

Honokiol radiosensitizes colorectal cancer cells: enhanced activity in cells with mismatch repair defects

Zhiyun He,^{1,2*} Dharmalingam Subramaniam,^{3*} Satish Ramalingam,³ Animesh Dhar,⁴ Russell G. Postier,⁵ Shahid Umar,² Youcheng Zhang,¹ and Shrikant Anant^{3,6}

¹Department of Medicine, Lanzhou University Second Hospital, Lanzhou, Gansu Province, China, Departments of ²Medicine and ⁵Surgery, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; Departments of ³Molecular and Integrative Physiology and ⁴Cancer Biology and ⁶The University of Kansas Cancer Center, University of Kansas Medical Center, Kansas City, Kansas

Submitted 25 April 2011; accepted in final form 4 August 2011

He Z, Subramaniam D, Ramalingam S, Dhar A, Postier RG, Umar S, Zhang Y, Anant S. Honokiol radiosensitizes colorectal cancer cells: enhanced activity in cells with mismatch repair defects. *Am J Physiol Gastrointest Liver Physiol* 301: G929–G937, 2011. First published August 11, 2011; doi:10.1152/ajpgi.00159.2011.—DNA mismatch repair is required for correcting any mismatches that are created during replication and recombination, and a defective mismatch repair system contributes to DNA damage-induced growth arrest. The colorectal cancer cell line HCT116 is known to have a mutation in the hMLH1 mismatch repair gene resulting in microsatellite instability and defective mismatch repair. Honokiol is a biphenolic compound that has been used in traditional Chinese medicine for treating various ailments including cancer. This study was designed to test the hypothesis that honokiol enhances the radiosensitivity of cancer cells with mismatch repair defect (HCT116) compared with those that are mismatch repair proficient (HCT116-CH3). We first determined that the combination of honokiol and γ -irradiation treatment resulted in dose-dependent inhibition of proliferation and colony formation in both cell lines. However, the effects were more pronounced in HCT116 cells. Similarly, the combination induced higher levels of apoptosis (caspase 3 activation, Bax to Bcl2 ratio) in the HCT116 cells compared with HCT116-CH3 cells. Cell cycle analyses revealed higher levels of dead cells in HCT116 cells. The combination treatment reduced expression of cyclin A1 and D1 and increased phosphorylated p53 in both cell lines, although there were significantly lower amounts of phosphorylated p53 in the HCT116-CH3 cells, suggesting that high levels of hMLH1 reduce radiosensitivity. These data demonstrate that honokiol is highly effective in radiosensitizing colorectal cancer cells, especially those with a mismatch repair defect.

radiation; mismatch repair; cell cycle; p53; apoptosis

COLORECTAL CANCER is a leading cause of adult cancer-related deaths and is a major health problem globally (41). Chemotherapeutic compounds currently being used for the treatment of colorectal cancer include 5-fluorouracil, oxaliplatin, gemcitabine, and irinotecan (26). Because conventional therapies, including surgical resection, chemotherapy, and radiation, are often inadequate in treating this disease, new treatment options are critically needed. Among the conventional therapies, radiotherapy is one of the most popular triplet therapies for the colorectal cancer (17). In Asian countries, most of those colorectal cancer patients are diagnosed or detected at a late

stage with local invasion or metastasis, and some patients with tumor recurrence, reducing the chances of surgical intervention. Therefore, these patients need a more effective radiation therapy. Ionizing radiation is a potent inducer of DNA damage, and it is thought that the ineffective repair of DNA double-strand breaks contributes most strongly to cytotoxicity (38). Cancer cells have been shown to contain inherited mutations resulting in their inability to reduce DNA replication following a genotoxic insult such as exposure to ionizing radiation (IR), thereby leading to radioresistance (12). The magnitude of this problem mandates the need to increase the sensitivity of the tumor cell to IR.

Honokiol (Fig. 1A) is a biphenolic compound present in the leaves, bark, and root of *Magnolia officinalis* (Chinese name is Houpu). It has been used in traditional Chinese and Japanese medicine for the treatment of various ailments because of its muscle relaxant, anti-gastric ulcer, antiallergic, antibacterial, and antithrombotic properties (14, 25). The known pharmacological effects of honokiol include inhibition of platelet aggregation and protection of the myocardium, and recent studies have demonstrated that it has potent antitumor effects (13, 33). Moreover, previous studies have demonstrated that honokiol in combination with radiation produces synergistic antitumor efficacy without increasing toxicity (17). However, it is not clear whether the synergism is affected by the mismatch repair status of the cell.

Approximately 15% of colorectal cancers arise as a result of defective DNA mismatch repair resulting in chromosomal instability (19, 27). Mismatch repair-deficient colorectal cancers have distinct features that distinguish them from other colorectal cancer types (20). Mismatch repair is a highly conserved repair pathway that plays an important role in the detection and correction of DNA mismatches created during replication and recombination (31). Inactivating mutations in mismatch repair genes cause a greatly increased rate of spontaneous mutation and are the underlying defect in hereditary nonpolyposis colorectal cancer (10, 32, 34, 35, 39, 40). In addition, mismatch repair defects are associated with a significant proportion of sporadic cancers (31). Introduction of a wild-type hMLH1, hMSH2, or hMSH6 mismatch repair-related genes by chromosome transfer into the corresponding mismatch repair-deficient cell lines induces adequate protein expression, restores mismatch repair activity, and stabilizes microsatellite loci (16, 21, 36). Mismatch repair also modifies the cytotoxicity of various anticancer agents (32). However, the role of mismatch repair in radiosensitization of colon cancer by natural compounds such as honokiol is not known.

* Z. He and D. Subramaniam contributed equally to this study.

Address for reprint requests and other correspondence: S. Anant, Univ. of Kansas Medical Center, 3901 Rainbow Blvd., Mail Stop 3040, Kansas City, KS 66160 (e-mail: sanant@kumc.edu).

