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Synthetic Homoisoflavane Derivatives of Cremastranone Suppress Growth of Colorectal Cancer Cells through Cell Cycle Arrest and Induction of Apoptosis

Ha-Eun Shin[1,†], Seul Lee[2,†], Yeram Choi[1], Sangkyu Park[3], Sangil Kwon[2], Jun-Kyu Choi[3], Seung-Yong Seo[2,*] and Younghee Lee[1,3,*]

[1]Department of Biochemistry, College of Natural Sciences, Chungbuk National University, Cheongju 28644,
[2]College of Pharmacy, Gachon University, Incheon 21936,
[3]Biotechnology Research Institute, Chungbuk National University, Cheongju 28644, Republic of Korea

Abstract

Colorectal cancer is diagnosed as the third most prevalent cancer; thus, effective therapeutic agents are urgently required. In this study, we synthesized six homoisoflavane derivatives of cremastranone and investigated their cytotoxic effects on the human colorectal cancer cell lines HCT116 and LoVo. We further examined the related mechanisms of action using two of the potent compounds, SH-19027 and SHA-035. They substantially reduced the cell viability and proliferation in a dose-dependent manner. Treatment with SH-19027 and SHA-035 induced cell cycle arrest at the G2/M phase and increased expression of p21 both of which are implicated in cell cycle control. In addition, the apoptotic cell population and apoptosis-associated marker expression were accordingly increased. These results suggest that the synthesized cremastranone derivatives have anticancer effects through the suppression of cell proliferation and induction of apoptosis. Therefore, the synthesized cremastranone derivatives could be applied as novel therapeutic agents against colorectal cancer.

Key Words: Anticancer effect, Homoisoflavane derivatives of cremastranone, Cell cycle, Apoptosis, Colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers worldwide (Mármol *et al*., 2017). With an estimated incidences and deaths of more than 1.9 million and 935,000 in 2020, respectively, CRC ranks as the third diagnosed cancer and the second cause of cancer death globally (Sung *et al*., 2021). CRC patients could undergo surgical dissection or chemotherapy for treatment (Van Cutsem *et al*., 2014; Xie *et al*., 2020). CRCs are mainly diagnosed at the advanced stage with metastases which is not suitable for curative resection; thus, adequate chemotherapy is needed for these patients (Van Cutsem *et al*., 2014). Fluoropyrimidines (intravenous 5-fluorouracil (5-FU) or oral capecitabine) are usually used as the first-line CRC chemotherapy (Van Cutsem *et al*., 2004). The 5-FU is used alone or combined with other cytotoxic agents such as leucovorin (LV), oxaliplatin, and irinotecan to increase the response rate (de Gramont *et al*., 2000; Douillard *et al*., 2000). The combined therapy regimens of 5-FU/LV/oxaliplatin (FOLFOX), 5-FU/LV/irinotecan (FOLFIRI) or capecitabine/LV/oxaliplatin (CAPOX) are used as the main approaches to CRC chemotherapy (Van Cutsem *et al*., 2014; Mármol *et al*., 2017; Xie *et al*., 2020). However, chemotherapy for CRCs still has many side effects, such as cytotoxicity on normal cells, and a limited efficacy including chemoresistance and recurrence (Xie *et al*., 2020). Fluoropyrimidines have been reported to show adverse effects in CRC patients, including suppression of bone marrow and gastrointestinal events (diarrhea, nausea, stomatitis, and vomiting) (Saif *et al*., 2008; Latchman *et al*., 2014). Considering the prevalence and lethality of CRC and the limitation of chemotherapy, continuous development of novel therapeutic agents is necessary.

Natural products from plants have been the major source of drugs over the past century (Mishra and Tiwari, 2011).

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***Corresponding Authors**
E-mail: yhl4177@cbnu.ac.kr (Lee Y), syseo@gachon.ac.kr (Seo SY)
Tel: +82-43-261-3387 (Lee Y), +82-32-820-4949 (Seo SY)
Fax: +82-43-267-2306 (Lee Y), +82-32-820-4829 (Seo SY)
[†]The first two authors contributed equally to this work.

The pharmacological activities of natural products and their chemical derivatives have been studied for the cancer therapy (Demain and Vaishnav, 2011; Mishra and Tiwari, 2011). Homoisoflavanones are natural compounds that contain one additional carbon atom on the isoflavanone (Lin *et al*., 2014). The homoisoflavanones are grouped according to their basic structure, 3-benzyl-4-chromanones, 3-benzylidene-4-chromanones, 3-benzyl-3-hydroxy-4-chromanones and scillascillins (du Toit *et al*., 2010). Their biological activities include anti-inflammatory, anti-bacterial, anti-oxidative, and anti-cancer effects, and so on (du Toit *et al*., 2010; Lin *et al*., 2014). Cremastranone (5,7-dihydroxy-3-(3-hydroxy-4-methoxybenzyl)-6-methoxychroman-4-one), a homoisoflavanone isolated from *Cremastra appendiculata*, is one of the 3-benzyl-4-chromanone types of homoisoflavanones, and it was reported that cremastranone has an anti-angiogenic and anti-proliferative activity in human umbilical vein endothelial cells (HUVEC) through cell cycle arrest and a decrease of the cdc2 expression (Shim *et al*., 2004; Kim *et al*., 2007, 2008). In addition, cremastranone inhibits neovascularization in oxygen-induced retinopathy (OIR) (Kim *et al*., 2007) and laser-photocoagulation-induced choroidal neovascularization (CNV) mouse models (Kim *et al*., 2008), representing features of ocular angiogenesis. The first synthetic cremastranone blocks cell proliferation and tube formation activity in endothelial cells (Lee *et al*., 2014). SH-11052, a synthetic derivative of cremastranone, inhibits angiogenic activity by blocking TNF-α and VEGF mediated pathways in human retinal endothelial cells (HREC) (Basavarajappa *et al*., 2014). Another homoisoflavanone, disporopsin, exhibits cytotoxicity to a wide range of cancer cell lines including HCT15, which is a CRC cell line (Nguyen *et al*., 2006).

Apoptosis, one of the types of programmed cell death, is a highly regulated mechanism that eliminates unnecessary or damaged cells in multicellular organisms. Apoptosis is induced by various physiological and pathological stresses including DNA damage, radiation and hypoxia (Kerr *et al*., 1972; Elmore, 2007). Unlike normal cells, cancer cells can evade apoptosis by modulating the expression of anti- or pro-apoptotic proteins for abnormal growth and survival (Fernald and Kurokawa, 2013). Thus, inducing apoptosis in cancer cells could be an effective strategy for cancer therapy, and many anti-cancer drugs target various phases of the intrinsic and extrinsic apoptotic pathway (Pfeffer and Singh, 2018).

Given the pharmacological activity of homoisoflavanones, it is expected that cremastranone derivatives have suppressive activity in cancer cells, and these compounds can be novel cancer therapeutic agents. In this study, we synthesized and evaluated six homoisoflavane derivatives of cremastranone as new anticancer agents and confirmed that four of them have potent anticancer effects in CRC cells. We also investigated the underlying mechanisms involved in their growth suppression using two of the compounds, SH-19027 and SHA-035.

