JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Novel Antimitotic Activity of 2-Hydroxy-4-methoxy-2′,3′-benzochalcone (HymnPro) through the Inhibition of Tubulin Polymerization

Soon Young Shin,[†,‡] Ji-Ho Kim,[†] Hyuk Yoon,[#] Yang-Kyu Choi,[⊥] Dongsoo Koh,[§] Yoongho Lim,*[,#] and Young Han Lee*[,†,‡]

[†]Department of Biological Sciences, College of Biological Science and Biotechnology, Cancer and Metabolism Institute, Konkuk University, Seoul 143-701, Republic of Korea

[‡]Institute of Clinical Medicine, Konkuk University Medical Center, Seoul 143-729, Republic of Korea

[#]Division of Bioscience and Biotechnology, BMIC, Konkuk University, Seoul 143-701, Republic of Korea

[⊥]Department of Laboratory Animal Medicine, College of Veterinary Medicine, Konkuk University, Seoul 143-701, Republic of Korea

[§]Department of Applied Chemistry, Dongduk Women's University, Seoul 136-714, Republic of Korea

ABSTRACT: The natural chalcones and their derivatives exhibit many biological activities, such as anti-inflammatory and antitumoral. However, the precise mechanisms of action of benzochalcone derivatives are currently unknown. Here, a set of benzochalcones was synthesized, and the molecular mechanisms underlying inhibition of tumor growth were investigated. Colony-forming assays revealed that among tested compounds, 2-hydroxy-4-methoxy-2′,3′-benzochalcone (HymnPro) most effectively inhibited the clonogenicity of Capan-1 human pancreatic cancer cells. HymnPro inhibited cell proliferation in several human solid tumor cell lines and suppressed xenografted tumor growth in nude mice. Mechanistically, HymnPro induced cell cycle arrest at the G_2/M phase, followed by an increase in apoptotic cell death. These events were associated with the inhibition of tubulin polymerization through binding of HymnPro to tubulin, leading to the formation of abnormal mono- or multipolar mitotic microtubule structures accompanied by spherical arrangement of multinucleated chromosomes. Furthermore, HymnPro activated caspase-2, caspase-9, caspase-3, and caspase-7 and increased the cleavage of poly(ADP-ribose) polymerase (PARP). HymnPro increased the phosphorylation of JNK1/2, Erk1/2, and p38 kinase. Pretreatment with SP600125, U0126, or SB600125 abrogated HymnPro-induced activation of caspases-3 and caspase-7 and the cleavage of PARP, suggesting that MAPK signalings are involved in HymnPro-induced apoptosis. It was concluded that a novel HymnPro compound exerts antitumor activity by disrupting microtubule assembly, which leads to mitotic arrest and sequential activation of the caspase pathway, resulting in apoptosis.

KEYWORDS: *benzochalcone, tubulin, G2/M arrest, caspase, apoptosis*

INTRODUCTION

Many clinically successful anticancer agents are derived from naturally occurring compounds. Flavonoids are polyphenolic compounds found in many edible plants, for example, fruits and vegetables. Flavonoids display diverse pharmacologic activities, including antioxidative, anti-inflammatory, and antitumorigenic properties.[1] Flavonoids contain a common C6−C3−C6 skeleton, which consists of three rings (A, C, and B rings), and they can be divided into several classes, for example, chalcones, flavonols, flavones, flavanones, anthocyanidins, and isoflavonoids, on the basis of their structural substituents. Of these, chalcones (1,3-diphenyl-2-propene-1-ones) are open-chain flavonoids bearing two aromatic rings (A and B rings) linked by a three-carbon enone moiety.[2] Several naturally occurring or synthesized forms of chalcone have multiple biological properties, including antitumor activities.[2] As chalcones are easy to synthesize and contain excellent leading skeletons, intensive studies have documented synthetic modifications of chalcones to enhance their bioactivity and therapeutic efficacy.[2] However, very little is known about the antitumor activity of benzochalcone derivatives.

Microtubules are long, hollow, cylindrical polymers composed of α- and β-tubulin dimers. They are highly dynamic structures that are regulated by polymerization and depolymerization of tubulin, and this dynamic instability property is important for carrying out many cellular functions of microtubules.[2] In interphase cells, microtubules are involved in multiple cellular processes, such as maintenance of cell structure, cell motility, and vesicle transportation. When cells enter into mitosis, microtubules are reorganized into a mitotic spindle, which is required for the congression of chromosomes. Bipolar attachment of mitotic microtubules to sister kinetochores aligns chromosomes at the cell equator during metaphase, and depolymerization of tubulin plays an essential role in accurate segregation of sister chromatids during anaphase. Disruption of microtubule dynamics or formation of abnormal mitotic microtubules prevents rearrangement of