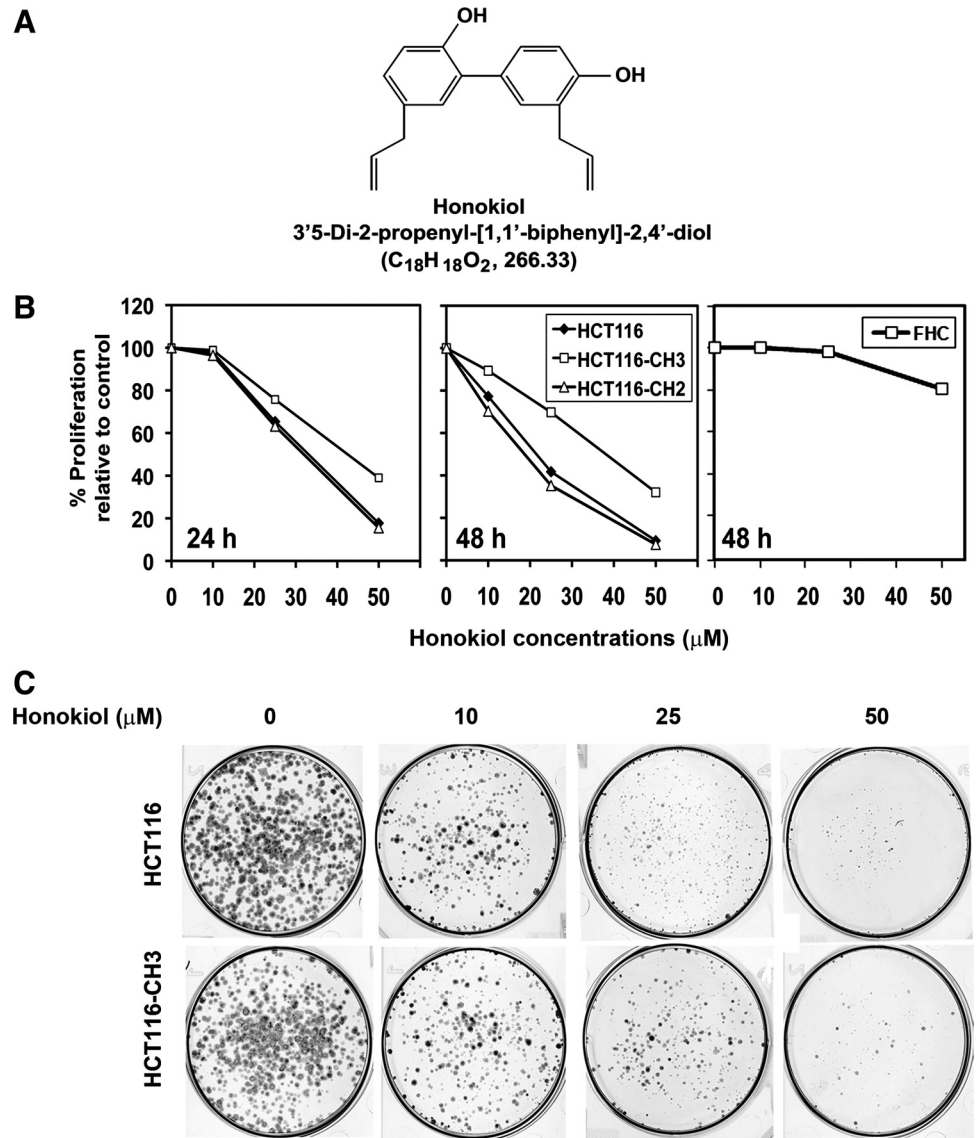


Fig. 1. Honokiol inhibits colorectal cancer growth. *A*: topological structure of honokiol. *B*: HCT116, HCT116-CH2, and HCT116-CH3 cells were incubated with increasing doses of honokiol (0–50 μM), and proliferation was determined by hexosaminidase assay at 24 and 48 h. Honokiol inhibited the growth of HCT116 cells. A similar level of inhibition is seen with HCT116-CH2 cells. In contrast, proliferation of HCT116-CH3 cell was not affected to the same level. On the other hand, up to 25 μM honokiol does not inhibit proliferation of FHC normal colonic epithelial cells. Here the cells were incubated with increasing concentrations of honokiol (0–50 μM) for 48 h and then proliferation levels were determined. *C*: honokiol inhibits colony formation. Cells were treated with increasing doses of honokiol (0–50 μM) for 24 h. Colonies were allowed to form by incubating in regular media containing 10% FBS for an additional 10 days. There was a reduction in colony formation in both cell lines but was more pronounced in HCT116 cells.

Accordingly, in this article, to determine whether honokiol enhances radiosensitivity of colon cancer cells with mismatch repair defect, we have used the model cell line HCT116, which is hMLH1 deficient, and compared it with HCT116-CH3, where chromosome 3 is stably reconstituted. The HCT116-CH3 cells are proficient in mismatch repair.

MATERIALS AND METHODS

Cells and reagents. HCT116 (American Type Culture Collection, Manassas, VA), HCT116-CH2, and HCT116-CH3 cells (21) transfected with chromosome 2 or chromosome 3, respectively, were kindly provided by Dr. Richard Boland and grown in DMEM containing 10% heat-inactivated fetal bovine serum (Sigma Chemical, St. Louis, MO) and 1% antibiotic-antimycotic solution (Mediatech, Herndon, VA) at 37°C in a humidified atmosphere of 5% CO₂. Normal colon epithelial cells (FHC, CRL-1831) were purchased from American Type Culture Collection, Manassas, VA and grown in Ham's F12 medium 45%, Dulbecco's modified Eagle's medium 45%, 25 mM HEPES, 10 ng/ml cholera toxin, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 100 ng/ml hydrocortisone, 10% fetal bovine serum (Sigma Chemical), and 1% antibiotic-antimycotic solution (Mediatech, Her-

ndon, VA) at 37°C in a humidified atmosphere of 5% CO₂. Honokiol was purchased from LKT Laboratories, St. Paul, MN.

Proliferation and apoptosis assays. To assess proliferation, cells were seeded onto 96-well plates and grown overnight. The cells were then treated with increasing doses of honokiol in DMEM containing 10% FBS and, after 4 h, the cells were exposed to γ-irradiation (2.5 and 5.0 Gy) in a gamma cell 40-cesium irradiator at 0.90 Gy/min. Analysis of cell proliferation was performed at 24 and 48 h following IR exposure by the hexosaminidase enzymatic assay as described previously (23). For apoptosis, caspase 3/7 activity was measured by use of the Apo-one Homogeneous Caspase-3/7 assay kit (Promega, Madison, WI).

Colony-formation assay. Briefly, six-well dishes were seeded with 500 viable cells/well in DMEM medium and allowed to grow overnight. The cells were then treated with increasing doses of honokiol (10–50 μM) in 10% FBS-containing DMEM. After 48 h, the honokiol-containing medium was removed and the cells were washed in PBS and incubated for an additional 10 days in complete medium. For combination of honokiol with radiation, the cells were grown overnight, then treated with honokiol (25 μM) in 10% FBS-containing DMEM for 4 h and then exposed to 2.5 and 5 Gy IR. After 48 h following radiation exposure, the honokiol-containing medium was

removed, and the cells were washed in PBS and incubated for an additional 10 days in complete medium. Each treatment was done in triplicate. The colonies obtained were washed with PBS and fixed in 10% formalin for 10 min at room temperature and then washed with PBS followed by staining with hematoxylin. The colonies were counted and compared with untreated cells.

Cell cycle analysis. HCT116 and HCT116-CH3 cells were plated at a density of 5×10^5 cells per well on six-well plates. After treatment with honokiol and 2.5 Gy IR, cells were allowed to grow for 48 h. Both floating and attached cells were collected and centrifuged at 1,000 rpm for 5 min. The pellets were washed with PBS. The cells were resuspended in PBS and fixed in ice-cold 70% ethanol. The cells were washed with PBS and 10 mg/ml RNase A was added. Propidium iodide was added to the tubes at a final concentration of 0.05 mg/ml and incubated at 37°C for 30 min in the dark. Cell cycle analysis was performed with a Becton Dickinson FACScan by using an FL2 detector with a band-pass filter at specifications of 585 ± 21 nm. In each analysis, 10,000 events were recorded. Results were analyzed with ModFit LT software (Verity Software House, Topsham, ME).

Western blot analysis. Cell lysates were subjected to polyacrylamide gel electrophoresis and blotted onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Cyclin A1, cleaved Caspase 3, Bcl2, Bax, and phospho-specific p53 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Cyclin D1 and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and specific proteins were detected by the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ).

