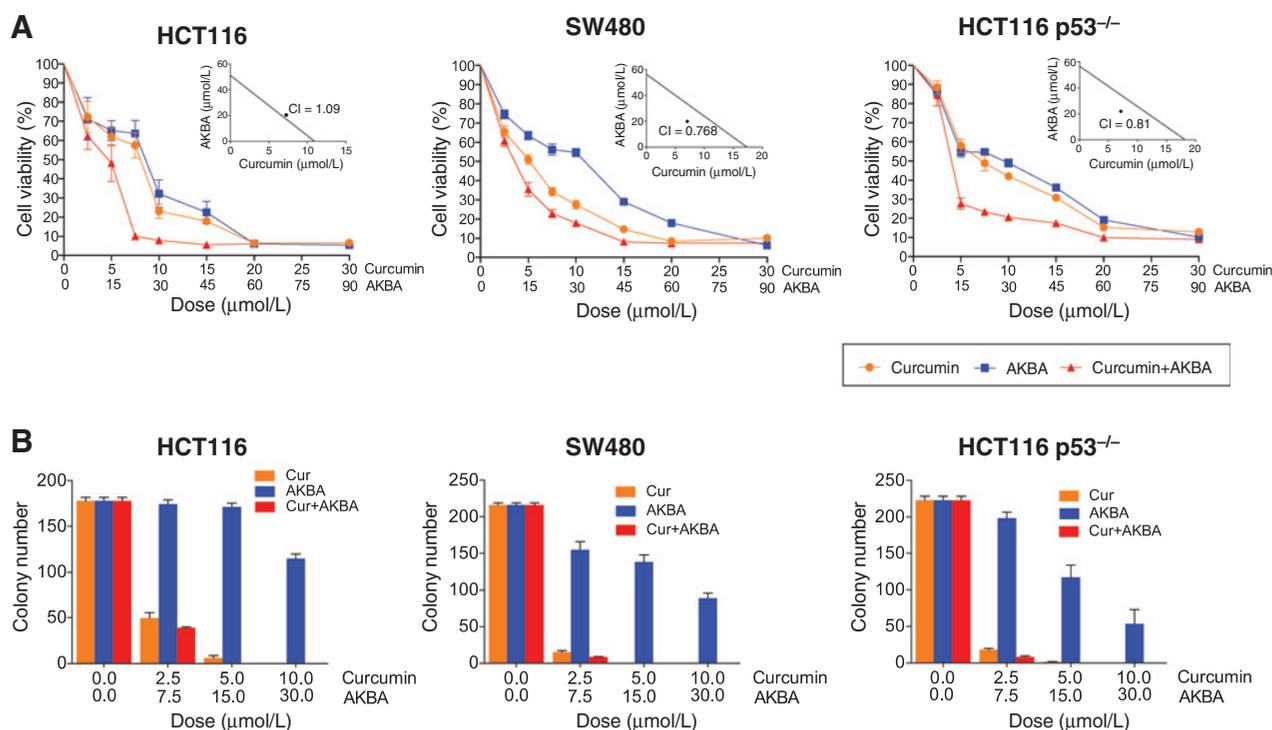


Figure 1.

Curcumin and AKBA exert cytotoxicity and suppress colony formation on colorectal cancer cell lines. A, cytotoxicity of different doses of curcumin and/or AKBA on HCT116, HCT116p53^{-/-}, and SW480 cell lines. Inset, synergy between curcumin and AKBA was calculated by use of the CI. B, colony formation assay of colorectal cancer cell lines treated with various concentrations of curcumin and/or AKBA.

and survival, cellular growth and proliferation, and cell cycle (Fig. 2D). Because cell cycle is related to both cell death and proliferation, we next focused on systematic evaluation of genes involved in cell-cycle regulation among the subset of 355 differentially expressed genes. Subsequently, we identified *MYC*, *CCND1*, *CCNE1*, and *CDK6* as potential target genes of the combined curcumin and AKBA treatment (Fig. 2C).

Curcumin and AKBA induce cell-cycle arrest, apoptosis, and inhibit proliferation in 3D cultures

We next investigated the mechanism of cell-cycle regulation by curcumin and AKBA alone and in combination in colorectal cancer cells. Curcumin treatment induced G₂-M arrest in HCT116 and HCT116p53^{-/-} cell lines, but not in the SW480 cell line, while AKBA induced G₁ arrest in HCT116 and SW480 cell lines. When cells were treated with curcumin and AKBA together, G₁ arrest was induced in HCT116 and SW480 cell lines, while G₂-M arrest was predominantly observed in the HCT116p53^{-/-} cells (Fig. 3A). These data indicate that combinatorial treatment with curcumin and AKBA exerts antitumorigenic effects through cell-cycle regulation. Next, we determined whether curcumin and AKBA enhanced apoptosis in colorectal cancer cell lines. Both curcumin and AKBA treatment increased cellular apoptosis in all three cell lines, while the two botanicals together further elevated apoptotic cell population regardless of the cell line analyzed (Fig. 3B). We then used the DAPI nuclear staining to confirm that both curcumin and AKBA enhanced cellular apoptosis (Fig. 3C). In somatic cells, the Bax:Bcl ratio serves as a regulator for cell susceptibility to apoptosis (32); hence, we first assessed whether