Received: August 5, 2013
Revised: December 4, 2013
Accepted: December 5, 2013
Published: December 5, 2013

dx.doi.org/10.1021/jf4034688 | J. Agric. Food Chem. 2013, 61, 12588−12597

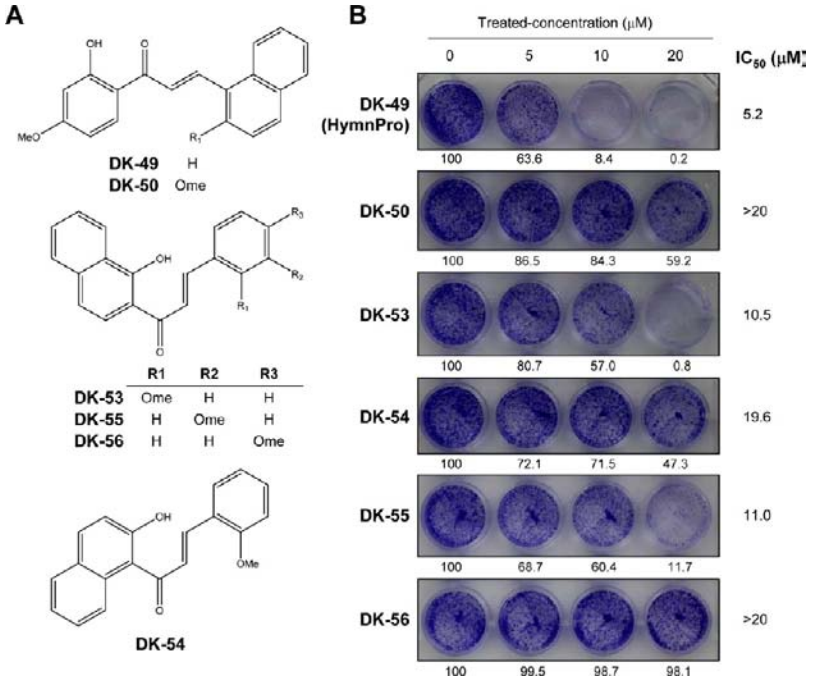

Figure 1. Effect of benzochalcone derivatives on the inhibition of clonogenicity of Capan-1 cells. (A) Structures of novel benzochalcone derivatives. (B) Capan-1 cells (5×10^3/well) were seeded and cultured in the absence or presence of benzochalcone derivatives (0, 5, 10, and 20 μM). After 7 days of treatment, colonies were stained with crystal violet and quantified by densitometry. Similar results were obtained from two other independent experiments.

the microtubule network, which can induce cell cycle arrest in M phase and trigger cell death.[3] One of the approaches to developing anticancer drugs is to inhibit mitosis by disrupting microtubule networks because continuous mitotic division of proliferating cancer cells is more sensitive to inhibition of mitosis than normal cells.[4] Antimitotic agents interacting with microtubules can be grouped into three major classes, as distinguished by the binding site on tubulin: taxane, vinca alkaloids, and colchicines sites. Taxanes stabilize tubulin polymers by blocking disassembly of tubulin, whereas vinca alkaloids and colchicines prevent the assembly of tubulin. A large number of antimitotic agents targeting tubulin are in active clinical development.[5]

In the present study, we examined the molecular basis of the antitumor activity of six benzochalcone derivatives and found that (*E*)-1-(2-hydroxy-4-methoxyphenyl)-3-(naphthalen-1-yl)-prop-2-en-1-one (2-hydroxy-4-methoxy-2′,3′-benzochalcone; designated HymnPro) can inhibit microtubule polymerization and thereby disrupt microtubule dynamics during mitosis, exerting an antitumor activity in vitro and in vivo. We also obtained evidence that caspase activation is followed by mitotic arrest and plays an important role in HymnPro-induced cell death. These novel effects of the HymnPro compound may provide new insights into the molecular mechanisms responsible for the antitumor effects of benzochalcone derivatives.

MATERIALS AND METHODS

Reagents. Benzochalcone derivatives were synthesized as described previously.[6] The pan-caspase inhibitor zVAD-fmk (Z-Val-Ala-Asp [OMe]-FMK), the MEK1 inhibitor U0126, the JNK inhibitor SP600125, and the p38 kinase inhibitor SB203580 were purchased from Calbiochem (San Diego, CA, USA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Clonogenic Long-Term Survival Assay. Capan-1 cells were counted and plated onto 24-well tissue culture plates (BD Falcon; Becton Dickson Immunocytometry System) (5×10^3 cells/well) in DMEM supplemented with 10% FBS. After attachment, the cells were treated with benzochalcone derivatives for 7 days. The cells were then fixed with 6% glutaraldehyde and stained with 0.1% crystal violet, as described previously.[7]

Cell Viability Assay. Cells were seeded onto 96-well plates (2×10^3 cells/well) and treated with various concentrations of HymnPro for the indicated lengths of time. Cell viability was measured using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Xenograft Tumor Growth in Nude Mice. Capan-1 cells (3×10^6 cells in 200 μL of 2% dimethyl sulfoxide (DMSO)/8% ethanol) were inoculated subcutaneously into the right flank of 5−6-week-old athymic nude mice (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea). Tumor growth was measured at intervals of 2−5 days with calipers. The tumor volume was calculated as $(L \times W^2)/2$, where L is the length and W is the width in millimeters. When the tumors reached a mean volume of approximately 100 mm^3, 100 μL of vehicle (2% DMSO/8% ethanol) or HymnPro (10 mg/kg in 2% DMSO/8% ethanol) was injected intraperitoneally daily. Mice were killed by exposure to CO_2 on day 42 to compare the size of cutaneous xenograft tumors.