Real-time reverse-transcription polymerase chain reaction analysis. Total RNA isolated from HCT116 and HCT116-CH3 cells treated with honokiol and IR by use of TRIzol reagent was reverse transcribed with Superscript II reverse transcriptase in the presence of random hexanucleotide primers (all from Invitrogen, Carlsbad, CA). Complementary DNAs were then used for real-time PCR using Jumpstart *Taq* DNA polymerase (Sigma Chemical) and SYBR Green nucleic acid stain (Molecular Probes, Eugene, OR). Crossing threshold values for individual genes were normalized to β -actin. Changes in mRNA expression were expressed as fold change relative to control. Primers used in this study were as follows: β -actin, 5'-GCTGATC-CACATCTGCTGG-3' and 5'-ATCATTTGCTCCTCCTCAGCG-3'; NM_000546.4 Homo sapiens tumor p53 transcript variant 1 mRNA, 5'-ATGGAGGAGCCGCAG-3' and 5'-AAATCATCCAT-TGCTT-3'.

Statistical analysis. All values are expressed as means \pm SE. Data were analyzed via an unpaired two-tailed *t*-test. *P* value of less than 0.05 was considered statistically significant. For comparing multiple groups, one-way ANOVA was performed with Bonferroni corrections for multiple comparisons. To be considered significant, the *P* value must be less than 0.017. The SPSS V17 statistical software was used for ANOVA analysis.

RESULTS

Mismatch repair status affects the ability of colorectal cancer cell survival against the combination of honokiol and IR. Genomic instability, resulting in defects of cell cycle checkpoints and DNA mismatch repair, has been implicated in lack of therapeutic response in patients with advanced disease. Previous studies have demonstrated that honokiol has anticancer effects on many cell types, but the exact mechanism of action is not well understood (24). In this study, we sought to determine whether honokiol might have a more potent effect on colorectal cancer cells with mismatch repair defects. For this, we used the MLH1-deficient HCT116 cell line and compared it to the mismatch-proficient HCT116-CH3 subline. To confirm that changes seen are due to correction of the repair

defect and not due to transfecting a chromosome, we also used the HCT116-CH2 subline in which the mismatch repair deficiency continues to persist (21). We first determined the effect of honokiol on the three cell lines. Honokiol alone inhibited the proliferation of all three cell lines (Fig. 1B). This antiproliferative effect was seen within 24 h at a dose of 25 μ M, which continued to significantly increase up to 48 h. However, the effects were less pronounced in the HCT116-CH3 cells, where the mismatch repair defect was corrected. Honokiol does not affect the proliferation of normal colonic epithelial cells for up to 25 μ M concentrations (Fig. 1B). To further determine that the effect of honokiol is long term, we performed a colony formation assay in which cells were treated with increasing concentrations of honokiol (10–50 μ M) for 24 h, following which the cells were allowed to grow. There was a significant difference in the number of colonies formed after treatment with 25 μ M honokiol, with fewer colonies observed in HCT116 cells compared with HCT116-CH3 cells (Fig. 1C). These data suggest that honokiol has greater effects on colorectal cancer cells that have mismatch repair defects.

Radiation is a form of therapy commonly used in colon and rectal cancers either before or after surgery. However, high doses of radiation have significant side effects. Hence identifying means to enhance radiosensitivity of tumors is important. Given that honokiol reduced growth of HCT116 cells, we next determined its effects on radiation treatment. First, we determined the effect of honokiol and radiation on cell proliferation. Honokiol significantly enhanced IR-mediated suppression of proliferation of all three cell lines in a dose- and time-dependent manner (Fig. 2A). More importantly, the effects were greater in the mismatch-deficient HCT116 and HCT116-CH2 cells compared with mismatch-proficient HCT116-CH3 cells. These data suggest that the combination of honokiol and IR is more potent in treatment of MLH1 mismatch repair-deficient cells. To determine the long-term effect of the combination of honokiol and IR treatment, HCT116 and HCT116-CH3 cells were treated with honokiol for 4 h before exposure to 2.5 and 5.0 Gy IR. The cells were treated with honokiol for an additional 24 h before they were allowed to grow in normal medium. Lower numbers of colonies were observed in the HCT116 compared with HCT116-CH3 cells, both when the cells were treated with honokiol and IR alone and when they were treated with the combination (Fig. 2B). These data suggest that mismatch repair proficiency protects cells from effects of honokiol and radiation therapy.

Alteration of G₁/S transition is an underlying event in the development and progression of many cancers and a key protein that regulates this transition is cyclin D1. This cyclin forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for G₁/S transition. Cyclin D1 also functions as a cofactor for several transcription factors (22). Cyclin D1 overexpression has been linked to the development and progression of cancer (2). In both HCT116 and HCT116-CH3 cells, honokiol and IR treatment resulted in reduced cyclin D1 expression, with the combination having an additive effect (Fig. 2C). However, the suppression was more pronounced in the HCT116 cells compared with HCT116-CH3 cells (Fig. 2C). Similar results were obtained with cyclin A1, an S/G₂ checkpoint-related protein, suggesting that the combination of honokiol and IR could potentially slow down the progression of cells out of S phase (Fig. 2C).