curcumin and AKBA regulate cell survival proteins, Bcl-2 and Bcl-x_L. Although Bcl-2 and Bcl-x_L were downregulated by both curcumin and AKBA alone, the effect of the dual treatment augmented Bcl-2 and Bcl-x_L inhibition in all three colorectal cancer cell lines (Fig. 3D). We then showed that both curcumin and AKBA upregulated proapoptotic protein Bax, while the two treatments together elevated the expression of Bax (Fig. 3D). In addition, poly (ADP-ribose) polymerase 1 (PARP1), a DNA single-strand breaks repair protein and a suppressor of the caspase pathway, was downregulated by curcumin and AKBA, both individually and in combination. Collectively, in line with microarray data, we confirmed that the combination of curcumin and AKBA results in increased cell-cycle arrest and apoptosis. In addition, to confirm the antitumorigenic properties of these botanicals, we used 3D cultures to determine whether curcumin and/or AKBA could interfere with colorectal cancer tumor growth (Supplementary Fig. S3). Although both curcumin and AKBA inhibited cellular growth individually, combined treatment with curcumin and AKBA further inhibited the growth in HCT116 cells. Together, we demonstrated that curcumin and AKBA exert antitumorigenic effects through cell-cycle arrest and apoptosis, while suppressing cellular growth in both 2D and 3D *in vitro* models.

Curcumin and AKBA control cell cycle-regulating miRNAs and their downstream target genes

Using the genes identified by the microarray analysis, we used TargetScan (Ver 6.2) and miRTargetBase to determine potential miRNAs regulated by curcumin and AKBA. We initially selected miR-34a, miR-145, and miR-16 as potential candidates based on

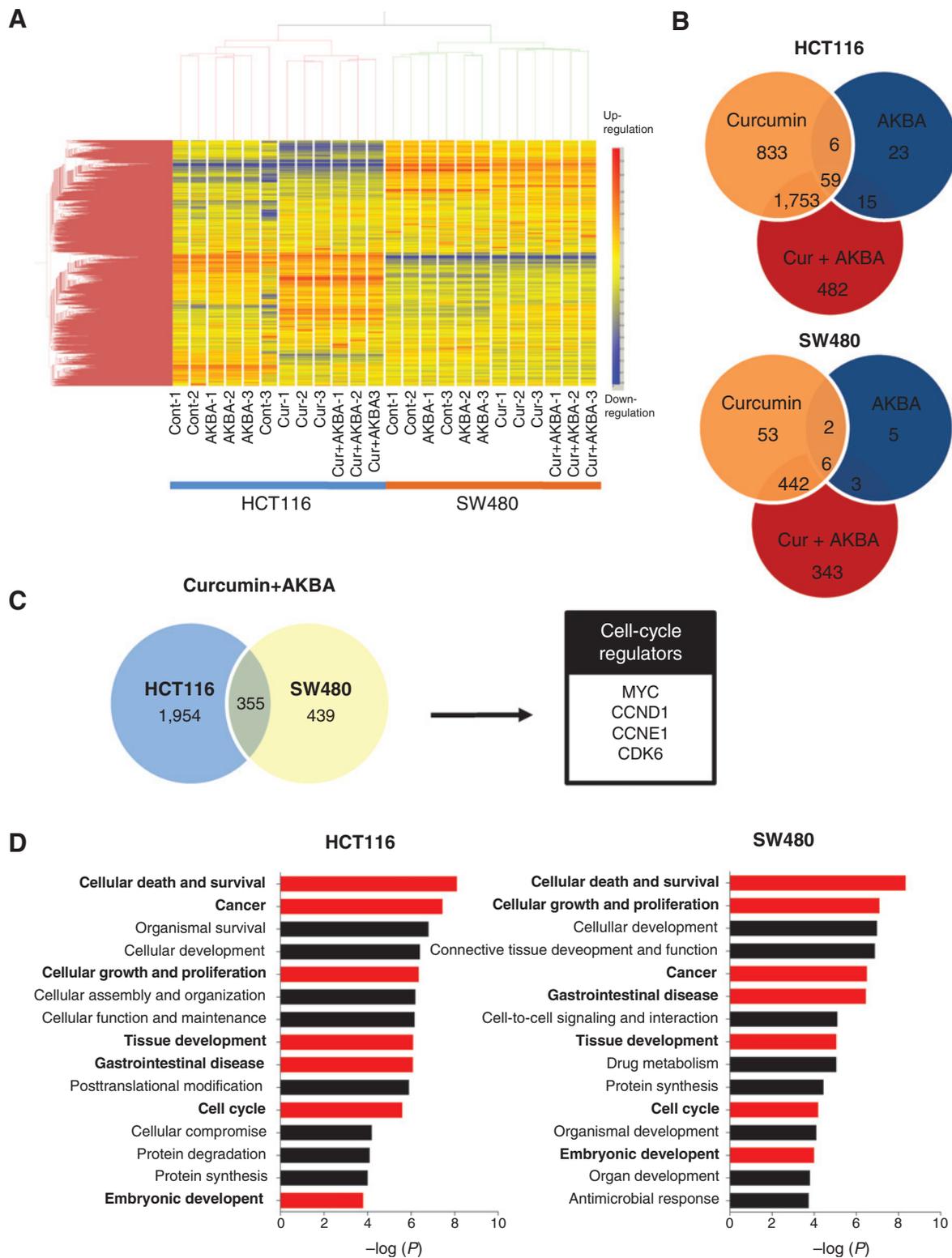


Figure 2. Curcumin and AKBA modulate apoptosis, antiproliferation, and cell-cycle regulation. HCT116 and SW480 cell lines were treated with curcumin, AKBA alone, or together followed by gene-expression microarray and bioinformatic analyses. A, heatmap of gene expression in colorectal cancer cell lines treated with curcumin and/or AKBA. B, Venn diagram showing number of commonly regulated genes shared by curcumin, AKBA, and the combined treatment in HCT116 and SW480 cell lines. C, commonly regulated genes shared by the combined treatment for HCT116 and SW480. D, predicted molecular functions of genes regulated by curcumin- and AKBA-treated colorectal cancer cells determined by the IPA.