Cell Cycle Analysis. Cellular DNA content was analyzed by flow cytometry as described previously.[8] Briefly, Capan-1 cells were collected after 12 or 24 h of exposure to 20 μM HymnPro, fixed in 70% ethanol, washed twice with phosphate-buffered saline (PBS), and stained with a 50 μg/mL propidium iodide (PI) solution containing 0.1% Triton X-100, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 50 μg/mL RNase A. Fluorescence was measured and analyzed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Western Blot Analysis. Cells were lysed in a buffer consisting of 20 mM HEPES (pH 7.2), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 10 μg/mL leupeptin, and 1 mM phenylmethanesulfonyl fluoride (PMSF). The protein extracts (20 μg each) were separated by 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-

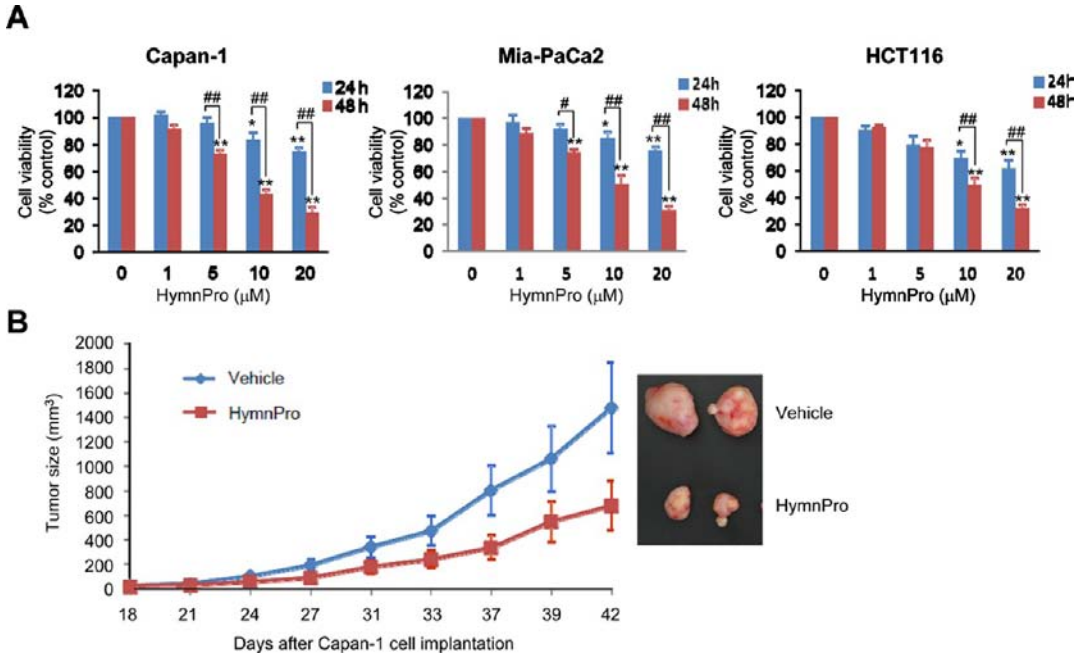

Figure 2. Effect of HymnPro on tumor growth. (A) Cell viability assay. Capan-1, Mia-PaCa2, and HCT116 cells were treated with different concentrations (1−20 μM) of HymnPro for 24 or 48 h, and cell viability was measured using a Cell Counting Kit-8. Statistical difference was determined by the Turkey−Kramer multiple-comparisons test. Error bars indicate SD; *, $P < 0.05$; **, $P < 0.001$ compared with untreated control (0 μM); #, $P < 0.05$; ##, $P < 0.001$. (B) Xenograft tumor growth in vivo. Capan-1 cells were injected subcutaneously into the right flank of each nude mouse. After 18 days, mice were administered vehicle (2% DMSO/8% ethanol) or HymnPro (20 mg/kg) daily. The mean volumes of xenograft tumors were measured at intervals of 2−5 days with calipers. Error bars indicate SD. Tumor-bearing nude mice were killed, and tumors were isolated 42 days postimplantation (inset).

PAGE) and transferred to nitrocellulose membranes. The blots were incubated with the corresponding primary antibodies and developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Microtubule Assembly Assay. The in vitro polymerization of tubulin was examined by the HTS-Tubulin Polymerization Assay kit (Cytoskeleton, Denver, CO, USA) according to the manufacturer's instructions. Tubulin polymerization was monitored spectrophotometrically at 340 nm with gentle plate agitation. The absorbance values were measured at 30 s intervals for 60 min using a SpectraMAX 190 plate reader (Molecular Devices, Toronto, ON, Canada). For measurement of in vivo microtubule assembly, soluble tubulin dimers and insoluble polymerized tubulin were separated and subjected to Western blotting as described previously.[9]

Immunofluorescence Microscopy. Capan-1 cells plated on coverslips were treated with either DMSO (vehicle) or 20 μM HymnPro for 24 h. They were then fixed with 4% paraformaldehyde and permeabilized using 0.1% Triton X-100, as described previously.[8] Primary antibody specific to α-tubulin, pericentrin, or cleaved caspase-7 (Asp198) was added and incubated for 2 h followed by an AlexaFluor 488-conjugated α-tubulin antibody (Invitrogen, Carlsbad, CA, USA) or AlexaFluor 555-conjugated pericentrin or cleaved caspase-7 (Invitrogen) secondary antibody incubation for 30 min. Nuclear DNA was stained with Hoechst 33258 (Sigma-Aldrich). Labeled cells were examined under an EVOSf1 fluorescence microscope (Advanced Microscopy Group, Bothell, WA, USA).