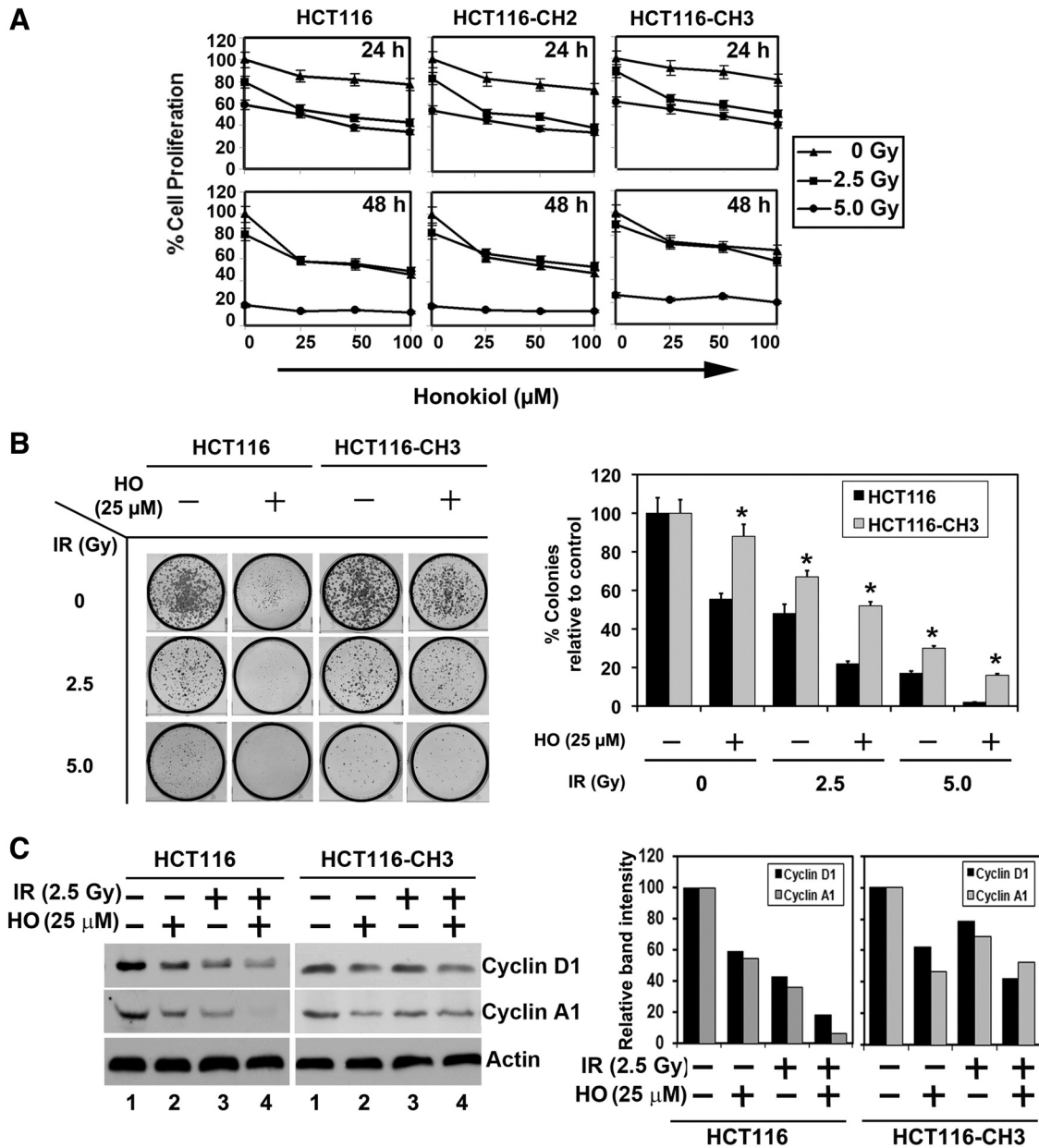


Fig. 2. Mismatch repair status affects the ability of colorectal cancer cell growth against the combination of honokiol and ionizing radiation (IR). **A**: HCT116, HCT116-CH2, and HCT116-CH3 cells were incubated with increasing doses of honokiol (0–100 μ M) and subsequently irradiated with increasing doses (0–5 Gy) of IR. Cell proliferation was determined at 24 and 48 h by use of hexosaminidase enzyme activity. The combination of honokiol with IR resulted in a significant dose- and time-dependent decrease in cell proliferation in all the 3 cell lines compared with untreated controls. Moreover, compared with HCT116-CH3, proliferation effects in HCT116 and HCT116-CH2 were significantly lower ($P < 0.017$). **B**: combination of honokiol (HO) and IR inhibits colony formation. HCT116 and HCT116-CH3 cells were incubated with 25 μ M honokiol for 24 h and subjected to 2.5 and 5.0 Gy IR. Following this, the cells were allowed to grow and form colonies. Treatment with honokiol and IR inhibited colony formation and showed that additive effect by ANOVA (right). Results are representative of 3 independent experiments. * $P < 0.017$ for HCT116-CH3 compared with HCT116 cells. **C**: lysates from HCT116 and HCT116-CH3 cells incubated with 25 μ M of honokiol and exposed to 2.5 Gy IR were analyzed by Western blotting for cyclin D1 and cyclin A1. At right, band intensities are plotted compared with control. The combination of honokiol and IR inhibits cyclin D1 and cyclin A1 expression.

Combination of honokiol and IR induces higher levels of cell death in mismatch repair-defective cells. Given the effects of honokiol and IR on cyclin expression, we next determined whether the combination of honokiol (25 μ M) with IR (2.5 Gy) affects cell cycle progression. Both honokiol and IR treatment affected cell cycle progression (Fig. 3, A and B). Treatment with honokiol alone resulted in an increase in G_0/G_1 phase in both the cell lines. In contrast, IR induced a

G_2/M phase arrest in the cells (Fig. 3A). When the two treatments were combined, we observed increased amounts of dead cells (Fig. 3A). Moreover, the number of dead cells in the HCT116 cells was higher compared with HCT116-CH3 cells. These data suggest that the ability of the combination of honokiol with radiation in inducing cell death is higher in those that have a mismatch repair defect.

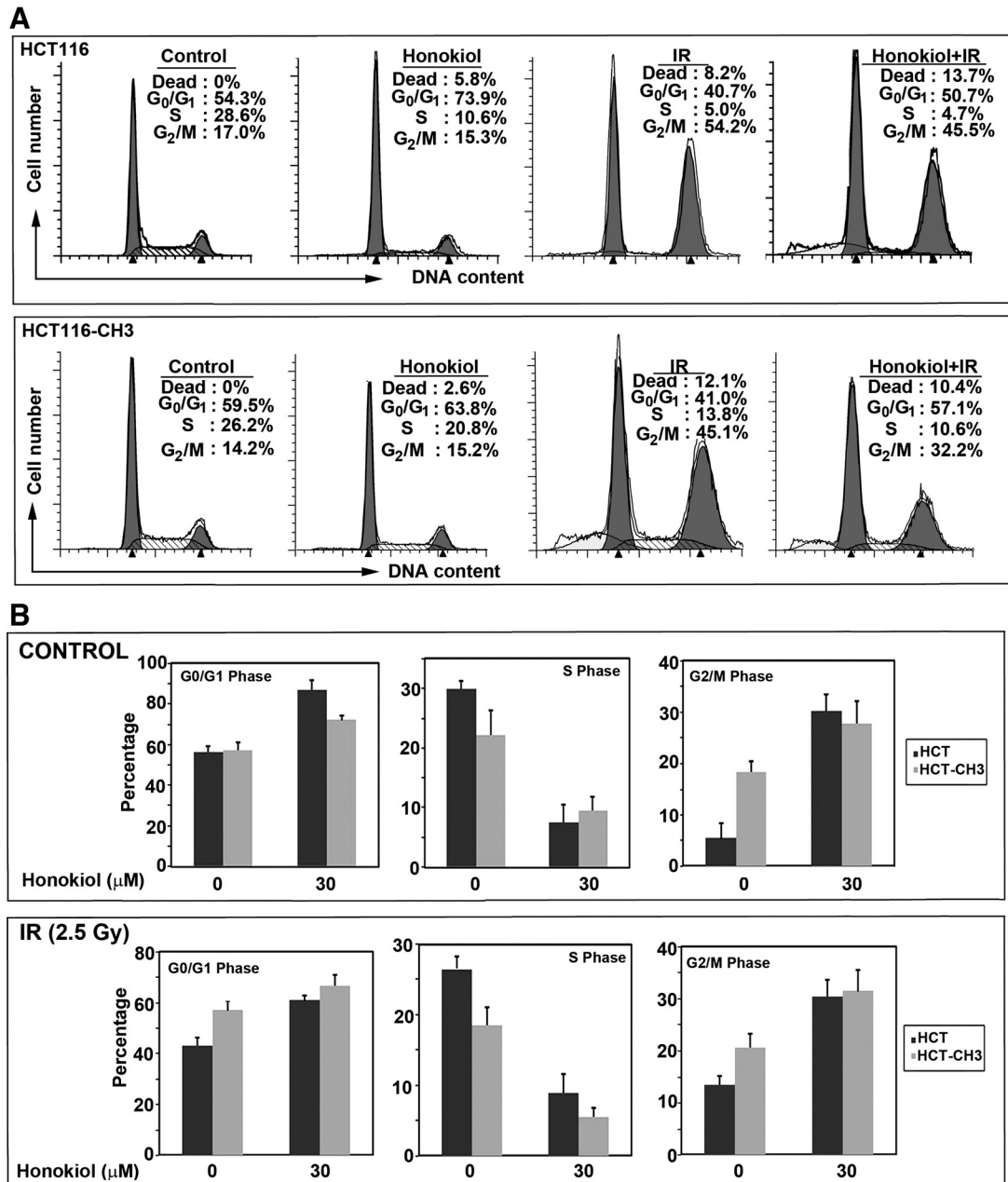


Fig. 3. Combination of honokiol and IR induces cell death. *A*: cell cycle profiles of HCT116 and HCT116-CH3 cells treated with 25 μ M honokiol and subjected to 2.5 Gy IR. After 48 h, cells were analyzed by flow cytometry using propidium iodide for DNA content. Honokiol treatment alone increased G₀/G₁ phase of cell cycle, whereas IR increased cell death of HCT116 cells, which is reduced in HCT116-CH3 cells. *B*: graphs are representative of data collected from 3 independent experiments after treatment with 25 μ M honokiol.