MATERIALS AND METHODS

Synthetic compounds

Six homoisoflavane derivatives of cremastranone were synthesized according to the methods developed previously (Basavarajappa *et al*., 2015; Heo *et al*., 2019). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded using Bruker 600 MHz spectrometer as solution. Chemical shifts are expressed in parts per million (ppm, δ) downfield from tetramethylsilane and are referenced to the deuterated solvent ($CDCl_3$, ^{1}H δ 7.26, ^{13}C δ 77.16; DMSO-d_6, ^{1}H δ 2.50, ^{13}C δ 39.52; CD_2Cl_2, ^{1}H δ 5.32, ^{13}C δ 54.00). ^{1}H NMR data were reported in the order of chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet and/or multiplet resonances), coupling constant (*J* value) in hertz (Hz), and number of protons. High-resolution mass spectra (HRMS) were recorded on a JEOL (Peabody, MA, USA) JMS-700 MStation™ (EI or FAB mode) and an Agilent (Santa Clara, CA, USA) 6530 Q-TOF LC/MS/MS system (ESI mode). **SH-17059:** ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.78 (dd, *J*=5.1, 3.0 Hz, 2H), 6.66 (dd, *J*=8.2, 2.0 Hz, 1H), 6.19 (s, 1H), 5.60 (s, 1H), 4.09 (ddd, *J*=10.5, 2.9, 1.5 Hz, 1H), 3.88 (s, 3H), 3.85 (s, 3H), 3.79 (s, 6H), 3.72 (dd, *J*=10.5, 8.3 Hz, 1H), 2.76 (ddd, *J*=16.4, 5.4, 1.2 Hz, 1H), 2.61–2.53 (m, 2H), 2.32 (dd, *J*=16.5, 8.4 Hz, 1H), 2.23–2.16 (m, 1H). ^{13}C{^{1}H} NMR (150 MHz, $CDCl_3$) δ 152.3, 151.8, 150.8, 145.7, 145.2, 136.0, 132.9, 120.5, 115.3, 110.7, 107.4, 96.1, 69.8, 61.2, 60.7, 56.1, 56.0, 37.7, 33.7, 25.6. HRMS (EI+): calculated for $C_{20}H_{24}O_6$ [M^+] 360.1573, found 360.1574. **SH-19017:** ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.81 (d, *J*=2.1 Hz, 1H), 6.79 (d, *J*=8.2 Hz, 1H), 6.71 (dd, *J*=8.2, 2.1 Hz, 1H), 6.28 (s, 1H), 5.59 (s, 1H), 5.45 (s, 1H), 4.27 (dd, *J*=11.3, 4.2 Hz, 1H), 4.09 (dd, *J*=11.3, 7.7 Hz, 1H), 3.94 (s, 3H), 3.92 (s, 3H), 3.88 (s, 3H), 3.17 (dd, *J*=14.0, 4.4 Hz, 1H), 2.77–2.71 (m, 1H), 2.60 (dd, *J*=14.0, 10.7 Hz, 1H). ^{13}C{^{1}H} NMR (150 MHz, $CDCl_3$) δ 191.6, 157.3, 153.9, 146.3, 145.8, 145.4, 133.9, 131.8, 120.7, 115.4, 110.9, 108.1, 96.1, 69.3, 61.9, 56.4, 56.1, 48.4, 32.4. HRMS (EI+): calculated for $C_{19}H_{20}O_7$ [M^+] 360.1209, found 360.1206. **SH-19021:** ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.78 (dd, *J*=5.1, 3.0 Hz, 2H), 6.66 (dd, *J*=8.2, 2.1 Hz, 1H), 6.17 (s, 1H), 5.61 (s, 1H), 4.08 (ddd, *J*=10.6, 3.1, 1.6 Hz, 1H), 3.87 (d, *J*=1.6 Hz, 6H), 3.77 (s, 3H), 3.74–3.69 (m, 3H), 2.74 (ddd, *J*=16.5, 5.5, 1.6 Hz, 1H), 2.60–2.54 (m, 2H), 2.31 (dd, *J*=16.5, 8.5 Hz, 1H), 2.22–2.15 (m, 1H), 1.30–1.23 (m, 1H), 0.56 (dd, *J*=8.0, 1.4 Hz, 2H), 0.31–0.25 (m, 2H). ^{13}C{^{1}H} NMR (150 MHz, $CDCl_3$) δ 152.6, 152.0, 150.7, 145.6, 145.2, 135.0, 132.9, 120.5, 115.3, 110.7, 107.3, 96.0, 78.6, 69.8, 60.7, 56.1, 55.9, 37.7, 33.8, 25.6, 11.2, 3.2. HRMS (EI+): calculated for $C_{23}H_{28}O_6$ [M^+] 400.1886, found 400.1884. **SH-19026:** ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.85 (d, *J*=8.4 Hz, 1H), 6.72–6.68 (m, 2H), 6.19 (s, 1H), 4.12–4.08 (m, 1H), 3.88 (s, 3H), 3.85 (s, 3H), 3.80 (s, 3H), 3.80 (s, 3H), 3.74 (dd, *J*=10.8, 8.4 Hz ,1H), 2.76 (ddd, *J*=16.8, 5.4, 1.2 Hz, 1H), 2.64–2.56 (m, 2H), 2.34 (q, *J*=8.4 Hz, 1H), 2.23–2.16 (m, 1H); ^{13}C{^{1}H} NMR (150 MHz, $CDCl_3$) δ 152.3, 151.6, 150.7, 146.5, 144.0, 135.9, 131.3, 121.7, 114.3, 111.4, 107.2, 96.0, 69.7, 61.0, 60.6, 56.0, 55.9, 37.9, 33.8, 25.5. HRMS (EI+): calculated for $C_{20}H_{24}O_6$ [M_+] 360.1573, found 360.1571. **SH-19027:** ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.94–6.88 (m, 3H), 6.19 (s, 1H), 4.09–4.06 (m, 1H), 3.88 (s, 3H), 3.85 (s, 3H), 3.80 (s, 3H), 3.79 (s, 3H), 3.73 (dd, *J*=10.8, 8.4 Hz, 1H), 2.77–2.73 (m, 1H), 2.60 (d, *J*=9.0 Hz, 2H), 2.34 (q, *J*=8.4 Hz, 1H), 2.22–2.17 (m, 1H); ^{13}C{^{1}H} NMR (150 MHz, $CDCl_3$) δ 152.3, 151.6, 150.6, 146.1, 135.9, 132.6, 124.6, 116.7, 116.6, 113.5, 106.9, 95.9, 69.4, 61.0, 60.6, 56.4, 55.9, 37.1, 33.6, 25.4. HRMS (EI+): calculated for $C_{20}H_{23}FO_5$ [M^+] 362.1530, found 360.1528. **SHA-035:** ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.93 (dd, *J*=8.4, 2.4 Hz, 1H), 6.88 (d, *J*=2.4 Hz, 1H), 6.82 (d, *J*=8.4 Hz, 1H), 6.11(s, 1H), 5.30 (t, *J*=6.0 Hz, 1H), 4.15 (t, *J*=5.4 Hz, 2H), 3.99–4.03(m, 1H), 3.78 (s, 3H), 3.76 (s, 3H), 3.72 (s, 3H), 3.71 (s, 3H), 3.66 (dd, *J*=10.2, 7.8 Hz, 1H), 3.46 (dd, *J*=10.8, 5.4 Hz, 2H), 2.69 (ddd, *J*=16.2, 5.4, 1.2 Hz, 1H),

2.50–2.56 (m, 2H), 2.31 (q, *J*=7.8 Hz, 2H), 2.26 (q, *J*=8.4 Hz, 1H), 2.10–2.17 (m, 1H), 1.10 (t, *J*=7.8 Hz, 1H); ^{13}C {^{1}H} NMR (150 MHz, $CDCl_3$) δ 174.4, 154.3, 152.2, 151.6, 150.6, 150.0, 139.7, 135.9, 132.0, 126.9, 123.7, 112.4, 107.1, 96.0, 69.4, 63.2, 61.0, 60.6, 56.0, 55., 40.5, 37.1, 33.5, 27.4, 25.5, 9.1. HRMS (EI+): calculated for $C_{26}H_{33}NO_9$ [M^+]: 503.2155, found 503.2150.