Quantification of Apoptotic Cells by Annexin V Staining. Capan-1 cells (1×10^6 cells/sample) were treated with 20 μM HymnPro for 24, 48, or 72 h. The cells were incubated with Cy3-conjugated Annexin V according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA). Cy3-positive cells (apoptotic cells) were analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems).

DNA Fragmentation Assay. For detecting DNA fragmentation using agarose gel electrophoresis, cells were treated with 20 μM HymnPro for 12, 24, or 48 h. Fragmented DNA was prepared using the Quick Apoptosis DNA Ladder Detection Kit (BioVision Inc., Mountain View, CA, USA) according to the manufacturer's instructions. After precipitation with 0.3 M sodium acetate (pH 5.5) and ethanol, DNA was dissolved in 10 mM Tris/EDTA (pH 8.0) buffer and then subjected to 1.2% agarose gel electrophoresis. DNA was stained with ethidium bromide and visualized with UV light illumination. For detection of in situ internucleosomal DNA fragmentation, Capan-1 cells grown on glass coverslips were treated with 20 μM HymnPro for 48 h. Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assays were performed using the ApoBrdU in situ DNA Fragmentation Assay Kit (BioVision Inc.) according to the manufacturer's instructions. The images were acquired using an EVOSf1 fluorescence microscope.

Statistical Analysis. Each experiment was performed at least three times. The data are plotted as means with SD. Statistical comparisons were made by a one-way ANOVA followed by the Turkey−Kramer multipl- comparisons test using GraphPad InStat v3.0 software. A p value of <0.05 was considered statistically significant.

RESULTS

Effect of Benzochalcone Derivatives on the Clonogenicity of Capan-1 Pancreatic Cancer Cells. To assess benzochalcones for antitumor activity, six benzochalcone derivatives that have not been evaluated before were synthesized (Figure 1A) and tested for their capacity to inhibit tumor cell growth by determining long-term clonogenic survival. Exponentially growing Capan-1 cells were exposed to different concentrations of benzochalcones for 7 days, and the ability of individual cells to proliferate into viable colonies was examined. Colony formation assays revealed that 2-hydroxy-4-methoxy-2′,3′-benzochalcone (DK-49) exhibited the most potent activity, with an IC_{50} value of 5.2 μM (Figure 1B). In contrast, 2-hydroxy-4,6′-dimethoxy-2′,3′-benzochalcone (DK-50), 2-hydroxy-2′-methoxy-5,6-benzochalcone (DK-54), and 2-

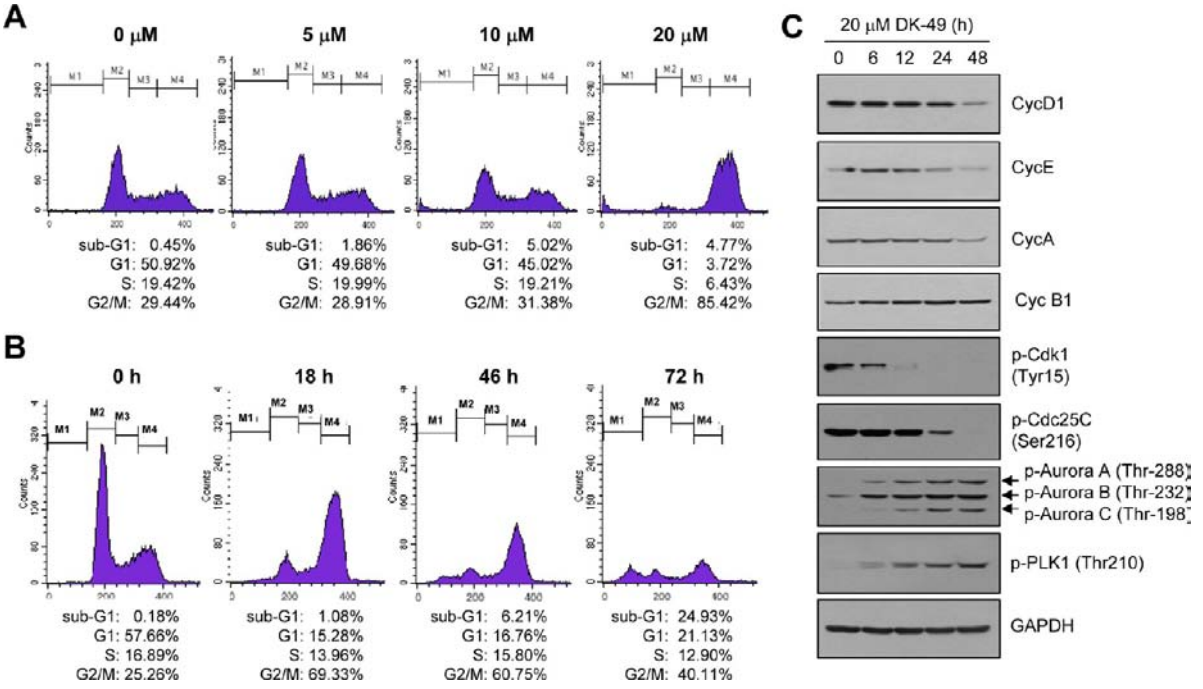

Figure 3. Effect of HymnPro on cell cycle progression. (A, B) Capan-1 cells (1×10^5 cells/sample) were treated with increasing concentrations of HymnPro for 24 h or with 20 μM HymnPro for various lengths of time. Cells were harvested, fixed with ethanol, and stained with propidium iodide (PI). The cellular DNA content was then determined by flow cytometry. M1, sub-G1 phase; M2, G1 phase; M3, S phase; M4, G2/M phase. (C) Capan-1 cells were treated with 20 μM HymnPro for various lengths of time, and whole-cell lysates were subjected to Western blotting using antibodies against the indicated proteins. GAPDH-specific antibody was used as an internal control to show equal protein loading.

