Suppression of the NF-κB signaling pathway in colon cancer cells by the natural compound Riccardin D from Dumortierahirsute

HUIPING LIU1, GUONING LI1, BIN ZHANG2, DEQING SUN2, JING WU2, FANG CHEN2, FENG KONG3, YUN LUAN3, WEN JIANG3, RONGMEI WANG2 and XIA XUE2

1Shandong Luye Pharmaceutical Co., Ltd., Yantai, Shandong 264000; 2Department of Pharmacy, The Second Hospital of Shandong University; 3Central Laboratory, The Second Hospital of Shandong University, Jinan, Shandong 250033, P.R. China

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Abstract. Colorectal cancer (CRC) is a major cause of mortality and morbidity. Chronic inflammation is closely associated with the development, progression and prognosis of the majority of intestinal malignancies. In recent years, targeting the nuclear factor (NF)-κB signaling pathway for CRC therapy has become an attractive strategy. Riccardin D, a novel macrocyclicbis (bibenzyl) compound, was isolated from the Chinese liverwort plant. Previous studies have suggested that Riccardin D exerted chemo-preventative effects against the intestinal malignancy formation. In the present study, cell counting kit-8, Hoechst 33258 staining, mitochondria membrane permeability assay, western blotting analysis, reverse transcription-polymerase chain reaction, luciferase reporter gene assay and molecular modeling analysis were performed to detect the effect and mechanisms of Riccardin D on human colon cancer cells. The results demonstrated that Riccardin D significantly inhibited the growth of HT-29 cells. In addition, the cDNA expression of cyclooxygenase-2, and the protein expression and activity of NF-κB and tumor necrosis factor-α were downregulated; however, the protein expression of cleaved caspase-3 and -9, and cleaved poly (adenosine diphosphate-ribose) polymerase, and the B-cell lymphoma (Bcl)-2: Bcl-2-associated X protein ratio were upregulated. Furthermore, Auto Dock analysis identified binding sites between Riccardin D and NF-κB. These results indicated that Riccardin D may inhibit cell proliferation and induce apoptosis in HT-29 cells, which may be associated with the blocking of the NF-κB signaling pathway. Thus, Riccardin D should be investigated as an NF-κB inhibitor in cancer therapy.

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer mortalities worldwide, and is the third most common cancer type and the fifth leading cause of mortality in China (1-3). The majority of CRCs develop sporadically (70-80%), and the somatic loss of tumor suppressor gene, adenomatous polyposis coli (APC), was revealed to be present in 85% of patients with CRC and familial adenomatous polyposis (4-6). It has previously been demonstrated to have significant pro-apoptosis and anti-proliferative effects in several different types of cancers in vivo and in vitro (1-3). Recently, Riccardin D, a novel macrocyclicbis (bibenzyl) compound isolated from the Chinese liverwort plant Dumortiera hirsuta, has been observed to prevent the growth of intestinal polyps in adenomatous polyposis coli (APC)Min−/− mice, an animal model of human familial adenomatous polyposis, which spontaneously develops numerous polyps in the intestinal tract due to a mutation in the APC gene (6). This indicates that Riccardin D may serve an important role in the inhibition of CRC.

APC serves a critical role in regulating gene transcription through the Wnt signaling pathway (7). The APC gene mutation is the most common cause of colon cancer associated with the Wnt/β-catenin signaling pathway, which serves a critical role in the development of colon cancer (6-8). By contrast, the nuclear factor (NF)-κB-cyclooxygenase (COX)-2 signaling pathway has also been shown to affect the APC gene mutation in the human intestine and colon cells (9,10). In the APCMin−/− mouse, a higher expression of inflammation-associated cytokines has been reported (11,12). These results have suggested that there may be a connection between inflammation and tumor development. Our previous report also demonstrated that Riccardin D downregulated inflammatory factors, particularly those associated with the NF-κB signaling pathway, in the mouse model (6). NF-κB is hypothesized to promote tumorigenesis via pro-inflammatory transcription factors, which can be activated by bacterial products, such as lipopolysaccharides (LPS), and inflammatory cytokines. The transcriptional targets of NF-κB include genes encoding COX-2, intercellular adhesion molecule-1, vascular endothelial growth factor, and the inflammatory cytokines, interleukin (IL)-1β and tumor necrosis factor α (TNFα) (13,14).