Cell culture

HCT116 and LoVo (human colorectal carcinoma) cells were maintained in RPMI-1640 (Cytiva, Marlborough, MA, USA) with 10% fetal bovine serum (FBS, Cytiva) and 1X Antibiotic-Antimycotic (Biowest, Riverside, MO, USA) at 37°C in a 5% CO_2 humidified atmosphere.

Antibodies and chemicals

The monoclonal antibody anti-c-Myc (#5605) and the polyclonal antibodies anti-PARP (#9542), anti-cdc2 (#7705S) and anti-cleaved caspase 3 (#9661) were purchased from Cell Signaling Technology (Beverly, MA, USA). The polyclonal antibodies anti-cyclin D1 (sc-753) and anti-p21 (sc-397) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell viability assay

Cell viability was measured with the water-soluble tetrazolium salt (WST)-based EZ-Cytox assay kit (DoGen Bio, Seoul, Korea). HCT116 and LoVo cells (5×10^3 cells/well) were plated in 96-well culture plates and incubated overnight. The cells were treated with SH-17059, SH-19017, SH-19021, SH-19026, SH-19027, or SHA-035 at the indicated concentrations for the indicated time periods. After 10 μL of the EZ-Cytox assay reagent were added, the plates were incubated at 37°C for 2 h. The absorbance was measured at 450 nm using a microplate reader (BioTek, Winooski, VT, USA).

Cell proliferation assay

The cell proliferation rate was measured using the Cell Proliferation ELISA kit (Roche, Basel, Switzerland). HCT116 and LoVo cells (5×10^3 cells/well) were plated in 96-well culture plates and incubated overnight. After treatment with SH-19027 or SHA-035, the cells were labeled with the BrdU labeling solution for 2 h at 37°C. After fixation of the cells, anti-BrdU antibody conjugated with peroxidase was added into each well, and the cells were incubated for 90 min at room temperature. A colorimetric assay was conducted with a substrate solution, and the absorbance at 370 nm with a reference wavelength of 492 nm was measured using a microplate reader (BioTek).

Western blot analysis

HCT116 cells were harvested and lysed in lysis buffer (pH 8.0, 20 mM Tris-HCl, 10% glycerol, 137 mM NaCl, 10 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, phosphatase inhibitor, and protease inhibitor cocktail). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Cytiva). The membranes were blocked with 5% skim milk in phosphate-buffered saline-Tween-20 (PBS-T; 140 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 2 mM KH_2PO_4, and 0.05% Tween-20), incubated with primary antibodies and then horseradish peroxidase conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). Immunoreactive proteins were detected with enhanced chemiluminescence (ECL) solution (ATTO, Tokyo, Japan).

Cell cycle assay

HCT116 and LoVo cells (2×10^5 cells/well) were plated in 6-well plates and incubated overnight. The cells were treated with DMSO or the indicated concentration of SH-19027 or SHA-035 for 24 h. The cells were fixed and permeabilized with the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences, Franklin Lakes, NJ, USA) for 20 min at 4°C. After the cells were stained with the BD PI/RNase Staining Buffer, cell cycle analysis was performed with FACS Calibur (BD Biosciences). The data were analyzed using the FCS Express program (De Novo Software, Glendale, CA, USA).

Apoptosis assay

Apoptotic cells were detected with the Annexin-V-FLOUS Staining Kit (Roche) according to the manufacturer's instructions. After the HCT116 and LoVo cells (2×10^5 cells/well) were plated in 6-well plates and incubated overnight, the cells were treated with SH-19027 or SHA-035. The cells were harvested and stained with Annexin V-FITC and propidium iodide (PI), and the fluorescent signal was detected by FACSymphony A3 (BD Biosciences). The data were analyzed using the FCS Express program (De Novo Software).

Caspase activity assay

The Caspase Colorimetric Substrate Set II Plus kit (BioVision, Milpitas, CA, USA) was used to detect the caspase activity according to the manufacturer's instructions. Cell lysates were harvested from HCT116 cells treated with SH-19027 or SHA-035 and incubated with 200 μM of each caspase substrate at 37°C for 4 h. The absorbance values were measured at 405 nm with a microplate reader (BioTek).

Statistical analysis

The results are shown as the mean ± standard deviation (SD) from at least three independent experiments. Statistical significance of the differences between two sample groups was evaluated using Student's t-test. $p<0.05$ was considered statistically significant.

RESULTS

Design and synthesis of novel cremastranone derivatives

Cremastranone, an antiangiogenic natural homoisoflavonoid, has been isolated from the several plants such as *Cremastra appendiculata* (Shim *et al*., 2004) and synthesized for the first time by us (Lee *et al*., 2014). Encouraged by the potent anti-angiogenic and anti-proliferative activity against HUVEC and HREC, a variety of synthetic derivatives of cremastranone has been developed so far (Basavarajappa *et al*., 2014, 2015). Homoisoflavane derivative SH-17059 consists of 5,6,7-trimethoxy but not a C4-carbonyl group. SH-17059 exhibited a more potent inhibitory activity against HREC than homoisoflavanones like cremastranone (Heo *et al*., 2019; Schwikkard *et al*., 2019). Thus, to improve the biological activity and study the structure-activity relationship of SH-17059, some A-ring and B-ring-modified derivatives were designed and synthesized (Fig. 1). SH-19017 and SH-19021 are A-ring-modified homoisoflavane derivatives in which the C6-methoxy of SH-17059 was replaced by a hydroxy and

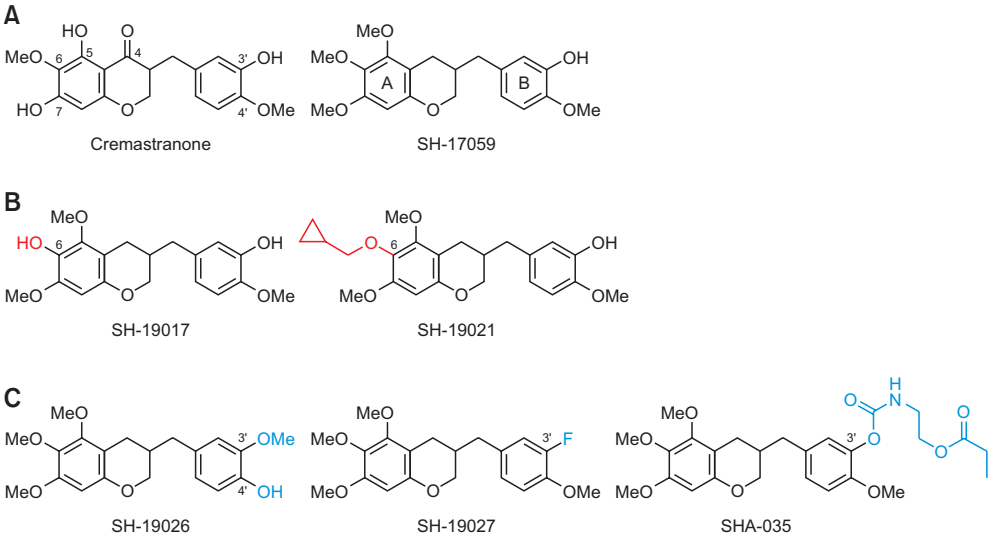

Fig. 1. Structure of cremastranone and the homoisoflavane derivatives. (A) Natural product cremastranone and synthetic homoisoflavane SH-17059, (B) A-ring modification of SH-17059, and (C) B-ring modification of SH-17059.