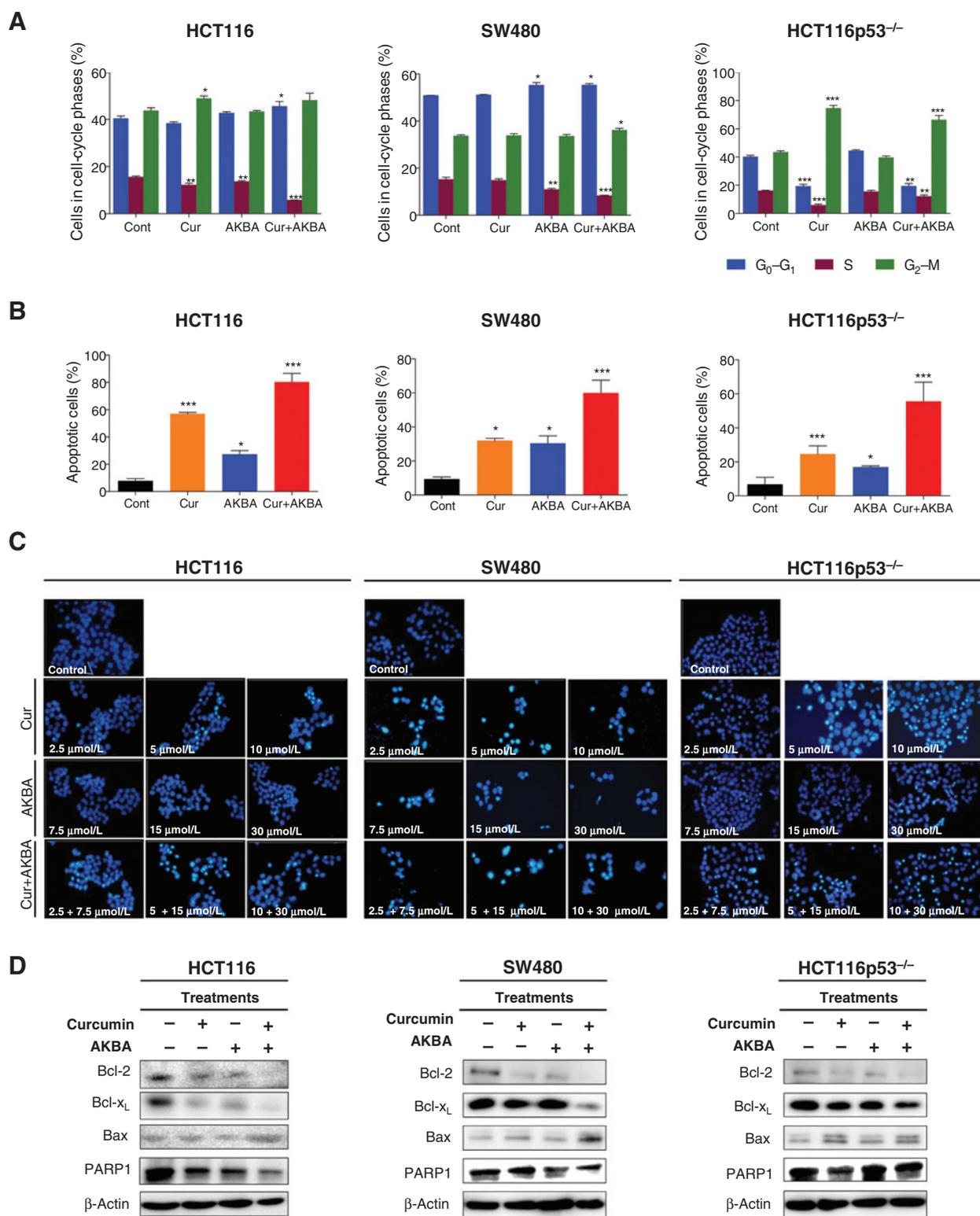
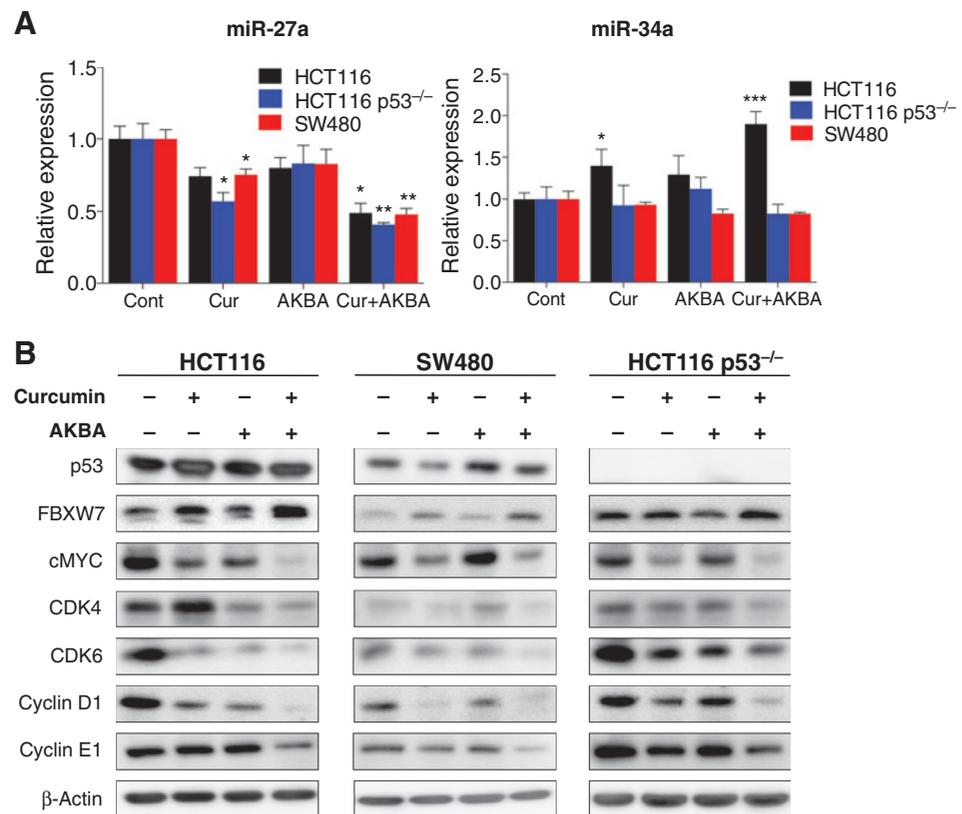