Mismatch repair-defective cells have higher levels of apoptosis in response to the combination of honokiol and IR. Caspase-3 and caspase-7 are key effector molecules in the apoptosis pathway involved in amplifying the signal from initiator caspases, such as caspase-8 and caspase-9 (8). To determine whether the cell death observed with honokiol and IR treatments is apoptosis, we determined whether caspase-3 and/or caspase-7 activity in HCT116 and HCT116-CH3 cells treated with either honokiol or IR is altered (Fig. 4A). Furthermore, although there was an increase in the caspase-3/7 activity following treatment with the combination of honokiol and IR in the HCT116 cells, the effects were less pronounced in the

MLH1-proficient HCT116-CH3 cells (Fig. 4A). This was further confirmed by Western blot analyses, where activated caspase-3 levels were observed to be higher in the HCT116 compared with the HCT116-CH3 cells (Fig. 4B). Further confirmation that the cells were undergoing apoptosis was obtained by Western blot analyses for the antiapoptotic Bcl2 and proapoptotic Bax proteins. Although either honokiol or IR alone inhibited the expression of Bcl2, there was a significant inhibition of Bcl2 expression when the two treatments were combined (Fig. 4C). On the other hand, there was a significant increase in Bax protein with either honokiol or IR treatment, with a further increase in response to the combination treat-

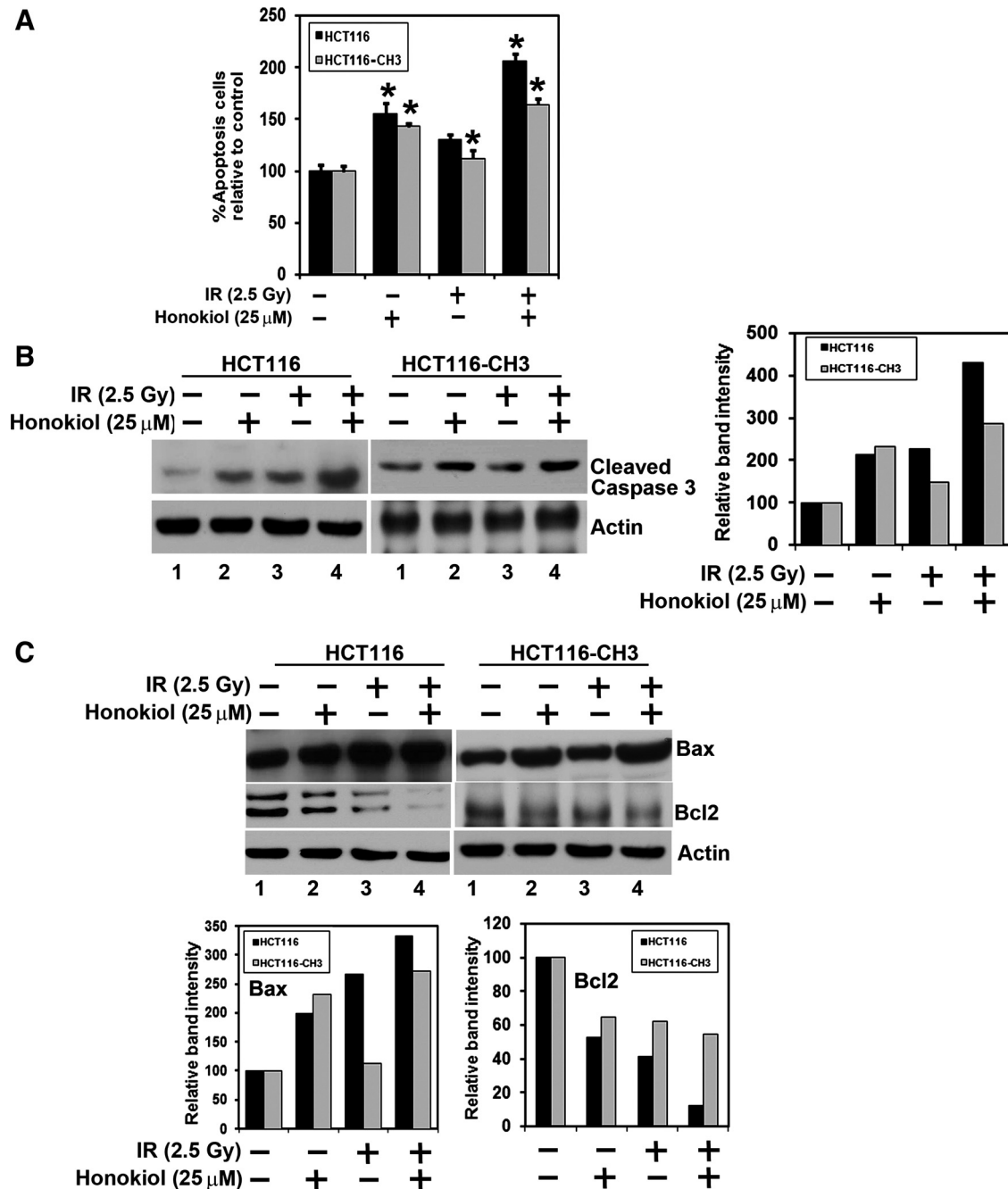


Fig. 4. Mismatch repair-defective cells have higher levels of apoptosis in response to the combination of honokiol and IR. **A**: HCT116 and HCT116-CH3 were treated with either honokiol (25 μ M) or 2.5 Gy IR or both and allowed to grow for 48 h. The combination of honokiol and IR induces apoptosis in both HCT116 and HCT116-CH3 cells, compared with untreated cells ($*P < 0.017$). **B**: combination of honokiol and IR induces caspase 3, an apoptosis mediator. HCT116 and HCT116-CH3 cells were treated with 25 μ M honokiol and 2.5 Gy IR. After 48 h, the lysates were analyzed by Western blotting for cleaved caspase 3 protein by using a rabbit anti-caspase 3 antibody. Band intensities were calculated and plotted as relative to control. The combination of honokiol with IR resulted in increased levels of cleaved caspase 3 compared with either treatment with honokiol or IR alone in HCT116 cells. Compared with HCT116 cells, there were lower levels of activated caspase 3 in HCT116-CH3 cells. **C**: lysates from a combination of honokiol with IR-treated HCT116 and HCT116-CH3 cells were analyzed by Western blotting for Bax and Bcl-2 proteins. Band intensities were calculated and plotted relative to control. The combination of honokiol with IR treatment induces proapoptotic protein Bax levels and reduces expression of antiapoptotic protein Bcl-2 in both the cells compared with untreated cells or with honokiol- or IR-treated cells.

ment. Similar results were obtained in HCT116-CH3 cells, although the effects were less pronounced. These data suggest that honokiol is a potent inducer of apoptosis and plays an important role in radiosensitizing the cells, especially those with a defect in the mismatch repair system.