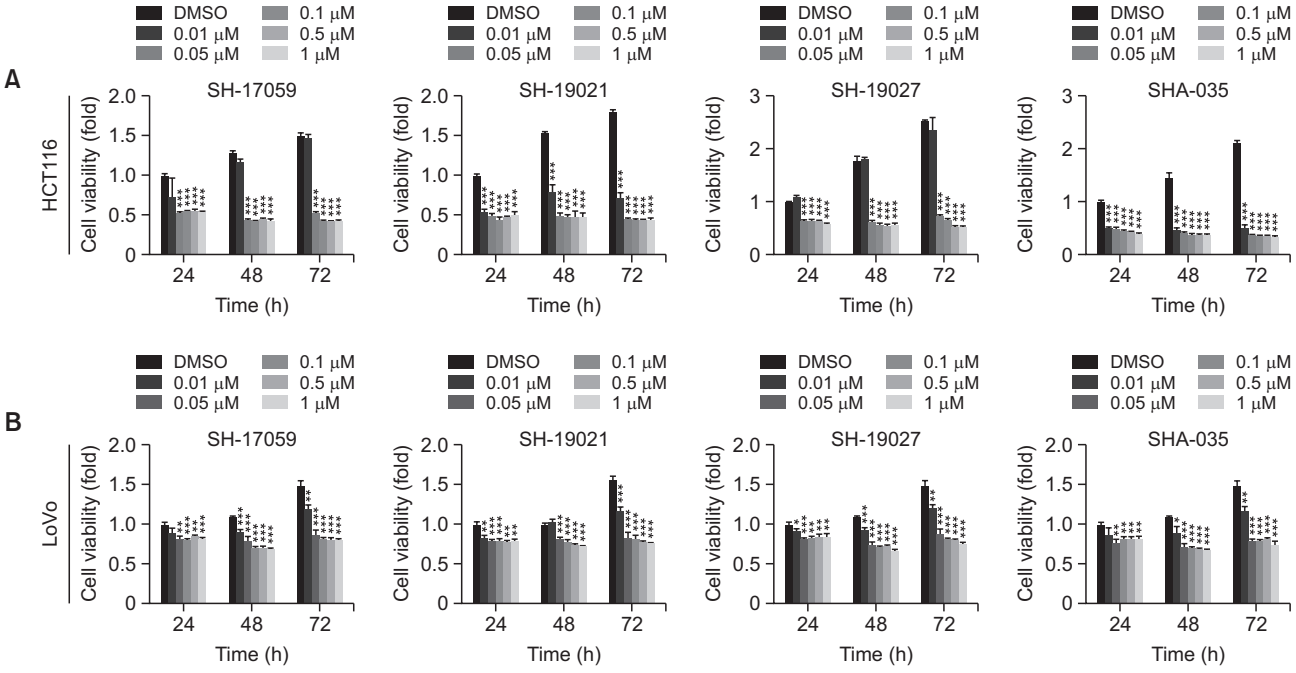

Fig. 2. Cell viability of colorectal cancer (CRC) cells is reduced by treatment with the homoisoflavane derivatives in a time- and dose-dependent manner. HCT116 (A) and LoVo (B) cells were treated with the indicated doses of SH-17059, SH-19021, SH-19027, and SHA-035 for the indicated times. A WST-based cell viability assay was performed. Values are the mean ± SD. *p<0.05, **p<0.01, ***p<0.005.

cyclopropylmethoxy, respectively. To modify the B-ring of SH-17059, SH-19026 and SH-19027 were synthesized. In SH-19026, the methoxy and hydroxy become inverted on the C3' and C4' positions, and SH-19027 has a fluoro group on the C3' position instead of the hydroxy. SHA-035 is a carbamate derivative in which a long-chain carbamoyl group is attached to the C3'-hydroxy.

Cytotoxic effect of the cremastranone derivatives on human CRC cells

To investigate the cytotoxic effects of the synthetic cremastranone derivatives, the human CRC cell lines HCT116 and LoVo and the mouse CRC cell line CT26 were treated with the compounds (Fig. 1), and then, the cell viability was measured. As shown in Fig. 2, treatment of the HCT116 and LoVo cells with SH-17059, SH-19021, SH-19027, and SHA-035 significantly reduced the cell viability in a dose- and time-dependent

manner. Similar results were also obtained in the CT26 cells when SH-19027 and SHA-035 were treated (Supplementary Fig. 1A). However, SH-19017 and SH-19026 had no effect in the HCT116 and LoVo cells (Supplementary Fig. 2). Therefore, we confirmed that some of the synthetic cremastranone derivatives have cytotoxic activity in CRC cells and the specific position of the substituents is critical for the activity. Both the 5,6,7-trimethoxy and 4'-methoxy moieties are essential for the cytotoxic activity while the fluoro and bulky carbamate moieties are beneficial as a surrogate of the hydroxy on the C3' position. Among these synthetic cremastranone derivatives, we selected SH-19027 and SHA-035 as potent chemicals using them in the subsequent experiments to investigate their mechanisms of action.

SH-19027 and SHA-035 inhibit proliferation and induce cell cycle arrest in CRC cells

Cell viability is related to the cell proliferation capacity and survival. Therefore, we first measured the cell proliferation rate with the BrdU incorporation assay in SH-19027 and SHA-035 treated cells. Treatment with SH-19027 and SHA-035 significantly inhibited the proliferation of the HCT116, LoVo, and CT26 cells compared to the DMSO control (Fig. 3A, Supplementary Fig. 1B). Subsequently, we detected the expression of the proliferation markers, c-Myc and cyclin D1 (Motokura and Arnold, 1993; Miller *et al*., 2012), through western blot analysis. We anticipated a decrease of expression of c-Myc and cyclin D1 when the cells were treated with SH-19027 and SHA-035. However, treatment with SH-19027 or SHA-035 increased the expression of c-Myc and cyclin D1 in the HCT116 cells (Fig. 3B). In the CT26 cells, the expression of cyclin D1 was also augmented in response to the treatment with SH-19027 or SHA-035, but the c-Myc expression was not significantly changed (Supplementary Fig. 1D). Previous studies reported that c-Myc could induce apoptosis or sensitize cancer cells to apoptosis (Pelengaris *et al*., 2002; Hoffman and Liebermann, 2008), and ectopic overexpression of cyclin D1 could have a role as an apoptosis inducer (Han *et*