Correspondence to: Dr Xia Xue, Department of Pharmacy, The Second Hospital of Shandong University, 247 Beiyuan Street, Jinan, Shandong 250033, P.R. China
E-mail: xue_1209@hotmail.com

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Previous reports have indicated that regular use of nonsteroidal anti-inflammatory drugs lowers the mortality associated with sporadic colon cancer and results in the regression of adenomas in patients with familial adenomatous polyposis, who have inherited a mutation in the APC gene (15,16). Pro-inflammatory cytokines, including TNFα, IL-6 and IL-1β, or transcription factors that are required by these cytokines for signaling, such as NF-κB and signal transducer and activator of transcription, have been identified as potential targets for anticancer therapy (17). In the present study, the mechanism underlying the anti-inflammation effects of Riccardin D in vitro were investigated. The results indicated that Riccardin D may exert its modulatory effects by blocking NF-κB activity in colon cancer cells. To the best of our knowledge, these results show, for the first time, the evaluation of macrocyclic bis(bibenzyls) against CRC associated with the inflammation pathway, suggesting its potential in the therapeutic intervention of intestinal cancers as a novel NF-κB inhibitor.

Materials and methods

Drugs. Riccardin D, a novel macrocyclic bis(bibenzyl) compound, was extracted from the Chinese liverwort plant Dumortiera hirsuta (previously collected from the Guizhou region, China), and its structure was identified as reported previously (18). The purity of Riccardin D, as measured by high performance liquid chromatography (18), was 98.6%. The compound was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 20 mM as stock solution for the in vitro study (18).

Cell lines and cell culture. The human colon cancer cell line HT-29 with a mutant APC gene, expressed as two C-terminal-truncated APC proteins of 100 and 200 kDa, was purchased from American Type Cell Culture Collection (Manassas, VA, USA) (12). The HCT-8 cell line expressing normal APC proteins was also obtained from American Type Cell Culture Collection. Cancer cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin (100 IU/ml), streptomycin (100 µg/ml) and 10 mM HEPES buffer at 37°C in a humid atmosphere (5% CO₂, 95% air).

Cell Counting kit (CCK)-8 assay. HCT-8 and HT-29 cells were seeded in 6-well plates (5x10⁴ cells/well) and incubated with increasing concentrations (2.5, 5, 10, 20, 40 and 60 µM) of Riccardin D for 24, 48 and 72 h at 37°C, respectively. The control cells were treated with an equal volume of the drug's vehicle DMSO. The cell viability was then determined using a CCK-8 kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan).

Hoechst 33258 staining. HT-29 cells were seeded in 6-well plates (3x10⁵ cells/well) and treated with 0, 5, 10 and 20 µM of Riccardin D for 24 h at 37°C; whereas control cells were treated with DMSO only. Cells were then fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min, stained with Hoechst 33258 (10 mg/l) for 1 h at 37°C, and then subjected to fluorescence microscopy (Nikon TE2000; Nikon Corporation, Tokyo, Japan). These data were obtained by eye via counting the number of apoptotic cells in five different fields of view for each group.