Figure 3. Curcumin and AKBA induce cell-cycle arrest and apoptosis. A, cell-cycle analysis. Cells were treated with 10 μmol/L curcumin and/or 30 μmol/L AKBA and then stained with propidium iodide and subjected to flow cytometry analysis to determine DNA content for phase of the cell cycle. B, cells were stained with Annexin V and 7-AAD and apoptotic cell number was determined by flow cytometry. Flow cytometry graphs and the corresponding percentage of apoptotic cells are shown in bar graphs. C, images of apoptotic cells treated with increasing doses of curcumin and/or AKBA determined by DAPI staining. D, immunoblot analysis of Bcl-2, Bcl-x_L, Bax, and PARP1. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 compared with the corresponding control.

Figure 4.

Curcumin and AKBA regulate miR-34a and miR-27a protein expression and their downstream targets. A, expression of miR-27a and miR-34a in colorectal cancer cells treated with curcumin and/or AKBA. miRNA expression was normalized to RNU6B. B, Western blot analyses of downstream targets of miRNAs. All colorectal cancer cell lines were treated with 10 $\mu\text{mol/L}$ curcumin and/or 30 $\mu\text{mol/L}$ AKBA for 24 hours. Cells were lysed and Western blot analyses were performed on putative downstream targets of miR-34a and miR-27a. β -Actin was run as a loading control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with the corresponding control.



their predicted target genes. miR-34a expression was upregulated by curcumin and further elevated by concurrent treatment with curcumin and AKBA in HCT116 cells (Fig. 4A), but had no significant effect in both SW480 and HCT116p53^{-/-} cell lines. Considering that miR-34a has been shown to be primarily activated by p53 (33), these data indicate that curcumin and AKBA modulate miR-34a expression through p53. This finding was consistent in RKO (p53 wild-type), Caco2, and HT29 (both p53-mutated) cell lines as well (Supplementary Fig. S4A). Next, we used Western blot analysis and qRT-PCR analyses to determine whether these botanicals suppressed expression of miR-34a target genes. Both curcumin and AKBA downregulated cMyc, CDK4, CDK6, and cyclin D1 expression, whereas the combined treatment further increased the inhibitory effects in the HCT116 cell line (Fig. 4B). To our surprise, miR-34a target gene expression was also downregulated in cell lines with p53 mutation/deficiency, indicating that these botanicals exert antitumorigenic effects only, in part, through miR-34a upregulation in colorectal cancer cells. Next, we investigated the expression of miR-145 and miR-16, both putative tumor-suppressive miRNA involved in modulation of cell cycle (34, 35) in colorectal cancer cell lines. Although miR-145 expression was increased by AKBA alone and with the combined treatment in the HCT116 cell line, curcumin treatment alone did not alter miR-145 expression in any of the colorectal cancer cell lines (Supplementary Fig. S4B). The combination of curcumin and AKBA treatment resulted in a significant increase in miR-16 levels in the HCT116 p53^{-/-} cells, while curcumin or AKBA alone did not significantly influence miR-16 expression in any cell line (Supplementary Fig. S4B). Therefore, these data collectively indicate that not all

tumor-suppressive miRNAs are regulated coordinately by curcumin, and that AKBA and curcumin target different miRNAs to exert their antitumorigenic effects.

Because our initial focus was on the identification of putative tumor-suppressive miRNAs, we investigated whether curcumin and AKBA can also regulate the expression of oncogenic miRNAs. Recent studies demonstrated that miR-27a, an oncogenic miRNA, was suppressed by curcumin in colorectal cancer cell lines (36, 37). Although we confirmed that miR-27a was downregulated by curcumin treatment alone in all three colorectal cancer cell lines, its expression was further inhibited by the combined treatment. These data indicate that unlike miR-34a, curcumin and AKBA inhibit miR-27a independently of p53 activation (Fig. 4A). Furthermore, we confirmed downregulation of miR-27a expression by the combined curcumin and AKBA treatment in RKO, HT29, and Caco2 cell lines (Supplementary Fig. S4A). We then investigated whether inhibition of miR-27a by curcumin and AKBA resulted in alteration of FBXW7, a tumor suppressor and a direct target of miR-27a (38). Although curcumin treatment alone upregulated FBXW7 expression, the combined treatment further enhanced FBXW7 expression (Fig. 4B; Supplementary Fig. S5). In addition, we demonstrated that both curcumin and AKBA individually and in combination suppressed cyclin D1 and cMyc, as well as downstream targets of FBXW7 (Fig. 4B; Supplementary Fig. S5).

miR-34a overexpression/miR-27a knockdown induces apoptosis and cell-cycle arrest and suppresses proliferation