hydroxy-4′-methoxy-3,4-benzochalcone (DK-56) showed no obvious inhibitory effects. We designated DK-49 as HymnPro, and further study focused on characterizing the molecular mechanism(s) underlying HymnPro-induced antitumor activity.

Effect of HymnPro on Tumor Cell Viability. To determine the antitumor activity of HymnPro in cancer cells, exponentially growing cancer cells, including human pancreatic cancer (Capan-1 and Mia-PaCa2) and human colon cancer (HCT116) cells, were exposed to various concentrations of HymnPro for 24 or 48 h, and cell viability assay was performed using a Cell Counting Kit-8. As shown in Figure 2A, HymnPro reduced cell viability in a dose- and time-dependent manner in pancreatic and colon cancer cells.

To evaluate whether HymnPro inhibits tumor growth in vivo, Capan-1 cells were inoculated subcutaneously into the flanks of athymic nude mice. At 18 days postimplantation, tumor-bearing mice were administered HymnPro (10 mg/kg) intraperitoneally once a day. Inhibition of tumor growth by HymnPro began to be observed after day 31, as compared to the vehicle-treated control group (Figure 2B). On day 42, the tumor sizes of the control and HymnPro-treated groups were 1479 ± 230 and 681 ± 78 mm^3, respectively, accounting for a 54% inhibition of tumor growth in the HymnPro-treated group.

Effect of HymnPro on Cell Cycle Progression. To investigate whether HymnPro affects cell cycle progression, the cell cycle profile of Capan-1 cells was analyzed by flow cytometry. Treatment with HymnPro for 24 h led to an accumulation of cells in the G_2/M phase, from 29.4 to 85.4%, with a concomitant decrease in G_1 and S phase cells (Figure 3A). A population of sub-G_1 phase cells, a characteristic of apoptotic cells, was evident at 46 h after exposure to 20 μM HymnPro; at time 0 h, only ~0.2% of the cells were in sub-G1 phase, whereas ~6.2 and ~24.9% of the cells were in sub-G1 phase after 46 and 72 h, respectively (Figure 3B). These data demonstrate that HymnPro induces cell cycle arrest at the G_2/M phase, followed by cell death, probably through induction of apoptosis in Capan-1 cells.

The Cdk1/cyclin B1 complex controls both entry into and exit from mitosis. Phosphorylation of Cdk1 on Tyr15 and phosphorylation of Cdc25C phosphatase on Ser216 negatively regulates the activation of the Cdk1/cyclin B1 complex.[10] To determine whether HymnPro-induced G_2/M arrest is associated with defects in G_2-to-M phase progression, the protein levels of cyclins and the phosphorylation statuses of Cdk1 and Cdc25C were examined by Western blotting. Treatment with 20 μM HymnPro increased cyclin B1 levels, but slowly reduced cyclin D1 and cyclin A levels for 48 h, whereas cyclin E levels were transiently increased (Figure 3C). Phosphorylation of Cdk1 (Tyr15) decreased markedly from 6 h, and almost none could be detected after 12 h of HymnPro treatment. Phosphorylation of Cdc25C (Ser216) also steadily decreased with HymnPro treatment. Notably, the phosphorylation of aurora kinases (Aurora A, B, and C) and polo-like kinase 1 (PLK1), hallmarks of the G_2/M phase, continuously increased after 6 h of HymnPro exposure. These data suggest that HymnPro-induced G_2/M arrest was not due to defects in G_2-to-M phase progression, but instead seemed to be associated with aberrant execution of mitosis.

Effect of HymnPro on the Inhibition of Tubulin Polymerization. Previous studies have demonstrated that the disruption of microtubule dynamics induces activation of the Cdk1/cyclin B1 complex and arrests the cell cycle in early mitosis.[3] Because several synthetic chalcones show inhibitory effects on tubulin polymerization,[11] we hypothesized that

HymnPro could affect tubulin polymerization to induce mitotic arrest.

To test this hypothesis, we tested the effect of HymnPro on the inhibition of tubulin assembly using an in vitro microtubule assembly assay. When tubulin was polymerized in the presence of GTP, the optical density at 340 nm increased in a time-dependent manner (Figure 4A). Addition of paclitaxel, a microtubule stabilizing agent, immediately stabilized microtubule assembly, whereas nocodazole, a microtubule destabilizing agent, depolymerized microtubules. Addition of HymnPro slowly inhibited tubulin polymerization. After hypotonic cellular lysis, polymerized tubulin can be separated into a particulate fraction from the soluble fraction, where disassembled tubulin dimers remain.[12] To validate the inhibition of tubulin polymerization in vivo by HymnPro, Western blotting was performed. A microtubule stabilizing agent (paclitaxel) and destabilizing agents (nocodazole and colchicines) were used as reference compounds. The results showed that HymnPro, nocodazole, and colchicine reduced, whereas paclitaxel strongly increased, the amount of α-tubulin in the particulate fraction (Figure 4B). These results suggest that HymnPro inhibits microtubule assembly.