Mitochondrial membrane permeability assay. The mitochondrial membrane potential (MMP) was investigated using JC-1 dye (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. The ratio of green to red fluorescence provides an estimate of the changes in MMP. Briefly, HT-29 cells seeded in 6-well plates (3x10⁵ cells/well) were exposed to 0, 5, 10 and 20 µM Riccardin D for 24 h at 37°C; whereas control cells were treated with DMSO only. Cells were then incubated with an equal volume of JC-1 staining solution (5 µg/ml) at 37°C for 20 min and rinsed twice with PBS. MMPs were monitored by determining the relative quantity of dual emissions from mitochondrial JC-1 monomers or aggregates using an Olympus fluorescent microscope under Argon-ion 488 nm laser excitation. Mitochondrial depolarization is indicated by an increase in the green/red fluorescence intensity ratio. Experiments were performed at least three times.

Western blotting analysis. Western blotting analysis was employed to evaluate the protein expression associated with tumor growth in human colon cancer cells. HT-29 cells (3x10⁵ cells/well) cultured in 6-well plates were incubated with 20 µM of Riccardin D for 48 h at 37°C. The cells were collected and washed thrice using PBS (Beyotime Institute of Biotechnology) to produce lysates using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology), and concentrations of proteins were determined using a bicinchoninic acid assay kit purchased from Beyotime Institute of Biotechnology. Protein samples (30 µg/lane) were separated by 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes. Non-specific binding was blocked via incubation with 5% non-fat milk for 2 h at room temperature, and membranes were then incubated for 1 h at room temperature using the following primary antibodies: Anti-NF-κB (1:800; cat. no. sc-8008; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-phospho (p)-NF-κB Ser²⁷⁶ (1:800; cat. no. sc-33020; Santa Cruz Biotechnology, Inc.), anti-caspase-3 (1:1,000; cat. no. 9662; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-caspase-9 (1:1,000; cat. no. 9502; Cell Signaling Technology, Inc.), anti-cleaved poly(adenosine diphosphate-ribose) polymerase (PARP; PARP; 1:1,000; cat. no. 9541; Cell Signaling Technology, Inc.), anti-B-cell lymphoma (Bcl) -2 (1:1,000; cat. no. 2772; Cell Signaling Technology, Inc.), anti-B-cell lymphoma (Bcl) -2-associated X protein (1:1,000; Bax; cat. no. 2772; Cell Signaling Technology, Inc.), TNFα (1:1,000; cat. no. 6962; Cell Signaling Technology, Inc.) and anti-β-actin (1:5,000; cat. no. ab6276; Abcam, Cambridge, MA, USA). The PVDF membranes were then washed with TBS containing 0.05% Tween-20 prior to incubation with horseradish peroxidase-conjugated secondary antibodies (1:1,000; cat. nos. ZDR-5306 and ZDR-5307; OriGene Technologies, Inc., Beijing, China) at room temperature for 1 h. The bound antibodies were detected using an enhanced chemiluminescence reagent (EMD Millipore, Billerica, MA, USA). Densitometry analysis was performed using an electrophoresis...
image analysis system (cat. no. FR980; Shanghai FuriScience & Technology Co., Ltd., Shanghai, China). Experiments were performed at least three times.

Reverse transcription-semi-quantitative polymerase chain reaction (RT-sqPCR). A RT-sqPCR assay was performed to analyze the expression of COX-2 in colon cancer cells. HT-29 cells (3x10³ cells/well) cultured in 6-well plates were administrated 0, 10 and 20 µM Riccardin D for 24 h at 37°C. Total RNA was extracted using an RNAeasy kit (Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. cDNA was synthesized from RNA using the First Strand cDNA Synthesis kit (Toyobo Life Science, Osaka, Japan). The following primers (Gene Core Biotech Co., Ltd., Shanghai, China) were used for amplification: COX-2 forward, 5'-TTCAAATGAGATTGTGGGAAAAATTGC-3' and reverse, 5'-AGATCATCCTCTGCCTGAGTGATCTT-3'; and β-actin forward, 5'-GGGTCAAGAGGATCTCTATG-3' and reverse, 5'-GCTCTCAAACATGATCTGCG-3'. The reverse transcription reaction was performed at 37°C for 15 min and 98°C for 5 min; and PCR was performed for 40 cycles at 92°C for 30 sec, 56°C for 30 sec and 68°C for 1 min. PCR products were run on 2% agarose gels containing 0.51 g of ethidium bromide and then photographed using an ultraviolet transilluminator (cat. no. FR980; Shanghai FuriScience & Technology Co., Ltd.). β-actin was used as the internal control and experiments were performed at least three times. Alpha Ease FC software was used to analyze relative light intensities (version 4.0.0.34; Protein Simple, San Jose, CA, USA).