To confirm that miR-34a upregulation contributes to the antitumorigenic effects of botanicals, we transiently overexpressed miR-34a and investigated its molecular function in the three

colorectal cancer cell lines (Supplementary Fig. S6A). Overexpression of miR-34a resulted in an enhanced apoptotic cell population in all three cell lines (Supplementary Fig. S6B); however, miR-34a-induced apoptosis appeared to be less prominent in p53-mutated/deficient cell lines. These results support the idea that there is a positive feedback loop between miR-34a and p53 (33). Furthermore, miR-34a overexpression resulted in G₁ arrest in all cell lines (Supplementary Fig. S6C), while it resulted in suppressed cell proliferation and colony formation (Supplementary Fig. S6D and S6E). Similarly, we investigated antitumorigenic activity of miR-27a inhibition in colorectal cancer cell lines. Transient knockdown of miR-27a enhanced cellular apoptosis in all three colorectal cancer cell lines (Supplementary Fig. S7A and S7B), while cell-cycle analysis showed that miR-27a knockdown resulted in an increase in G₂-M phase arrest in HCT116 and HCT116 p53^{-/-} cell lines, while prolonged S-phase fraction in the SW480 cell line (Supplementary Fig. S7C). miR-27a knockdown resulted in suppression of cell growth and colony formation in all three cell lines (Supplementary Fig. S7D and S7E).

Curcumin and AKBA inhibit tumor progression in a mouse xenograft model

To confirm the therapeutic potential of curcumin and AKBA *in vivo*, we generated mouse xenograft tumors by injecting HCT116 cells subcutaneously into nude athymic mice. We subsequently treated the animals with curcumin (25 mg/kg body weight) and/or AKBA (75 mg/kg b/w) for 20 days (Fig. 5A). Although previous studies used 100 mg/kg curcumin to attenuate tumor growth in the mouse xenograft model (39, 40), we purposely chose a lower dose of curcumin to mimic a more reasonable and physiologically achievable clinical dose of curcumin. The body weight of the animals was unaffected by the treatment during the experiment (Fig. 5B). The progressive growth in tumor volume showed that treatment with curcumin or AKBA suppressed tumor growth at a similar rate (Fig. 5C). Interestingly, treatment with botanicals appeared to influence tumor growth immediately, because the average tumor volume of treatment groups deviated from the control group 4 days from the initiation of treatment. The combined curcumin and AKBA treatment resulted in further inhibition in the growth of the xenograft tumors. Confirming our measurement for tumor volume, tumor weight positively correlated with changes the tumor volume (Fig. 5D). To determine whether combined treatments resulted in an enhanced suppression of tumor growth, we calculated the expected tumor volume using FTV of curcumin and AKBA (Fig. 5C). Interestingly, throughout the treatment period, curcumin and AKBA together resulted in synergistic tumor growth suppression (ratio of expected:observed FTV > 1). In addition, we wanted to ascertain whether these *in vivo* antitumorigenic effects of curcumin and AKBA corresponded with the expression alterations of miR-27a and miR-34a, as witnessed in cell culture studies. The expression of miR-27a was downregulated in tumors from mice treated with AKBA or curcumin and AKBA (Fig. 5E), while tumors from mice treated with curcumin and/or AKBA had increased miR-34a (Fig. 5F). In addition, we measured the expression of target genes of these miRNAs in the xenograft tumor tissues. We confirmed that FBXW7, cMyc, CDK6, and cyclin E1 were modulated by the combined treatment (Supplementary Fig. S8). Collectively, these data indicate that changes in expression of these two miRNAs in tumors, at least in part, contributed to tumor growth inhibition.

Curcumin and AKBA are significantly more cytotoxic against colorectal cancer cells than aspirin

Finally, to compare the antitumorigenic properties of both botanicals with a well-established chemopreventive drug, we used the MTT and colony formation assays to compare the potency of curcumin and AKBA with aspirin, a nonsteroidal anti-inflammatory drug (NSAID). A previous study showed that aspirin is more effective in PI3KCA-mutated cancers (41); hence, we compared the effects of these botanicals to aspirin in two cell lines HCT116 (PI3KCA-mutated) and SW480 (PI3KCA wild-type). Although both curcumin and AKBA treatments significantly inhibited colony formation at 2.5 μmol/L, we found that 5,000 μmol/L aspirin was required to moderately inhibit colony formation in both HCT116 and SW480 cell lines, indicating that curcumin was significantly more effective in inhibiting colony formation than aspirin (Fig. 6A). Similarly, results of the MTT assay indicated that 20,000 μmol/L aspirin was needed to exert a similar cellular cytotoxicity to the equivalent of the combined curcumin and AKBA treatment of 10 μmol/L for HCT116 cell line, while almost 30,000 μmol/L aspirin was needed for SW480 cell line (Fig. 6B). In addition, while PI3KA wild-type SW480 cell line appeared to be more resistant to aspirin than HCT116 cell line, the combined botanicals were just as potent in both cell lines. Although there are other parameters contributing to drug efficacy, such as bioavailability and drug retention time, these comparisons highlight the potency of these botanicals against colorectal cancer cells.