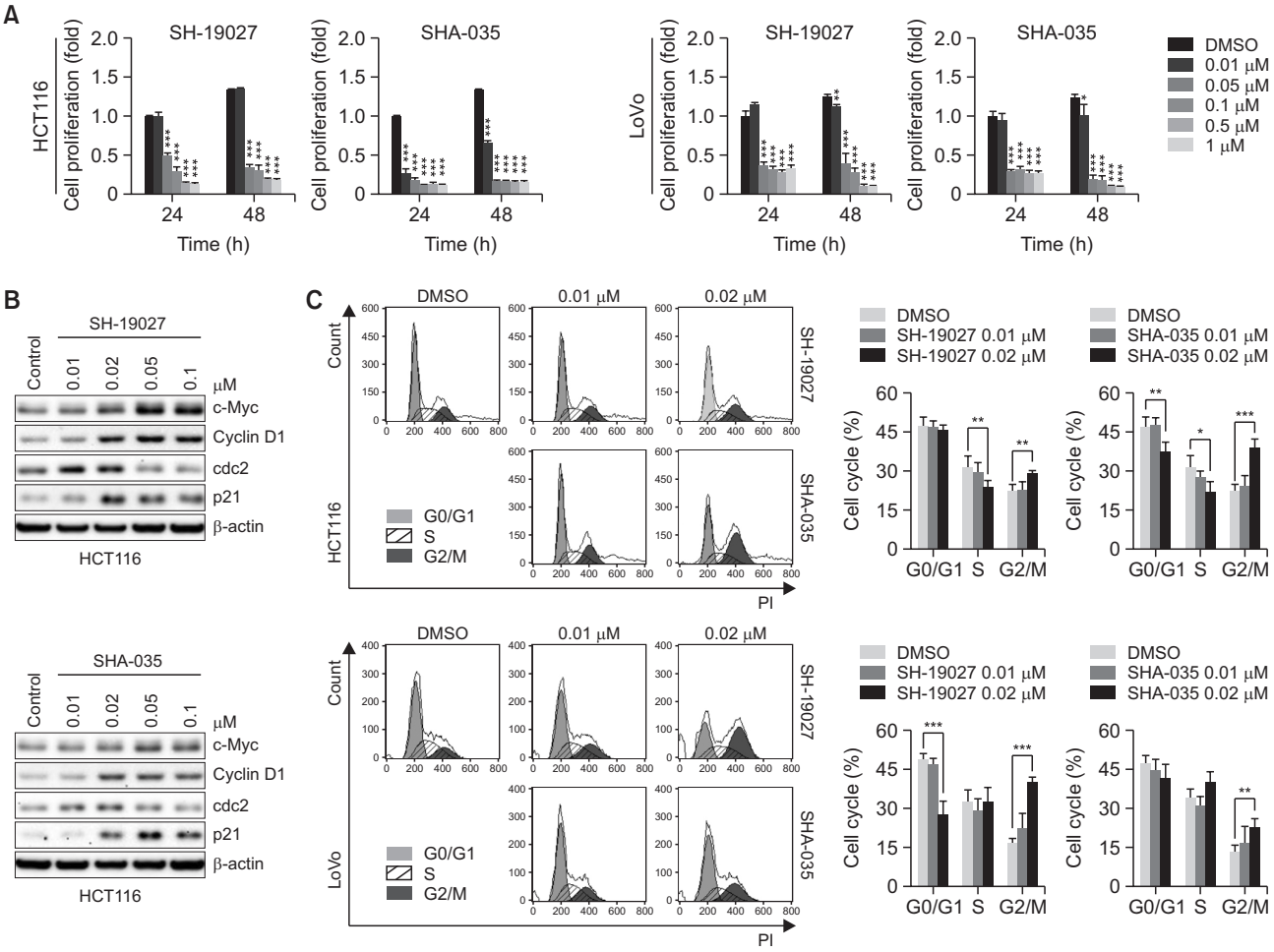

Fig. 3. Effects of SH-19027 and SHA-035 on proliferation and cell cycle in CRC cells. (A) BrdU colorimetric incorporation assay was performed in HCT116 and LoVo cells after treatment with the indicated doses of SH-19027 and SHA-035 for the indicated times. (B) HCT116 cells were treated with the indicated doses of SH-19027 or SHA-035 for 48 h, and the cell lysates were subjected to western blot analysis. β-actin was used as a loading control. (C) Cell cycle of HCT116 and LoVo cells were examined by flow cytometry after treatment with the indicated doses of SH-19027 or SHA-035 for 24 h (HCT116) or 48 h (LoVo). Percentages of cells in the G0/G1, S, and G2/M phase of the cell cycle are shown as a graph. Values are the mean ± SD. *p<0.05, **p<0.01, ***p<0.005.

al., 1999). In this regard, we thought that the upregulation of c-Myc and/or cyclin D1 expression by SH-19027 and SHA-035 treatment may lead to cell death by increasing the sensitivity to apoptosis.

In a previous study, cremastranone, from which SH-19027 and SHA-035 were derived, induced cell cycle arrest in the G2/M phase and increased the expression of p21 in HUVEC cells (Kim *et al*., 2007). To determine whether the anti-proliferative effect of SH-19027 and SHA-035 was associated with cell cycle arrest in CRC cells, HCT116 and LoVo cells were stained with PI and analyzed with flow cytometry. The treatment of SH-19027 and SHA-035 increased the proportion of cells in the G2/M phase (Fig. 3C). Because cell division control protein 2 (cdc2), also known as cyclin-dependent kinase 1 (CDK1), is required for G2/M transition in cell cycle and p21 could inactivate cyclin A/B-cdc2 complexes (Vermeulen *et al*., 2003; Matthews *et al*., 2022), we investigated the p21 and cdc2 expression. In the cells treated with a low concentration of SH-19027 or SHA-035, cdc2 expression was increased, but at higher concentrations of SH-19027 and SHA-035, cdc2 expression was decreased, and the expression of p21 was increased (Fig. 3B). These data suggest that SH-19027 and SHA-035 inhibit the proliferation of CRC cell through G2/M phase arrest, and this phenomenon accompanies the regulation of p21 and cdc2 expression.

Apoptosis was induced in CRC cells by SH-19027 and SHA-035

The decrease of cell viability may be related with apoptosis. Therefore, we investigated whether the treatment of the CRC cells with SH-19027 and SHA-035 induced apoptosis. Annexin V-FITC/PI staining was performed for detecting apoptosis. In comparison with the DMSO control, the percentages of apoptotic cells increased by the treatment with SH-19027 and SHA-035 in a dose-dependent manner (Fig. 4A, Supplementary Fig. 1C). In addition, we performed western blot analysis to detect the apoptosis markers cleaved poly(ADP-ribose) polymerase (PARP) (Gobeil *et al*., 2001) and cleaved caspase 3 (Fan *et al*., 2005). In response to the treatment of SH-19027 and SHA-035, the 89 kDa fragment of PARP and cleaved caspase 3 were observed by the SH-19027 and SHA-035 treatment (Fig. 4B, Supplementary Fig. 1D). To further confirm the