The combination of honokiol and IR induces higher levels of p53 phosphorylation in mismatch repair-defective cells. Tumor suppressor p53 protein plays a significant role in G₂/M transition and is activated in response to genotoxic injury (9). Since honokiol and IR induces G₂/M cell cycle arrest and subsequent

apoptosis, we next determined whether combination of honokiol with IR affected p53 expression. There was a significant increase in p53 mRNA levels in HCT116 cells but not in HCT116-CH3 cells (Fig. 5A). These data suggest that the combination of honokiol and IR induces apoptosis in a p53-independent manner in mismatch-proficient cells. We further confirmed this at the protein level. Phosphorylation of the NH₂-terminal end of p53 activates the protein. Higher levels of p53 phosphorylation at Ser15 were observed following IR treatment in both HCT116 and HCT116-CH3 cells (Fig. 5B). Furthermore, although the combination of honokiol and IR treatment demonstrated increased levels of phosphorylated p53 in HCT116 cells, this was significantly suppressed in the HCT116-CH3 cells. These data suggest that HCT116-CH3 cell is less sensitive to the treatment of honokiol and IR, again demonstrating that the combination of honokiol and IR is more potent in inducing p53 phosphorylation in cells with mismatch repair defects.

DISCUSSION

Colorectal cancer is a common malignancy in economically developed countries, particularly in North America, Europe, and Australia, and has emerged as one of the leading causes of

cancer-related deaths in the Western world. The significant morbidity of surgery, radiation, and chemotherapy for colorectal cancer has led to searches for less toxic alternative therapies. Concurrent or sequential combinations of radiotherapy or chemotherapy with chemopreventive agents have been suggested as promising alternatives to single-agent therapies. Recent reports from experimental and clinical studies have also proposed that combinations of radio- or chemotherapy agents with natural preventive agents have shown greater than additive effects resulting in increased efficacy while reducing potential side effects (4, 6). Moreover, the possibility exists that, with the addition of agents with low side effects, the dose of agents with potential side effects can be significantly reduced while keeping the clinical therapeutic outcome the same.

Our results indicate that the combination of honokiol with IR possesses potential as a promising anti-colorectal cancer therapeutic strategy. Many studies have shown a broad antitumor activity for honokiol (7, 13, 25). Unlike many other natural products, honokiol exhibits a desirable spectrum of bioavailability (13). The development of other polyphenolic agents has been hindered by poor absorption and rapid excretion (e.g., curcumin) (29, 30, 37). However, honokiol does not have this disability in that significant systemic levels of honokiol can be obtained in preclinical models and that honokiol can also cross the blood-brain barrier (13). Honokiol inhibits multiple facets of signal transduction (13). Currently, it is not known whether honokiol has a single major target or several targets. However, it has several activities that make it desirable as a therapeutic. First, it is orally bioavailable and crosses the blood-brain barrier. Second, it inhibits NF- κ B activity differently from other known inhibitors. This suggests that it can sensitize tumors to apoptosis in the face of conventional chemotherapy and radiation. Honokiol can also cause mitochondrial dysfunction (13). These data together suggest that honokiol could be used as an effective agent either alone or in combination with IR and/or chemotherapeutic drugs. Of course, there is a need to consider how the honokiol could be administered. For colorectal cancers, one mode could be oral administration in the form of a capsule, especially since it is water soluble and has been shown to be bioavailable. Another could be an intravenous route of administration. In this regard, a recent study demonstrated that a biodegradable self-assembled PEG-PCL-PEG micelle encapsulating honokiol can be administered intravenously, for effectively targeting colorectal cancers (15). More importantly, this method was found to be effective, stable, and safe.

Honokiol disrupts many of the characteristic cancer-promoting events. Previous studies have shown that honokiol radiosensitizes lung cancer cells in both dose- and time-dependent manner (18). Our present studies show a similar effect on colorectal cancer cells. We have observed that the combination of honokiol with IR inhibits proliferation of colorectal cancer cells and promotes apoptosis. Previous studies with LL/2 Lewis lung carcinoma cells showed that honokiol combined with radiotherapy can induce the cells to arrest in the G₀/G₁ phase and a corresponding decrease in the S-phase (18). Here we have determined that the combination of honokiol with IR results in an increase in cells undergoing arrest in G₂/M phase, along with an increase in the number of cells in sub-G₀ and a decrease in the number of cells in the S phase at 48 h. Our studies further suggest that there is significantly higher level of

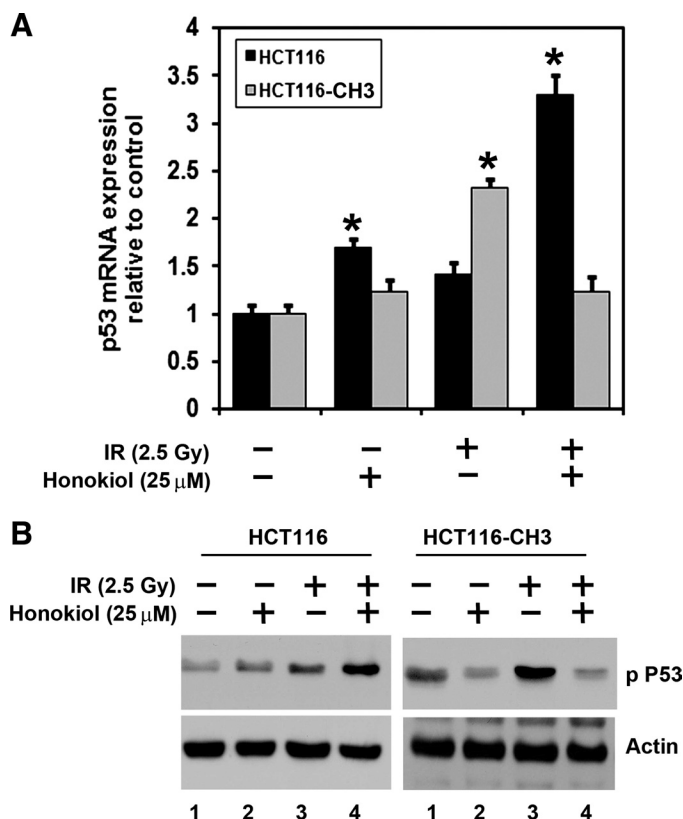


Fig. 5. Higher levels of p53 phosphorylation are observed in mismatch repair-defective cells in response to the combination of honokiol and IR. **A**: p53 mRNA levels were analyzed by real-time PCR. Analyses of total RNA from HCT116 and HCT116-CH3 cells following combination of honokiol and IR treatment for 48 h showed upregulation of p53 mRNA (* $P < 0.05$). **B**: lysates obtained from HCT116 cells treated with the combination of honokiol and IR caused significant upregulation of phosphorylated p53 protein levels. In contrast, there were significantly lower levels of phosphorylated p53 protein levels in HCT116-CH3 cells.

apoptosis. An intriguing observation is that cyclin D1 and cyclin A1 were reduced when cells were treated with honokiol and IR. A reduction in cyclin D1 is generally believed to result in cells undergoing G₀/G₁ arrest since the protein is responsible for progression through the G₁/S phase transition (3). However, our data suggest that the combination of honokiol and IR downregulated the expression of cyclin A1, a protein that regulates S/G₂ progression. Additional studies are required to determine whether specific cells within a pool of cells are undergoing G₁/S or S/G₂ checkpoint arrests. Taken together, these data suggest that combination of honokiol and IR can affect the various stages of cell cycle progression of the colorectal cancer cells, with the ultimate effect being induction of apoptosis during mitosis leading to mitotic catastrophe.