Discussion

Herein, we demonstrate for the first time that curcumin and AKBA can act synergistically to exert antitumorigenic effects in colorectal cancer cells, in both *in vitro* and *in vivo* experimental models. Analysis of gene-expression microarrays identified cell-cycle regulation, cellular apoptosis, and proliferation as the primary protective mechanisms by which curcumin and AKBA act in colorectal cancer cell lines, which were subsequently validated in a series of molecular and functional analyses during the course of this study. On the basis of data obtained from gene-expression microarrays, we illustrate that curcumin and AKBA regulated the expression of miR-34a and miR-27a, two miRNAs involved in cell-cycle regulation in cancer and modulated their downstream target genes. Finally, we used a mouse xenograft model to confirm our *in vitro* data and demonstrated that the combination of these botanicals resulted in a synergistic suppression of tumor growth.

miRNAs are a class of small noncoding RNAs that play critical roles in the regulation of gene expression. miR-34a is a well-established tumor suppressor that regulates both cell cycle and apoptosis pathways. Therefore, it is not surprising that miR-34a is primarily regulated by p53, a key cell cycle-regulating tumor suppressor (33). We demonstrated that curcumin and AKBA only upregulated miR-34a in the HCT116 cell line and not HCT116p53^{-/-} and SW480 cell lines, suggesting that these botanicals control miR-34a expression through p53 activation. miR-34 is one of the miRNAs identified for its chemotherapeutic use and it is currently being clinically analyzed with patients who have unresectable primary liver cancer and advanced or metastatic liver cancer (NCT01829971). We overexpressed miR-34a in colorectal cancer cell lines that resulted in inhibition of cell proliferation and colony formation; however, it was noted that the effect was less

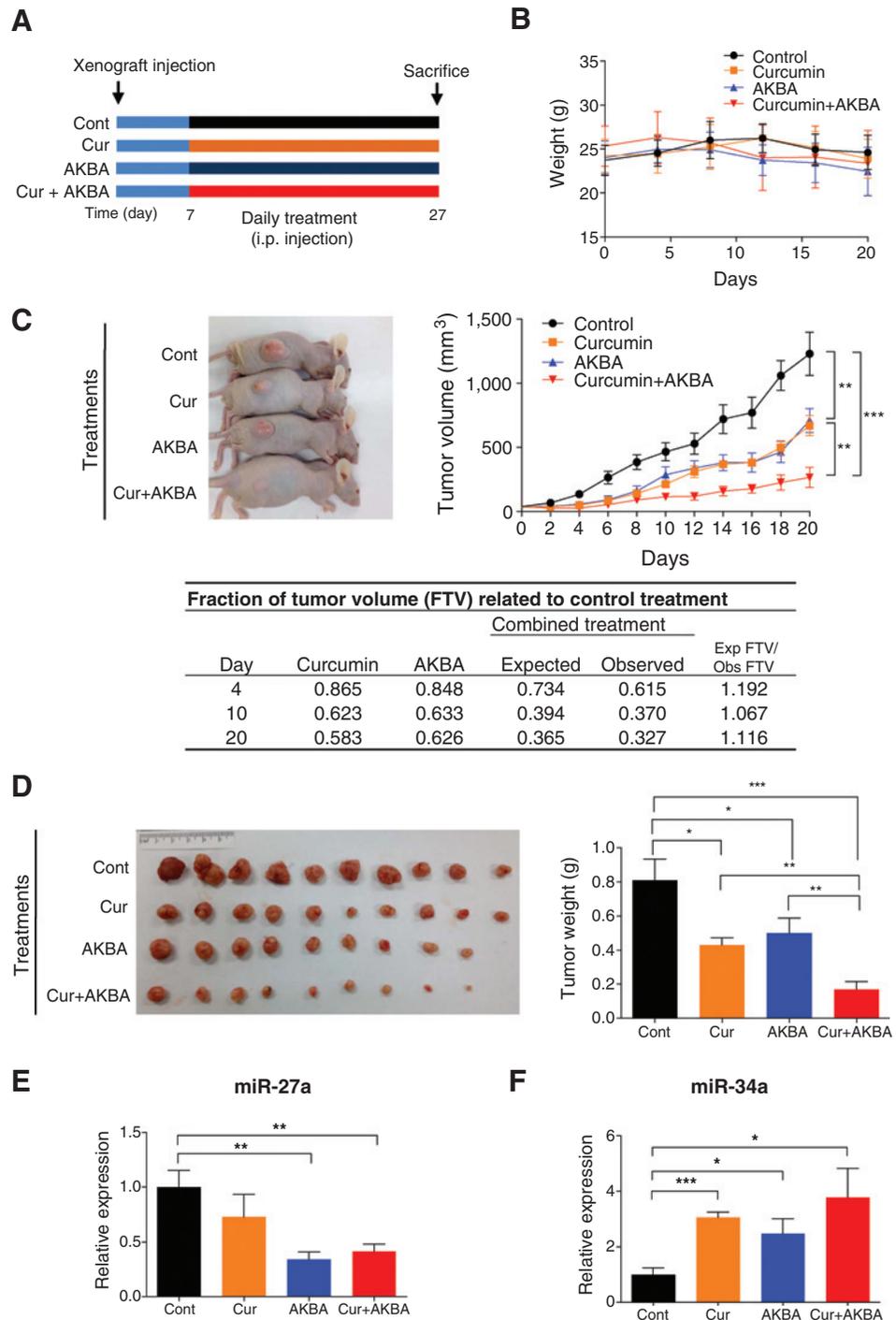


Figure 5. Curcumin and AKBA suppress xenograft tumor growth. A, the schematic diagram of the curcumin and AKBA treatment protocol. B, body weight of animals during the 20-day treatment period. C, representative animals demonstrating tumor sizes according to the different treatments and a graph showing the progressive tumor volume increase during treatment period. FTV was calculated for both curcumin and AKBA treatment alone in the table. Exp FTV was calculated by multiplying the FTV of curcumin and AKBA and then comparing it to the Obs FTV. Ratio of Exp FTV:Obs FTV > 1 indicates a synergistic effect. D, image of xenograft tumors collected from experimental animals and a graph depicting the average tumor weight by treatment. E, graph of the relative expression of miR-27a in xenograft tumors by treatment. F, graph of the relative expression of miR-34a in xenograft tumors by treatment (normalized to RNU6B) *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with the corresponding control.

pronounced in p53-mutated/deficient cell lines. These results suggest that interference in the miR-34a and p53-positive feedback loop may have contributed to the lack of efficacy of miR-34a-induced inhibition of cell proliferation and colony formation (33). Considering p53 mutation occurs in the latter stages of colorectal tumorigenesis (42), the role of botanicals in upregulating miR-34a during initiation and progression stages of colorectal cancer is of importance. With curcumin and AKBA suppressing validated miR-34a target genes, including cMyc,

cyclin D1, CDK4, CDK6, and Bcl-2, our data imply that these botanicals could be used therapeutically to upregulate miR-34a in colonic epithelial cells.