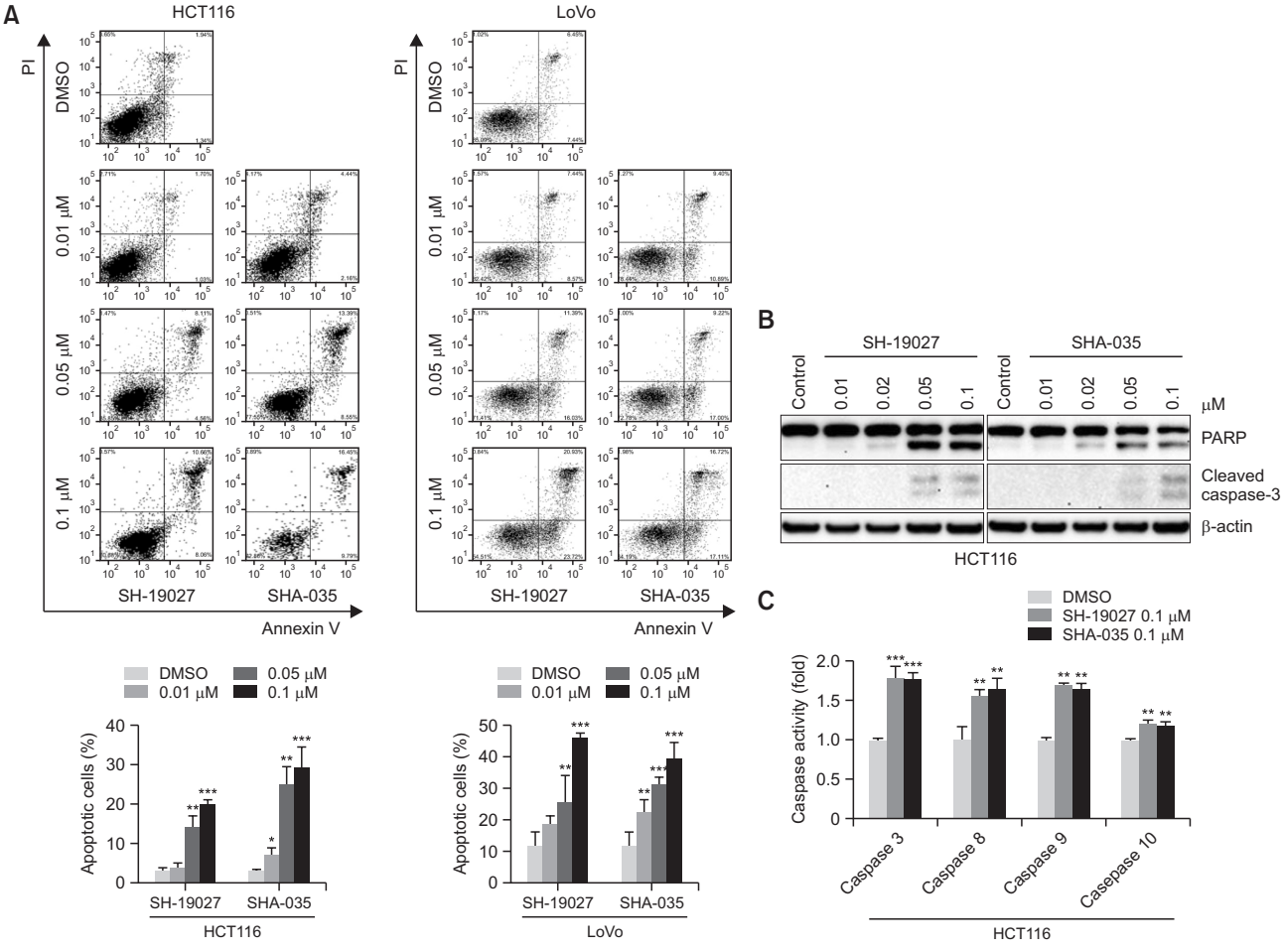

Fig. 4. SH-19027 and SHA-035 induce apoptosis in the CRC cells. (A) After treatment with SH-19027 or SHA-035 for 24 h in HCT116 and 48 h in LoVo cells, the apoptosis assay was performed by Annexin V-FITC/PI staining. Percentage of apoptotic cells is the sum of annexin V positive cells. (B) HCT116 cells were treated with SH-19027 or SHA-035 for 48 h, followed by western blot analysis performed for detecting apoptosis marker expression. β-actin was used as a loading control. (C) Caspase activity in SH-19027- and SHA-035-treated HCT116 cells was measured using caspase substrates. Values are the mean ± SD. **p*<0.05, ***p*<0.01, ****p*<0.005.

caspase activation, caspase activity assays were done with caspase substrates. We found that apoptosis executioner caspase 3 and initiator caspases 8, 9, and 10 were activated by the SH-19027 and SHA-035 treatment (Fig. 4C) (Fan *et al*., 2005). These results show that SH-19027 and SHA-035 induce apoptosis in CRC cells.

DISCUSSION

Homoisoflavanones are one of the natural compounds found in various plants, and their pharmacological activities including cytotoxicity in tumors have been reported (du Toit *et al*., 2010; Lin *et al*., 2014). Previous studies reported that cremastranone and its derivatives have anti-angiogenic and anti-proliferative activities in endothelial cells (Kim *et al*., 2008; Basavarajappa *et al*., 2014; Lee *et al*., 2014). In the present study, we demonstrated the anti-cancer effects of synthetic homoisoflavane derivatives of cremastranone in CRC cells and suggest that the cytotoxic effects are associated with cell cycle arrest and the induction of apoptosis.

For the synthesis of the cremastranone derivatives, it was considered that homoisoflavane SH-17059 has better synthetic accessibility and physicochemical properties compared to homoisoflavanone cremastranone (Basavarajappa *et al*., 2015; Schwikkard *et al*., 2019). The cytotoxic effects on colon cancer cell lines were evaluated for A-ring and B-ring-modified derivatives of SH-17059. Because SH-19017 and SH-19026 had no effect in the HCT116 and LoVo cells, it was suitable to maintain both the C6-substituent of the A-ring and the C4'-substituent of the B-ring as methoxy groups. Comparable activity was shown in SH-19027 with a C3'-fluoro of the B-ring with improved metabolic stability. SHA-035 in which carbamate was introduced at the C3'-hydroxy of the B-ring exhibited improved activity. Through these results, we confirmed that the C3'-hydroxy group is not necessarily essential for the cytotoxic effect. For further study, we selected SH-19027 and SHA-035 as potent chemicals and investigated their detailed effects in the CRC cells.

Homoisoflavanones isolated from *Disporopsis aspera* have a cytotoxic activity in several human cancer cell lines including CRC with an IC_{50} ranging from 22.9 to 64.2 μM (Nguyen *et al*., 2006). Another homoisoflavanone from *Ophiopogon japonicas* also showed cytotoxic effects on the lung cancer cell line A549 (IC_{50}=0.84-32.76 μM) (Zhou *et al*., 2013). Another study reported that homoisoflavanones extracted from *Bellevalia flexuosa* were tested for their anti-proliferative effects against the human melanoma cell line MDA-MB-435, breast cancer cell line MDA-MB-231, and ovarian cancer cell line Ovcar3 with the MTS assay (El-Elimat *et al*., 2018). In that study, the most potent homoisoflavanones had IC_{50} values of 1.6, 9.5, and 3.6 μM against those cell lines, respectively. Moreover, while there are several studies on the anti-cancer effects of homoisoflavonones in various cancer cells, there are few studies on their effects in CRC cells. In this study, we showed that the synthesized novel cremastranone derivatives SH-19027 and SHA-035 inhibited cell viability and proliferation at nanomolar concentrations in CRC cell lines HCT116 and LoVo. Therefore, SH-19027 and SHA-035, can be considered for novel anti-cancer drugs against CRC that can be used at lower concentrations, and these characteristics could lower the side effects such as hepatotoxicity.