Previous studies have shown that introduction of a single copy of chromosome 3 into HCT116 cells resulted in a correction of mismatch repair deficiency and microsatellite instability (21). In fact, one copy of the normal hMLH1 gene is sufficient to restore mismatch repair activity and microsatellite stability (21). Moreover, the transfer of a single intact chromosome ensures that the encoded genes are likely to be expressed at normal levels in the recipient cells (21). These cell lines have been useful for investigating drug sensitivity. Mismatch repair also modifies the cytotoxicity of various anticancer agents (32). In fact, the cells have been used previously to compare drug sensitivities between repair-deficient and repair-proficient isogenic cell lines. HCT116-CH3 cells are resistant to relatively high concentrations of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (16, 21). Furthermore, HCT116 cells have been shown to be more sensitive than HCT116-CH3 cells to anticancer agents such as 6-thioguanine, cisplatin, doxorubicin, 5-fluorouracil, etoposide, camptothecin, tegafur, streptozotocin, bleomycin, mitomycin C, vinblastine, vincristine, nimustine, Ara-C, chlorambucil, and epirubicin (1, 5, 11, 21, 32). These results indicated that the mismatch repair system is a critical determinant of the response to cytotoxic drugs. Although the potent radiosensitizing ability of chemotherapeutic agents has been well documented both in vitro and in vivo, their effects in isogenic cell lines with mismatch repair defects have not been documented.

In the present article, we have compared cells that differ in their ability to repair DNA mismatches and demonstrated that honokiol can induce greater radiosensitization of the mismatch repair-deficient HCT116 cells compared with the mismatch repair-proficient HCT116-CH3 cells. These fit well with the recent clinical report demonstrating that 5-fluorouracil-based adjuvant chemotherapy is beneficial for colorectal cancers with the mismatch repair-deficient phenotype but not for those with the mismatch repair-proficient phenotype (28). These data further support the notion that results obtained in vitro with the isogenic HCT116 cell lines (CH2 and CH3) can be consistent with therapeutic efficacy in vivo in human clinical trials. In conclusion, these data strongly suggest that combination of honokiol with IR has a promising potential for the therapeutic interventions of mismatch repair-deficient colorectal cancer.

Our studies show that the combination of honokiol with IR has more potent effect in inhibiting the growth of mismatch repair-deficient colorectal cancer cells compared with isogenic cells that are mismatch repair proficient. The combination not only suppressed cell proliferation but also induced a long-term effect by inhibiting the formation of colonies. Moreover, we

have determined that the cells are arrested in the G₂/M phase of the cell cycle and the cells are undergoing apoptosis. Given that we observed increased levels of phosphorylated p53, further studies are warranted to determine whether the cells are undergoing mitotic catastrophe. p53 is a critical player in G₂/M transition and subsequent apoptosis, especially in response to genotoxic injury. Another interesting question relates to the role of p53 and apoptosis and the status of mismatch repair of the cells. Our studies demonstrated that, although both HCT116 and HCT116-CH3 cells undergo apoptosis, there is significantly less apoptosis in the mismatch-proficient HCT116-CH3 cells. However, although p53 is phosphorylated in response to honokiol and IR in the HCT116 cells, it is not phosphorylated in HCT116-CH3 cells. These data suggest that enhanced cell death observed in response to honokiol and IR in the mismatch repair-defective cells is due to increased p53 phosphorylation. Further studies are required, however, to confirm this is the case in cells that are mismatch repair deficient and also lack p53. Nevertheless, it is intriguing to think that the combination of honokiol and IR can induce cell death by different mechanisms namely a p53-independent pathway in mismatch-deficient cells while a p53 independent mechanism in mismatch-proficient cells.

In conclusion, the present studies provide evidence that the combination of honokiol and IR treatment results in a dose-dependent inhibition of the growth of colon cancer cells, with a more potent effect observed in cells with a defect in mismatch repair deficiency. Furthermore, the combination treatment was more potent in inducing apoptosis in the cells with mismatch repair defect. There was also reduced expression of cyclin A1 and D1 and increased phosphorylation of p53 in the mismatch defect cells. These data demonstrate that honokiol is highly effective in radiosensitizing colorectal cancer cells, especially those with a mismatch repair defect. Future studies should focus on further characterizing the mechanism and determining the contribution of the various signaling pathways in the process. Furthermore, studies using various spontaneous and xenograft models will be needed to confirm the results in the in vivo setting.

ACKNOWLEDGMENTS

Technical assistance provided by Sivapriya Ponnuram for generating some of the data is acknowledged. We also thank Lauren Larsen for administrative support during the course of writing this manuscript. Reprint requests and other correspondence can also be addressed to Y. Zhang (zhangchmd@yahoo.com.cn).

GRANTS

This study was supported by National Institutes of Health Grants DK062265, CA109269, CA135559 to S. Anant and Thomas O'Sullivan Foundation grant to D. Subramaniam. S. Anant is an Eminent Scientist of the Kansas Biosciences Authority.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

1. Aebi S, Fink D, Gordon R, Kim HK, Zheng H, Fink JL, Howell SB. Resistance to cytotoxic drugs in DNA mismatch repair-deficient cells. *Clin Cancer Res* 3: 1763–1767, 1997.
2. Alao JP. The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. *Mol Cancer* 6: 24, 2007.