Luciferase reporter gene assay. The effect of Riccardin D on NF-κB-dependent reporter gene transcription induced by LPS was analyzed by NF-κB-luciferase assay using a Firefly Luciferase Reporter Gene Assay kit (Beyotime Institute of Biotechnology). Briefly, HT-29 cells (3x10³ cells/well) were plated in 6-well plates and transiently transfected using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) method with the pNF-κB-luc plasmid reporter gene (1 µg; Beyotime Institute of Biotechnology) containing four NF-κB binding motifs (5'-GGGAAATTC-3') and β-galactosidase (90 ng; BioVectorNTCCInc.,Beijing,China).Following 24 h post-transfection, cells were treated with Riccardin D and 10 µg/ml LPS for a further 20 h. Cells were then harvested in order to measure β-galactosidase and luciferase activity. Relative luciferase activity was normalized to the value of β-galactosidase to correct the transfection efficacy (β-galactosidase Assay kit; Beyotime Institute of Biotechnology). Triplicate experiments with triplicate samples were performed.

Molecular modeling analysis. Auto Dock Vina (version 4.0; Molecular Graphics Laboratory, The Scripps Research Institute, La Jolla, CA, USA) has previously been demonstrated to locate docking modes that are consistent with X-ray crystal structures (19). Auto Dock simulates interactions between substrates or drug candidates as ligands, and their macromolecular receptors with known three dimensional structures, allowing ligand flexibility described to a full extent. In order to investigate the binding mode of Riccardin D, a molecular model of Riccardin D docked into NF-κB was produced using SYBYL-X Suite by Certara USA, Inc. (Princeton, NJ, USA). The X-ray crystal structure of NF-κB was taken from the Protein Data Bank (1K3Z) and was used for docking studies. Computer software was used in the present study to mimic the structure of Riccardin D and NF-κB, in order to identify the main binding sites between Riccardin D and NF-κB through docking simulation. The default parameters as described in the Sybyl manual were used.

Statistical analysis. Statistical analysis was performed using PASW Statistics Windows 18 download (version 15.0; SPSS Inc.,Chicago, IL, USA). Data were presented as the mean ± standard deviation and were analyzed by Student's t-test or one-way analysis of variance followed by a Student-Newman-Keuls post hoc test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference. Triplicate experiments were performed using triplicate samples.

Results

Inhibition of colon cancer cell growth by Riccardin D. Human colon cancer HT-29 and HCT-8 cells were exposed to Riccardin D for 24, 48 and 72 h, and were then subjected to a proliferation assay. The inhibition rate (%) was calculated as (OD₅₇₀ value of control group - OD₅₇₀ value of drug treated group)/OD₅₇₀ value of control group x100. As shown in Fig. 1, Riccardin D, in a concentration range of 2.5-40 µM, inhibited HT-29 and HCT-8 cell growth in a dose-dependent manner. Notably, the anti-proliferation effects of Riccardin D on human colon cancer cells was more significant in HT-29 cells with the APC mutation (HT-29 vs. HCT-8: at 2.5 µM for 24 h, and at 60 µM for 48 and 72 h; P<0.05). These experiments also indirectly revealed the chemopreventative effects of Riccardin D on intestinal adenoma formation in APCMac™ mice; thus, the subsequent experiments were carried out using HT-29 cells only.