The cell cycle is regulated by cyclins, cyclin-dependent kinase (CDK), and cyclin-dependent kinase inhibitors (CKI) (Vermeulen *et al*., 2003; Matthews *et al*., 2022). The representative tumor suppressor protein p53 induces cell cycle arrest in response to various stresses primarily through transcriptional activation of p21, one of the CKIs (Taylor and Stark, 2001). p21 inhibits the activity of cyclin-CDK complexes including cyclin B-cdc2, which are major components for entry to mitosis in the cell cycle. Indeed, p21 blocks G2/M transition by directly binding to the cyclin B-cdc2 complex or inhibiting phosphorylation at Thr^{161} of cdc2, which is required for its maximum activity (Smits *et al*., 2000; Taylor and Stark, 2001; Kreis *et al*., 2015). p21 not only inhibits the activity of cdc2 but also reduces the transcriptional level of cdc2. It was reported that p21-overexpressing cells downregulate the transcription of genes involved in mitosis, including cdc2 (Chang *et al*., 2000). Considering that the treatment with SH-19027 and SHA-035 decreased the cdc2 expression and upregulated the p21 expression, cell cycle arrest at the G2/M phase by SH-19027 and SHA-035 may be associated with the regulation of p21 and cdc2.

The proto-oncogene c-*MYC* has key roles in various biological processes including cell growth, proliferation, and differentiation, and increased expression of c-*MYC* has been revealed in various types of human cancers (Miller *et al*., 2012). Cyclin D1 is mostly known as a key regulator of cell cycle at G1/S progression, and upregulated expression of cyclin D1 is involved in the abnormal proliferation of cancer cells (Motokura and Arnold, 1993). However, treatment of SH-19027 and SHA-035 increased the expression of c-Myc and cyclin D1 despite the decrease in cell proliferation. Several studies have reported that c-Myc and E1A sensitize cells to apoptosis (Han *et al*., 1999; Pelengaris *et al*., 2002; Hoffman and Liebermann, 2008). c-Myc possesses potential to drive apoptosis by activating Bax and releasing cytochrome c when tumor cells are exposed to apoptotic stimuli (Soucie *et al*., 2001; Cao *et al*., 2008; Cerquetti *et al*., 2015). Ectopic expression of cyclin D1 induces apoptosis via increase of Rb phosphorylation in rat fibroblast cells (Sofer-Levi and Resnitzky, 1996), endoplasmic reticulum stress in bortezomib-treated myeloma cells (Bustany *et al*., 2015) and releasing cytochrome c in retinoic acid-treated breast cancer cells (Niu *et al*., 2001). In addition, cyclin D1 overexpression amplified fenretinide-induced apoptosis in a breast cancer xenograft mouse model (Pirkmaier *et al*., 2003). In this respect, upregulation of c-Myc and cyclin D1 may act as an apoptosis inducer in some cytotoxic conditions. Therefore, increased expression of c-Myc and cyclin D1 by the SH-19027 and SHA-035 treatment may contribute to apoptosis induction in human CRC cells. Considering that the expression of c-Myc was not changed by the compounds in the mouse colorectal CT26 cells, regulation of c-Myc may not be a general property in CRC cell lines. A study on the detailed mechanisms involved in the induction of c-Myc and cyclin D1 and their possible contribution to apoptosis needs to be done in the near future.

Taken together, our results suggest that the novel compounds SH-19027 and SHA-035 can be considered as potent novel anti-cancer drugs against CRC considering that the induction of apoptosis is a major strategy of anti-cancer drugs (Pfeffer and Singh, 2018), and we believe that the results of this investigation contribute to a new strategy for cancer therapy.

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REFERENCES

Basavarajappa, H. D., Lee, B., Fei, X., Lim, D., Callaghan, B., Mund, J. A., Case, J., Rajashekhar, G., Seo, S.-Y. and Corson, T. W. (2014) Synthesis and mechanistic studies of a novel homoisoflavanone inhibitor of endothelial cell growth. *PLoS One* **9**, e95694.

Basavarajappa, H. D., Lee, B., Lee, H., Sulaiman, R. S., An, H., Magaña, C., Shadmand, M., Vayl, A., Rajashekhar, G., Kim, E. Y., Suh, Y. G., Lee, K., Seo, S. Y. and Corson, T. W. (2015) Synthesis and biological evaluation of novel homoisoflavonoids for retinal neovascularization. *J. Med. Chem.* **58**, 5015-5027.

Bustany, S., Cahu, J., Guardiola, P. and Sola, B. (2015) Cyclin D1 sensitizes myeloma cells to endoplasmic reticulum stress-mediated apoptosis by activating the unfolded protein response pathway. *BMC Cancer* **15**, 262.

Cao, X., Bennett, R. L. and May, W. S. (2008) c-Myc and caspase-2 are involved in activating Bax during cytotoxic drug-induced apoptosis. *J. Biol. Chem.* **283**, 14490-14496.

Cerquetti, L., Sampaoli, C., De Salvo, M., Bucci, B., Argese, N., Chimento, A., Vottari, S., Marchese, R., Pezzi, V., Toscano, V. and Stigliano, A. (2015) C-MYC modulation induces responsiveness to paclitaxel in adrenocortical cancer cell lines. *Int. J. Oncol.* **46**, 2231-2240.

Chang, B. D., Watanabe, K., Broude, E. V., Fang, J., Poole, J. C., Kalinichenko, T. V. and Roninson, I. B. (2000) Effects of p21Waf1/Cip1/Sdi1 on cellular gene expression: implications for carcinogenesis, senescence, and age-related diseases. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 4291-4296.

de Gramont, A., Figer, A., Seymour, M., Homerin, M., Hmissi, A., Cassidy, J., Boni, C., Cortes-Funes, H., Cervantes, A., Freyer, G., Papamichael, D., Le Bail, N., Louvet, C., Hendler, D., de Braud, F., Wilson, C., Morvan, F. and Bonetti, A. (2000) Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. *J. Clin. Oncol.* **18**, 2938-2947.

Demain, A. L. and Vaishnav, P. (2011) Natural products for cancer chemotherapy. *Microb. Biotechnol.* **4**, 687-699.

Douillard, J., Cunningham, D., Roth, A., Navarro, M., James, R., Karasek, P., Jandik, P., Iveson, T., Carmichael, J., Alakl, M., Gruia, G., Awad, L. and Rougier, P. (2000) Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. *Lancet* **355**, 1041-1047.

du Toit, K., Drewes, S. E. and Bodenstein, J. (2010) The chemical structures, plant origins, ethnobotany and biological activities of homoisoflavanones. *Nat. Prod. Res.* **24**, 457-490.

El-Elimat, T., Rivera-Chávez, J., Burdette, J. E., Czarnecki, A., Alhawarri, M. B., Al-Gharaibeh, M., Alali, F. and Oberlies, N. H. (2018) Cytotoxic homoisoflavonoids from the bulbs of Bellevalia flexuosa. *Fitoterapia* **127**, 201-206.

Elmore, S. (2007) Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* **35**, 495-516.

Fan, T. J., Han, L. H., Cong, R. S. and Liang, J. (2005) Caspase family proteases and apoptosis. *Acta Biochim. Biophys. Sin.* **37**, 719-727.

Fernald, K. and Kurokawa, M. (2013) Evading apoptosis in cancer. *Trends Cell Biol.* **23**, 620-633.

Gobeil, S., Boucher, C. C., Nadeau, D. and Poirier, G. G. (2001) Characterization of the necrotic cleavage of poly(ADP-ribose) polymerase (PARP-1): implication of lysosomal proteases. *Cell Death Differ.* **8**, 588-594.

Han, E. K., Ng, S. C., Arber, N., Begemann, M. and Weinstein, I. B. (1999) Roles of cyclin D1 and related genes in growth inhibition, senescence and apoptosis. *Apoptosis* **4**, 213-219.