3. Ashworth T, Roy AL. Phase specific functions of the transcription factor TFII-I during cell cycle. *Cell Cycle* 8: 596–605, 2009.
4. Bello L, Carrabba G, Giussani C, Lucini V, Cerutti F, Scaglione F, Landre J, Pluderi M, Tomei G, Villani R, Carroll RS, Black PM, Bikfalvi A. Low-dose chemotherapy combined with an antiangiogenic drug reduces human glioma growth in vivo. *Cancer Res* 61: 7501–7506, 2001.
5. Brattain MG, Fine WD, Khaled FM, Thompson J, Brattain DE. Heterogeneity of malignant cells from a human colonic carcinoma. *Cancer Res* 41: 1751–1756, 1981.
6. Burdelya LG, Komarova EA, Hill JE, Browder T, Tararova ND, Mavrakis L, DiCorleto PE, Folkman J, Gudkov AV. Inhibition of p53 response in tumor stroma improves efficacy of anticancer treatment by increasing antiangiogenic effects of chemotherapy and radiotherapy in mice. *Cancer Res* 66: 9356–9361, 2006.
7. Chen F, Wang T, Wu YF, Gu Y, Xu XL, Zheng S, Hu X. Honokiol: a potent chemotherapy candidate for human colorectal carcinoma. *World J Gastroenterol* 10: 3459–3463, 2004.
8. Cho SG, Choi EJ. Apoptotic signaling pathways: caspases and stress-activated protein kinases. *J Biochem Mol Biol* 35: 24–27, 2002.
9. Christophorou MA, Ringshausen I, Finch AJ, Swigart LB, Evan GI. The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. *Nature* 443: 214–217, 2006.
10. Davis TW, Wilson-Van Patten C, Meyers M, Kunugi KA, Cuthill S, Reznikoff C, Garces C, Boland CR, Kinsella TJ, Fishel R, Boothman DA. Defective expression of the DNA mismatch repair protein, MLH1, alters G2-M cell cycle checkpoint arrest following ionizing radiation. *Cancer Res* 58: 767–778, 1998.
11. Fink D, Nebel S, Norris PS, Aebi S, Kim HK, Haas M, Howell SB. The effect of different chemotherapeutic agents on the enrichment of DNA mismatch repair-deficient tumour cells. *Br J Cancer* 77: 703–708, 1998.
12. Flanagan SA, Krokosky CM, Mannava S, Nikiforov MA, Shewach DS. MLH1 deficiency enhances radiosensitization with 5-fluorodeoxyuridine by increasing DNA mismatches. *Mol Pharmacol* 74: 863–871, 2008.
13. Fried LE, Arbiser JL. Honokiol, a multifunctional antiangiogenic and antitumor agent. *Antioxid Redox Signal* 11: 1139–1148, 2009.
14. Fujita M, Itokawa H, Sashida Y. [Studies on the components of Magnolia obovata Thunb. 3. Occurrence of magnolol and honokiol in M obovata and other allied plants]. *Yakugaku Zasshi* 93: 429–434, 1973.
15. Gong C, Wei X, Wang X, Wang Y, Guo G, Mao Y, Luo F, Qian Z. Biodegradable self-assembled PEG-PCL-PEG micelles for hydrophobic honokiol delivery: I. Preparation and characterization. *Nanotechnology* 21: 215103, 2010.
16. Hawn MT, Umar A, Carethers JM, Marra G, Kunkel TA, Boland CR, Koi M. Evidence for a connection between the mismatch repair system and the G2 cell cycle checkpoint. *Cancer Res* 55: 3721–3725, 1995.
17. Hoffe SE, Shridhar R, Biagioli MC. Radiation therapy for rectal cancer: current status and future directions. *Cancer Control* 17: 25–34, 2010.
18. Hu J, Chen LJ, Liu L, Chen X, Chen PL, Yang G, Hou WL, Tang MH, Zhang F, Wang XH, Zhao X, Wei YQ. Liposomal honokiol, a potent anti-angiogenesis agent, in combination with radiotherapy produces a synergistic antitumor efficacy without increasing toxicity. *Exp Mol Med* 40: 617–628, 2008.
19. Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 363: 558–561, 1993.
20. Jover R, Paya A, Alenda C, Poveda MJ, Peiro G, Aranda FI, Perez-Mateo M. Defective mismatch-repair colorectal cancer: clinicopathologic characteristics and usefulness of immunohistochemical analysis for diagnosis. *Am J Clin Pathol* 122: 389–394, 2004.
21. Koi M, Umar A, Chauhan DP, Cherian SP, Carethers JM, Kunkel TA, Boland CR. Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N-methyl-N'-nitro-N-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. *Cancer Res* 54: 4308–4312, 1994.
22. Krecicki T, Smigiel R, Fraczek M, Kowalczyk M, Sasiadek MM. Studies of the cell cycle regulatory proteins P16, cyclin D1 and retinoblastoma protein in laryngeal carcinoma tissue. *J Laryngol Otol* 118: 676–680, 2004.
23. Landegren U. Measurement of cell numbers by means of the endogenous enzyme hexosaminidase. Applications to detection of lymphokines and cell surface antigens. *J Immunol Methods* 67: 379–388, 1984.
24. Lee SY, Yuk DY, Song HS, Yoon do Y, Jung JK, Moon DC, Lee BS, Hong JT. Growth inhibitory effects of obovatol through induction of apoptotic cell death in prostate and colon cancer by blocking of NF-kappaB. *Eur J Pharmacol* 582: 17–25, 2008.
25. Lee YJ, Lee YM, Lee CK, Jung JK, Han SB, Hong JT. Therapeutic applications of compounds in the Magnolia family. *Pharmacol Ther* 130: 157–176, 2011.
26. Patel BB, Majumdar AP. Synergistic role of curcumin with current therapeutics in colorectal cancer: minireview. *Nutr Cancer* 61: 842–846, 2009.
27. Poynter JN, Siegmund KD, Weisenberger DJ, Long TI, Thibodeau SN, Lindor N, Young J, Jenkins MA, Hopper JL, Baron JA, Buchanan D, Casey G, Levine AJ, Le Marchand L, Gallinger S, Bapat B, Potter JD, Newcomb PA, Haile RW, Laird PW. Molecular characterization of MSI-H colorectal cancer by MLH1 promoter methylation, immunohistochemistry, and mismatch repair germline mutation screening. *Cancer Epidemiol Biomarkers Prev* 17: 3208–3215, 2008.
28. Ribic CM, Sargent DJ, Moore MJ, Thibodeau SN, French AJ, Goldberg RM, Hamilton SR, Laurent-Puig P, Gryfe R, Shepherd LE, Tu D, Redston M, Gallinger S. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. *N Engl J Med* 349: 247–257, 2003.
29. Shehzad A, Khan S, Shehzad O, Lee YS. Curcumin therapeutic promises and bioavailability in colorectal cancer. *Drugs Today (Barc)* 46: 523–532, 2010.
30. Shehzad A, Wahid F, Lee YS. Curcumin in cancer chemoprevention: molecular targets, pharmacokinetics, bioavailability, and clinical trials. *Arch Pharm (Weinheim)* 343: 489–499, 2010.
31. Sinicrope FA. DNA mismatch repair and adjuvant chemotherapy in sporadic colon cancer. *Nat Rev Clin Oncol* 7: 174–177, 2010.
32. Takahashi T, Min Z, Uchida I, Arita M, Watanabe Y, Koi M, Hemmi H. Hypersensitivity in DNA mismatch repair-deficient colon carcinoma cells to DNA polymerase reaction inhibitors. *Cancer Lett* 220: 85–93, 2005.
33. Teng CM, Chen CC, Ko FN, Lee LG, Huang TF, Chen YP, Hsu HY. Two antiplatelet agents from Magnolia officinalis. *Thromb Res* 50: 757–765, 1988.
34. Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. *Science* 260: 816–819, 1993.
35. Toft NJ, Arends MJ. DNA mismatch repair and colorectal cancer. *J Pathol* 185: 123–129, 1998.
36. Umar A, Koi M, Risinger JI, Glaab WE, Tindall KR, Kolodner RD, Boland CR, Barrett JC, Kunkel TA. Correction of hypermutability, N-methyl-N'-nitro-N-nitrosoguanidine resistance, and defective DNA mismatch repair by introducing chromosome 2 into human tumor cells with mutations in MSH2 and MSH6. *Cancer Res* 57: 3949–3955, 1997.
37. Wahlstrom B, Blennow G. A study on the fate of curcumin in the rat. *Acta Pharmacol Toxicol (Copenh)* 43: 86–92, 1978.
38. Ward JF. The yield of DNA double-strand breaks produced intracellularly by ionizing radiation: a review. *Int J Radiat Biol* 57: 1141–1150, 1990.
39. Wheeler JM, Bodmer WF, Mortensen NJ. DNA mismatch repair genes and colorectal cancer. *Gut* 47: 148–153, 2000.
40. Wheeler JM, Loukola A, Aaltonen LA, Mortensen NJ, Bodmer WF. The role of hypermethylation of the hMLH1 promoter region in HNPCC versus MSI+ sporadic colorectal cancers. *J Med Genet* 37: 588–592, 2000.
41. Wilkinson N, Scott-Conner CE. Surgical therapy for colorectal adenocarcinoma. *Gastroenterol Clin North Am* 37: 253–267, ix, 2008.