Riccardin D induces HT-29 cell apoptosis. The induction of apoptosis was detected to evaluate the inhibitory effect of Riccardin D via a number of different assays. As indicated by Hocheast 33258 analysis in Fig. 2A, the number of apoptotic cells increased following 24 h incubation with 20 µM of Riccardin D. Treatment with Riccardin D, also revealed morphological changes using Hocheast 33258 staining, including decreased cell size and nuclear chromatin condensation.

The JC-1 fluorescence probe demonstrated that the MMP in HT-29 cells was markedly reduced following treatment with Riccardin D. As shown in Fig. 2B, the red fluorescence of JC-1 gradually decreased and the green fluorescence was correspondingly increased following Riccardin D administration. In the range of 5-20 µM, the ratio of green to red fluorescence increased in a dose-dependent manner. The results revealed that treatment with Riccardin D in HT-29 cells reduced the MMP.

Further studies of the apoptotic proteins in HT-29 cells were also undertaken. Riccardin D treatment activated the caspase cascade pathway, in agreement with in vivo data from our previous research in the APCMac™ mouse (6). As shown in Fig. 2C, the levels of cleaved caspase-3, caspase-9 and PARP were markedly increased in Riccardin D treated cells. Riccardin D also increased the Bax:Bel-2 ratio, as the protein level of Bax was markedly increased, which was accompanied
by a notable decrease in Bcl-2 in HT-29 cells treated with 20 µM Riccardin D.

**Suppression of inflammation via a decrease in COX-2 and NF-κB expression.** The results revealed that Riccardin D decreased the protein expression of NF-κB. Fig. 3A indicated that the level of total NF-κB p65 protein in HT-29 cells was markedly decreased by 53.4% (P<0.01) when compared with control. Further analysis of p-NF-κB Ser536 demonstrated that the active form of NF-κB, was markedly decreased by 47.2% (P<0.01; Fig. 3A) compared with the control.

The inhibitory effect of Riccardin D on COX-2 cDNA expression was also shown in HT-29 cells by RT-PCR analysis. As shown in Fig. 3B, following 24 h exposure with 10 and 20 µM Riccardin D, the levels of COX-2 mRNA were decreased by 35.0 and 66.7%, respectively (Fig. 3B). These results suggested that Riccardin D treatment reduced COX-2 expression at the DNA level.

To further substantiate the finding that Riccardin D may target NF-κB, the present study also investigated the effects of Riccardin D on NF-κB-mediated transcriptional activity using HT-29 cells, wherein 20 µM of Riccardin D reduced luciferase activity by 45.2% (P<0.01) compared with control cells, which were treated with LPS and 0 µM Riccardin D (Fig. 3C). These results provided additional insight into the suppression of intestinal tumorigenesis by Riccardin D.

In addition, the theoretical binding mode of Riccardin D to NF-κB-p65 is presented in Fig. 4. Two binding sites were identified between Riccardin D and NF-κB-p65 protein.

**Discussion**

Patients with chronic inflammatory bowel disease are more susceptible to developing CRC, and a number of studies have indicated that chronic inflammation may affect intestinal tumorigenesis (20-22). It is also believed that aberrant Wnt/β-catenin signaling following the loss of APC function may initiate colon adenoma formation (21,23). The APC gene mutation mouse model (APC<sup>Min/+</sup>) has been considered to be the standard experimental model for research into intestinal carcinogenesis as tumors grow spontaneously in the intestinal tract (4). Our previous study demonstrated that the administration of Riccardin D caused the inhibition of polyps in the intestine of APC<sup>Min/+</sup> mice through its anti-proliferative, apoptotic and anti-inflammatory effects (6). Therefore, the present study investigated the anti-cancer effects of Riccardin D using human colon cancer cell lines in vitro and aimed to further understand the underlying mechanisms involved in the NF-κB signaling pathway in HT-29 cells with the APC mutation. The results revealed that Riccardin D markedly inhibited the proliferation of the human colon cancer cell line HT-29 with a mutant carboxy-truncated APC gene.