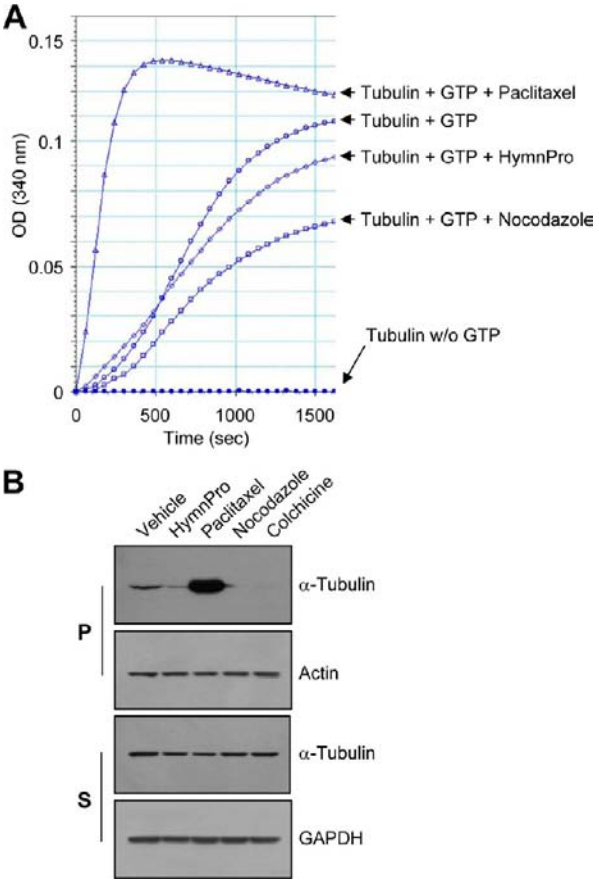

Figure 4. Effect of HymnPro on the inhibition of tubulin polymerization. (A) In vitro tubulin polymerization assay. Tubulin in reaction buffer was incubated at 37 °C without (w/o) or with 1 mM GTP either in the absence or in the presence of 20 μM HymnPro. Paclitaxel (5 μM) or nocodazole (5 μM) was used as a positive or negative reference compound, respectively. The increase in absorbance was measured at 340 nm as a function of time by spectrophotometry. (B) Capan-1 cells were treated with 0.1% DMSO (vehicle) or 20 μM HymnPro, 2 μM paclitaxel, 2 μM nocodazole, or 50 nM colchicines for 24 h. The cells were lysed in hypotonic buffer followed by centrifugation at 12000 rpm for 30 min. The particulate fraction (P) containing insoluble polymerized tubulin and the supernatant fraction (S) containing unpolymerized tubulin were separated and subjected to Western blotting with anti-α-tubulin antibody. Actin was used as a control for the particulate fraction, and GAPDH was the control for the soluble fraction.

Effect of HymnPro on the Cellular Microtubule Network. We next addressed whether HymnPro affects microtubule structure. Morphological features of the microtubules were analyzed by immunofluorescence staining using an anti-α-tubulin antibody. Microtubule networks in control cells (0 h) were intact (Figure 5A, top panels). At 12 h of HymnPro treatment, most microtubule bundles occurred around the nucleus (Figure 5A, middle panels). However, at 24 h of HymnPro treatment, dense compact microtubules were seen in the nucleus, but were absent from the cell periphery and were accompanied by fragmented or multinucleated chromosomes (Figure 5A, bottom panels).

To determine whether dense nuclear staining of tubulin by HymnPro is associated with abnormal microtubule structures, spindle features were analyzed in mitotic cells. Typical bipolar spindles with chromosomes arranged along the metaphase plate were seen in normal mitotic cells (Figure 5B, a–c). However, treatment with HymnPro for 24 h induced abnormal microtubules, which appeared as spindle-like astral monopolar or multipolar microtubules (Figure 5B, d–i; arrows) and a disorganized spherical arrangement of chromosomes (Figure 5B, e, h, and k). Some cells had asymmetric microtubules with chromosomes that remained at the spindle pole (Figure 5B, j–l; arrows).

To determine whether these astral microtubules were mitotic spindles, we analyzed the location of pericentrin, a maker of mitotic centrosomes. In normal mitotic cells, typical pericentrin dots appeared at the spindle poles (Figure 5C, top panels). However, treatment with HymnPro induced singlet or doublet pericentrin dots that were enclosed within spherical chromosomes (arrows). As pericentrin is expressed at centrosomes associated with spindle microtubules,[13] these results suggest that HymnPro caused the failure of mitotic spindle formation or induced a delay in attachment of spindles to the chromosomes, resulting in defects in chromosome congression to the metaphase plate and aberrant kinetochore–microtubule assembly. This would be associated with the blocking of mitosis at the prometaphase or metaphase–anaphase transition.