In contrast, miR-27a has been identified recently as a putative oncogene in colorectal cancer (38). miR-27a facilitates proliferation through Sp protein repressor ZBTB10 and FBXW7 (38, 43) and this study confirmed the oncogenic role of miR-27a in colorectal cancer through series of knockdown experiments. Previous studies have demonstrated that

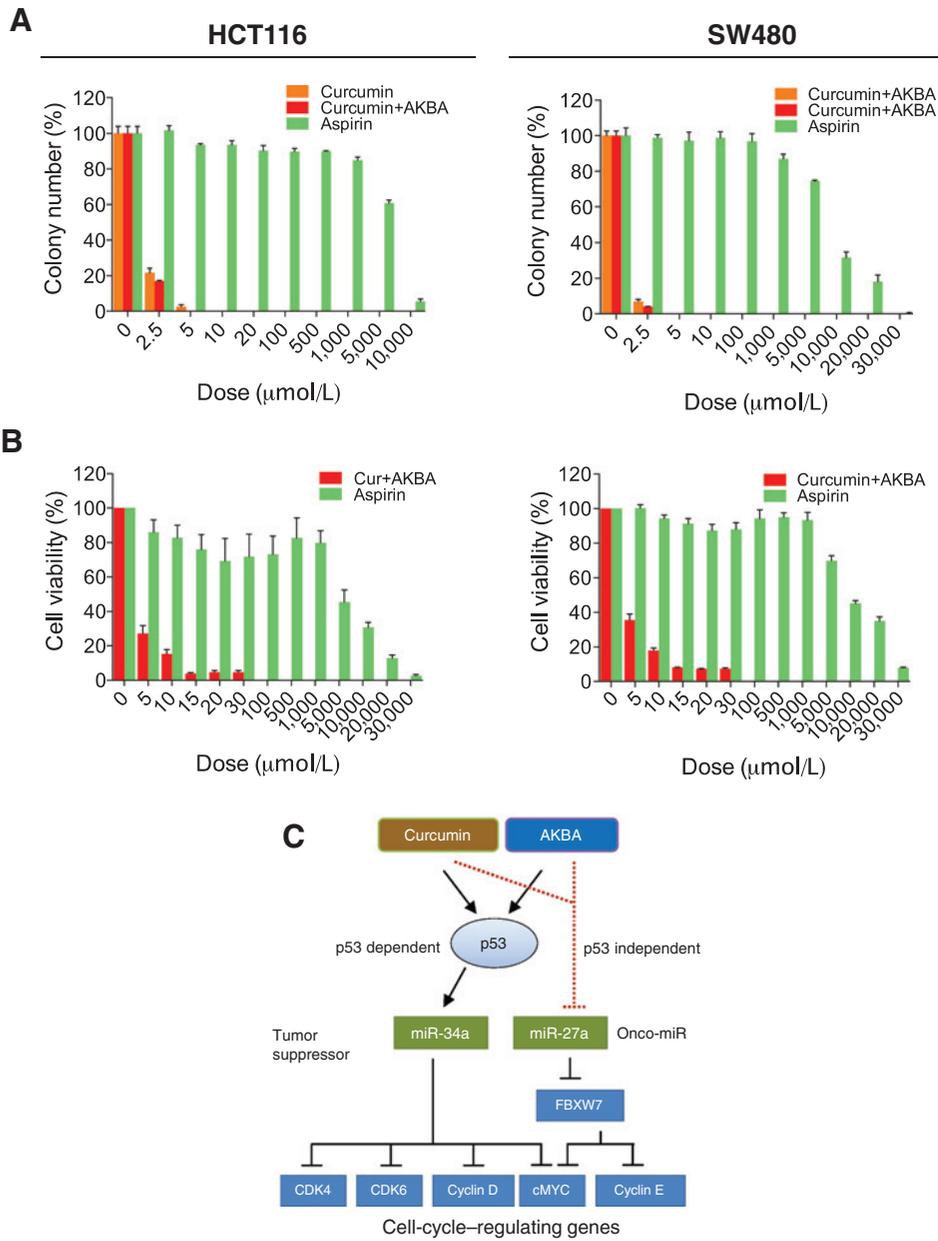


Figure 6. Comparison of the effects of curcumin and AKBA to aspirin on colorectal cancer cells. A, the effects of aspirin on colony formation was compared with curcumin alone, and curcumin and AKBA on HCT116 and SW480 cell lines. B, the cytotoxic effects of aspirin compared with the combination of curcumin and AKBA treatment on HCT116 and SW480 cell lines. C, schematic diagram depicting the role of curcumin and AKBA on modulation of miR-34a and miR-27a and subsequent cell-cycle regulation.