The NF-κB transcription factor pathway is a crucial regulator of a number of normal cellular functions. Under physiological conditions, NF-κB may coordinate the transcription of cytokines including COX-2 (24), cyclin D1 (25) and numerous other factors (26). The constitutive activation of NF-κB contributes to multiple cellular outcomes and pathophysiological conditions, including rheumatoid arthritis, asthma, inflammatory bowel disease, acquired immunodeficiency syndrome and cancer, through interactions with multiple pathways including the cell cycle, apoptosis and proliferation (27,28). Aberrant NF-κB signaling has been identified in a number of different types of cancer; NF-κB regulates the transcription of target genes that promote cell survival and proliferation, inhibit apoptosis, and mediate invasion and metastasis (29). Loss of APC function can lead to the activation of β-catenin signaling, which is the first step in the oncogenic pathway leading to CRC development (30). It has been reported that TNF receptor superfamily member 19 (TNFRSF19) is a β-catenin target gene and TNFRSF19 receptor molecule-associated activation of NF-κB signaling

Figure 1. Inhibition of the human colon cancer cell lines HT-29 and HCT-8 induced by treatment with Riccardin D. HT-29 and HCT-8 cells were exposed to increasing concentrations of Riccardin D or an equal volume of the drug's vehicle (DMSO) for 24, 48 and 72 h. Viable cells were evaluated by a Cell Counting kit-8 assay and were recorded as a percentage of the untreated controls at the corresponding time point. The results are presented as the mean ± standard deviation of 3 repeated experiments. *P<0.05 HT-29 vs. HCT-8 cells.
has demonstrated that β-catenin may regulate NF-κB activity via TNFRSF19; activation of NF-κB activity has also been observed in the APC<sup>Min</sup> mice model, which was inhibited by Riccardin D as shown in our previous study (4). NF-κB inhibition is also considered to be an important therapeutic target in CRC (14,31). Thus, it may be hypothesized that the inhibition of NF-κB by Riccardin D maybe a pivotal mechanism of its effects in chemotherapy for CRC with the APC mutation. The present study then detected the expression and activity of NF-κB, revealing that Riccardin D markedly reduced NF-κB protein expression in HT-29 cells; the active form of NF-κB, p-NF-κBSer<sup>536</sup>, was also markedly suppressed. The NF-κB signaling pathway is a complex network that regulates cellular pathways involved in a myriad of physiological and pathological conditions. In addition, the accumulation of nuclear NF-κB increases the aberrant activation of the COX-2 gene (14). In the present study, Riccardin D inhibited the expression of NF-κB, COX-2 and TNFα, as well as the transcriptional activity of NF-κB induced by LPS. Riccardin D may also be directly associated with the transcriptional activity of NF-κB.
may be associated with the inhibition of NF-κB signaling and that Riccardin D may be a novel NF-κB inhibitor, inhibiting colon cancer cell proliferation and inducing cell apoptosis. The critical role of regulating the NF-κB signaling pathway in apoptosis, tumor promotion and tumor maintenance, suggests that Riccardin D maybe a promising candidate in cancer therapy. Further studies investigating the mechanism of Riccardin D and its therapeutic effects on clinical tumors are still required. However, based on these results, Riccardin D may be an effective NF-κB inhibitor in cancer therapy.

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Availability of data and materials

All of the materials used in the present study are commercially available and all data included in the present study were obtained by the co-authors.

Authors' contributions

HL, DS, BZ, FK and RW designed the study. HL, XX, JW and FC performed the experiments. HL, GL, XX, YL and WJ analyzed the data. HL, GL and XX wrote the manuscript. HL, DS, BZ, FK and RW designed the study. HL, GL, XX, YL and WJ analyzed the data. HL, GL and XX wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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