Effect of HymnPro on Apoptosis. Metaphase catastrophe could cause the induction of apoptosis. We observed that HymnPro-induced apoptosis was followed by G_2/M arrest (Figure 3B). To gain more insight into the functional consequences of HymnPro-induced mitotic arrest, we analyzed the apoptotic status of the cells. One of the earliest cellular changes during apoptosis is translocation of phosphatidylserine (PS), a membrane phospholipid, from the inner to the outer membrane. To determine whether PS appeared in the outer membrane after HymnPro treatment, Annexin V-Cy3 staining was performed. As expected, the population of Annexin V-stained cells strongly increased after 48 h of HymnPro treatment (Figure 6A). In addition, drastic increases in DNA laddering were detected after 48 h of HymnPro treatment (Figure 6B), suggesting the induction of DNA fragmentation, a characteristic of cells undergoing apoptosis, by HymnPro exposure. TUNEL assays confirmed DNA fragmentation in situ as revealed by incorporation of bromodeoxyuridine (BrdU)

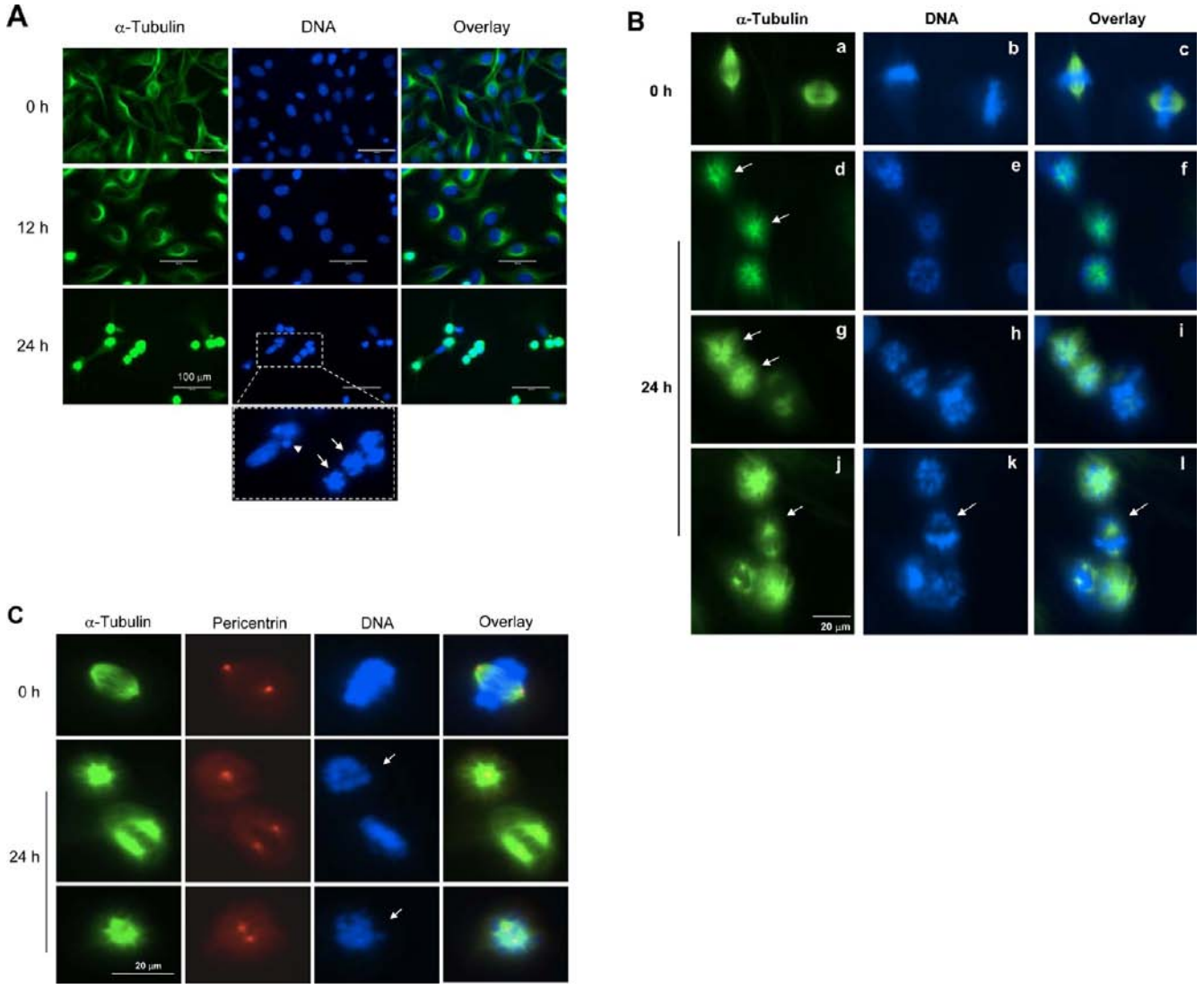

Figure 5. Effect of HymnPro on the cellular microtubule network. (A) Capan-1 cells were cultured on coverslips and treated with 20 μM HymnPro for 12 or 24 h. The cells were fixed and incubated with an antibody against α-tubulin for 2 h, followed by incubation with an AlexaFluor 488-conjugated secondary antibody (green signal) for 30 min. Nuclear DNA was stained with 1 μg/mL Hoechst 33258 for 10 min (blue signal). Overlay images are shown on the right. Fluorescence-positive cells were examined under an EVOSf1 fluorescence microscope. Arrowheads indicate fragmented chromosomes. Arrows indicate multinucleated chromosomes. Scale bar = 100 μm. (B) Capan-1 cells were treated with HymnPro for 24 h followed by incubation with anti-α-tubulin antibody and Hoechst 33258 for 10 min. M phase cells were selected and analyzed in the microtubule network. Arrows in panels d and g indicate abnormal mono- or multipolar microtubules, respectively. Arrows in panels j–l denote asymmetric microtubules (j) with chromosomes at the spindle pole (k, l). Scale bar = 20 μm. (C) Effect of HymnPro on the mitotic spindles. Capan-1 cells were treated with HymnPro for 24 