curcumin has been shown to downregulate miR-27a in colorectal cancer cell lines (36, 37). Although we confirmed these findings, we also demonstrated that curcumin and AKBA in combination can further suppress miR-27a expression in colorectal cancer cell lines. One of the oncogenic functions of miR-27a in colorectal cancer is through direct inhibition of the tumor-suppressor FBXW7 (38). FBXW7 is a member of F-box family of proteins that interacts with Skp1 and CUL1 to form a ubiquitin ligase complex (44) that play a pivotal role in the regulating the cell cycle by degrading key proteins in cell division and determination of cell fate, including cMyc, cyclin E1, c-Jun, and Notch (45). This study demonstrated that treatment of colorectal cancer cells with curcumin and AKBA upregulated FBXW7, resulting in downregulation of its downstream target genes. Collectively, our data indicate that these botani-

cals play an important role in regulation of the miR-27a-FBXW7 axis. On the basis of the findings from this study, we propose a mechanism by which curcumin and ABKA control cell-cycle regulation in colorectal cancer cells (Fig. 6C). Curcumin and AKBA can modulate miRNAs, such as miR-34a and miR-27a, in the presence of p53 to control cell-cycle regulation. However, these botanicals can still maintain cell-cycle regulation through miRNA regulation in the absence of p53. Although additional investigations are required to fully understand the complex mechanisms involved in miRNA regulation by curcumin and AKBA, this study highlights the therapeutic potential of these botanicals to coordinate modulation of cancer-related miRNAs.

Mutations in p53 are found in nearly all types of cancers and p53 is the most frequently mutated gene in colorectal cancer (46).

p53 is known for its role in maintaining genomic integrity by regulating cell cycle and cell death pathways (47). This study has shown that the combination of curcumin and AKBA induced synergistic cytotoxicity in p53-deficient/mutated cell lines, but not p53 wild-type cell lines. Although curcumin has been shown to induce apoptosis through p53 independently, it primarily induces apoptosis through stabilization of p53 through inhibition of MDM2 (48). We suspect that the synergism we observed between curcumin and AKBA in this study is a result of AKBA compensating for lack of curcumin's cytotoxicity in these cell lines. Considering all cancers are heterogeneous, combining antitumorogenic agents may increase effectiveness of these agents in various human tumors. Although further investigation is needed to clarify how these botanicals interact to overcome mutations in colorectal cancer, these compounds have a promising potential to provide safe and cost-effective adjunctive treatment alongside conventional therapies.

To confirm the antitumorogenic properties of curcumin and AKBA *in vivo*, we generated xenograft tumors using human colorectal cancer cells in mice and treated these mice with curcumin and/or AKBA. As expected, treatment of mice with curcumin or AKBA resulted in decreased tumor proliferation. However, to our surprise, the combined treatment resulted in synergistic suppression of tumor growth. Although the synergistic tumor-suppressive effect observed for the combined treatment was modest, this finding is important considering that consumption of these two botanicals concurrently is extremely safe. Given that curcumin and AKBA are both readily available and affordable, there is a significant potential for use of these botanicals to prevent colorectal cancer development.

Finally, to highlight the potency of these botanicals with more conventional chemopreventive drugs, we compared the antitumorogenic properties of curcumin and AKBA to aspirin. Recently, a clinical observational study concluded that daily consumption of aspirin, a NSAID, lowers the risk of colorectal cancer (49). The cytotoxicity of curcumin was over 2,000 times more than aspirin *in vitro*, while it suppressed cellular proliferation at a significantly lower dose than aspirin demonstrating that antitumorogenic potential of these botanicals far exceeded that of aspirin. Although our data along with many preclinical studies confirmed the antitumorogenic effects of botanicals, the poor bioavailability associated with both curcumin and AKBA has been perceived as a potential hindrance for their use in prevention trials (50, 51). However, more recently, there is increasing research focus on improving bioavailability of botanicals, including curcumin, through development of new formulations (52). In addition, *in vivo* studies have demonstrated that curcumin exerts antitumorogenic effects when consumed orally (25, 53). Similarly, we have also demonstrated previously that gavaging of AKBA resulted in attenuation of tumor growth in a mouse xenograft model (23). Although this study focused on antitumorogenic synergism between these botanicals through

intraperitoneal administration, our study provides strong evidence for these two botanicals as potential chemotherapeutic agents. Furthermore, unlike aspirin, high levels of curcumin can be consumed safely without increasing the risk of gastrointestinal bleeding and peptic ulcer—the risks associated with continuous administration of aspirin (49). In addition, daily consumption of these botanicals can provide added health benefits as curcumin is effective against other diseases such as rheumatoid arthritis and depression (54, 55).

In summary, we for the first time demonstrate that individual and combined treatment with curcumin and AKBA exerted their antitumorogenic effects, at least in part, by regulation of miRNAs and their downstream target genes involved in cell-cycle regulation. Furthermore, using a mouse xenograft model, we confirmed that curcumin or AKBA suppressed tumor growth, while the combination resulted in synergistic tumor suppression. Collectively, this study highlights the novel therapeutic potential of using the combined treatment of two botanicals, curcumin and AKBA, to inhibit colorectal cancer tumor growth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Toden, Y. Okugawa, D. Nattamai, N. Baldwin, M. Shakibaei, A. Goel

Writing, review, and/or revision of the manuscript: S. Toden, Y. Okugawa, D. Nattamai, C.R. Boland, A. Goel

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Anguiano, A. Goel

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Other (funded the project and supervised the laboratory): C.R. Boland

Acknowledgments

The authors thank Ms. Patricia Kraehe for technical assistance. The authors also thank Margaret Hinshelwood, manager of the Office of Scientific Publications, Baylor Charles A. Sammons Cancer Center, Dallas, for critical suggestions and editing to further improve the quality of this article.

Grant Support

This study was supported by the grants R01 CA72851, CA 181572, and U01 CA187956 from the National Cancer Institute, NIH, and funds from the Baylor Research Institute to A. Goel.

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Received October 13, 2014; revised February 5, 2015; accepted February 9, 2015; published OnlineFirst February 23, 2015.

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