Heo, M., Lee, B., Sishtla, K., Fei, X., Lee, S., Park, S., Yuan, Y., Lee, S., Kwon, S., Lee, J., Kim, S., Corson, T. W. and Seo, S. Y. (2019) Enantioselective synthesis of homoisoflavanones by asymmetric transfer hydrogenation and their biological evaluation for antiangiogenic activity. *J. Org. Chem.* **84**, 9995-10011.

Hoffman, B. and Liebermann, D. A. (2008) Apoptotic signaling by c-MYC. *Oncogene* **27**, 6462-6472.

Kerr, J. F., Wyllie, A. H. and Currie, A. R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**, 239-257.

Kim, J. H., Kim, J. H., Yu, Y. S., Jun, H.-O., Kwon, H. J., Park, K. H. and Kim, K.-W. (2008) Inhibition of choroidal neovascularization by homoisoflavanone, a new angiogenesis inhibitor. *Mol. Vis.* **14**, 556-561.

Kim, J. H., Kim, K. H., Kim, J. H., Yu, Y. S., Kim, Y.-M., Kim, K.-W. and Kwon, H. J. (2007) Homoisoflavanone inhibits retinal neovascularization through cell cycle arrest with decrease of cdc2 expression. *Biochem. Biophys. Res. Commun.* **362**, 848-852.

Kreis, N. N., Louwen, F. and Yuan, J. (2015) Less understood issues: p21Cip1 in mitosis and its therapeutic potential. *Oncogene* **34**, 1758-1767.

Latchman, J., Guastella, A. M. and Tofthagen, C. (2014) 5-Fluorouracil toxicity and dihydropyrimidine dehydrogenase enzyme: implications for practice. *Clin. J. Oncol. Nurs.* **18**, 581-585.

Lee, B., Basavarajappa, H. D., Sulaiman, R. S., Fei, X., Seo, S.-Y. and Corson, T. W. (2014) The first synthesis of the antiangiogenic homoisoflavanone, cremastranone. *Org. Biomol. Chem.* **12**, 7673-7677.

Lin, L.-G., Liu, Q.-Y. and Ye, Y. (2014) Naturally occurring homoisoflavonoids and their pharmacological activities. *Planta Med.* **80**, 1053-1066.

Mármol, I., Sánchez-de-Diego, C., Pradilla Dieste, A., Cerrada, E. and Rodriguez Yoldi, M. J. (2017) Colorectal carcinoma: a general overview and future perspectives in colorectal cancer. *Int. J. Mol. Sci.* **18**, 197.

Matthews, H. K., Bertoli, C. and de Bruin, R. A. M. (2022) Cell cycle control in cancer. *Nat. Rev. Mol. Cell Biol.* **23**, 74-88.

Miller, D. M., Thomas, S. D., Islam, A., Muench, D. and Sedoris, K. (2012) c-Myc and cancer metabolism. *Clin. Cancer Res.* **18**, 5546-5553.

Mishra, B. B. and Tiwari, V. K. (2011) Natural products: an evolving role in future drug discovery. *Eur. J. Med. Chem.* **46**, 4769-4807.

Motokura, T. and Arnold, A. (1993) Cyclin D and oncogenesis. *Curr. Opin. Genet. Dev.* **3**, 5-10.

Nguyen, A.-T., Fontaine, J., Malonne, H. and Duez, P. (2006) Homoisoflavanones from Disporopsis aspera. *Phytochemistry* **67**, 2159-2163.

Niu, M. Y., Ménard, M., Reed, J. C., Krajewski, S. and Pratt, M. A. (2001) Ectopic expression of cyclin D1 amplifies a retinoic acid-induced mitochondrial death pathway in breast cancer cells. *Oncogene* **20**, 3506-3518.

Pelengaris, S., Khan, M. and Evan, G. (2002) c-MYC: more than just a matter of life and death. *Nat. Rev. Cancer* **2**, 764-776.

Pfeffer, C. M. and Singh, A. T. (2018) Apoptosis: a target for anticancer therapy. *Int. J. Mol. Sci.* **19**, 448.

Pirkmaier, A., Yuen, K., Hendley, J., O'Connell, M. and Germain, D. (2003) Cyclin D1 overexpression sensitizes breast cancer cells to fenretinide. *Clin. Cancer Res.* **9**, 1877-1884.

Saif, M. W., Katirtzoglou, N. A. and Syrigos, K. N. (2008) Capecitabine: an overview of the side effects and their management. *Anti-Cancer Drugs* **19**, 447-464.

Schwikkard, S., Whitmore, H., Sishtla, K., Sulaiman, R. S., Shetty, T., Basavarajappa, H. D., Waller, C., Alqahtani, A., Frankemoelle, L., Chapman, A., Crouch, N., Wetschnig, W., Knirsch, W., Andriantiana, J., Mas-Claret, E., Langat, M. K., Mulholland, D. and Corson, T. W. (2019) The antiangiogenic activity of naturally occurring and synthetic homoisoflavonoids from the Hyacinthaceae (sensu APG II). *J. Nat. Prod.* **82**, 1227-1239.

Shim, J. S., Kim, J. H., Lee, J., Kim, S. N. and Kwon, H. J. (2004) Anti-angiogenic activity of a homoisoflavanone from Cremastra appendiculata. *Planta Med.* **70**, 171-173.

Smits, V. A. J., Klompmaker, R., Vallenius, T., Rijksen, G., Mäkelä, T. P. and Medema, R. H. (2000) p21 inhibits Thr161 phosphorylation of Cdc2 to enforce the G2 DNA damage checkpoint. *J. Biol. Chem.* **275**, 30638-30643.

Sofer-Levi, Y. and Resnitzky, D. (1996) Apoptosis induced by ectopic expression of cyclin D1 but not cyclin E. *Oncogene* **13**, 2431-2437.

Soucie, E. L., Annis, M. G., Sedivy, J., Filmus, J., Leber, B., Andrews, D. W. and Penn, L. Z. (2001) Myc potentiates apoptosis by stimulating Bax activity at the mitochondria. *Mol. Cell. Biol.* **21**, 4725-4736.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A. and Bray, F. (2021) Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **71**, 209-249.

Taylor, W. R. and Stark, G. R. (2001) Regulation of the G2/M transition by p53. *Oncogene* **20**, 1803-1815.

Van Cutsem, E., Cervantes, A., Nordlinger, B. and Arnold, D.; ESMO Guidelines Working Group (2014) Metastatic colorectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* **25**, iii1-iii9.

Van Cutsem, E., Hoff, P., Harper, P., Bukowski, R., Cunningham, D., Dufour, P., Graeven, U., Lokich, J., Madajewicz, S., Maroun, J., Marshall, J. L., Mitchell, E. P., Perez-Manga, G., Rougier, P., Schmiegel, W., Schoelmerich, J., Sobrero, A. and Schilsky, R. L. (2004) Oral capecitabine vs intravenous 5-fluorouracil and leucovorin: integrated efficacy data and novel analyses from two large, randomised, phase III trials. *Br. J. Cancer* **90**, 1190-1197.

Vermeulen, K., Van Bockstaele, D. R. and Berneman, Z. N. (2003) The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif.* **36**, 131-149.

Xie, Y.-H., Chen, Y.-X. and Fang, J.-Y. (2020) Comprehensive review of targeted therapy for colorectal cancer. *Signal Transduct. Target. Ther.* **5**, 22.

Zhou, C. X., Zou, L., Mo, J. X., Wang, X. Y., Yang, B., He, Q. J. and Gan, L. S. (2013) Homoisoflavonoids from Ophiopogon japonicus. *Helv. Chim. Acta* **96**, 1397-1405.