h followed by incubation with anti-α-tubulin or anti-pericentrin antibody. Nuclear DNA was stained with Hoechst 33258. M phase cells were selected and analyzed for the status of mitotic spindles. Fluorescence-positive cells were examined under an EVOSf1 fluorescence microscope. Scale bar = 20 μm. Arrows indicate mono- or bipolar multinucleated chromosomes.

into the spherical chromosomes (Figure 6C). These data suggest that HymnPro induces apoptosis after M phase arrest.

Effect of HymnPro on the Activation of the Caspase Pathway. Disruption of the microtubule network, such as depolymerization or polymerization of tubulin, results in apoptosis.[3] Caspases are cysteinyl aspartate proteinases that cleave substrate proteins at aspartate residues. Upon receiving an apoptotic signal, the precursor caspases undergo proteolytic processing to generate an active subunit, leading to the induction of apoptosis. Previous studies have demonstrated that apoptosis occurring during the metaphase/anaphase transition is characterized by the activation of caspase-2.[14] We therefore investigated the possible involvement of caspase-2 in HymnPro-induced apoptosis. The activation of caspases was monitored by detection of their proteolytic cleavage. Treatment of Capan-1 cells with 20 μM HymnPro promoted time-dependent cleavage of caspase-2, caspase-9, caspase-3, and caspase-7 (Figure 7A). The DNA repair enzyme poly(ADP-ribose) polymerase (PARP) is cleaved by caspase-3 and caspase-7 from its full-length 116 kDa form to an inactive 85-kDa form. We also observed that PARP cleavage was detectable at 24 h and was more extensive by 48 h of HymnPro treatment. Furthermore, pretreatment with a pan-caspase inhibitor, zVAD-fmk, abrogated HymnPro-dependent cleavage of caspase-7, caspase-3, and PARP (Figure 7B). Among caspases, caspase-7 is known to translocate to the nucleus, where its substrate PARP is localized, and regulate chromatin compaction.[15] Fluorescence microscopy revealed that cleaved caspase-7 was detected at the

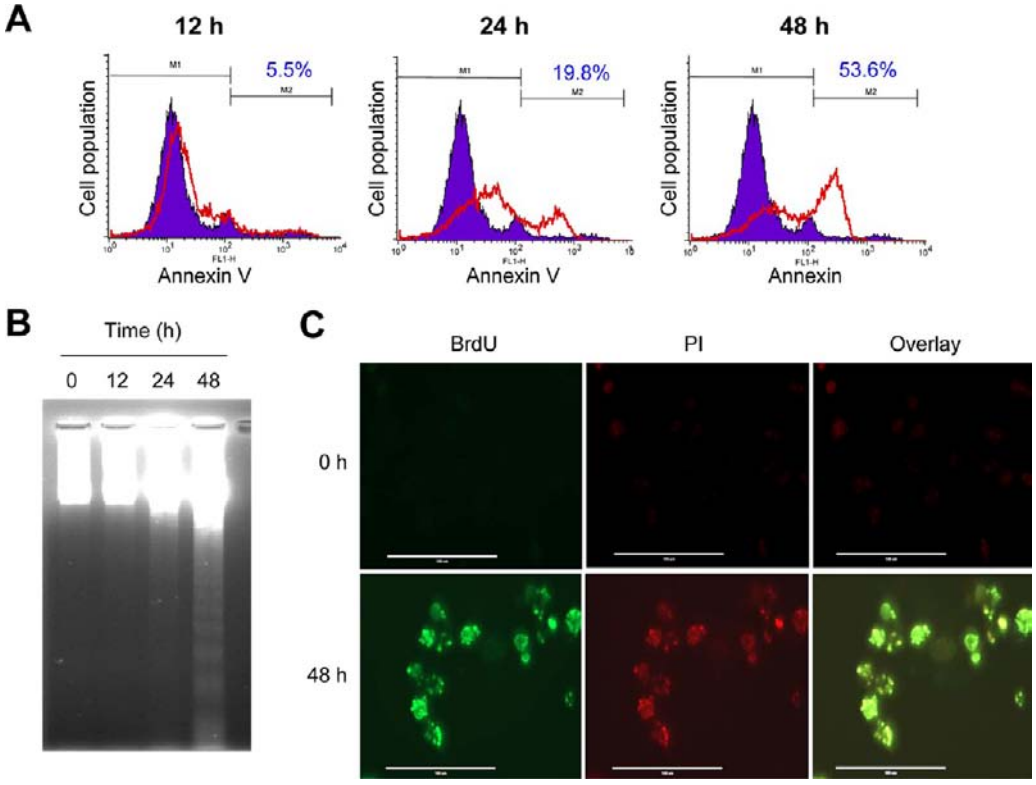

Figure 6. Effect of HymnPro on apoptosis. (A) Capan-1 cells were treated with 20 μM HymnPro for the indicated time. Translocation of phosphatidylserine was detected with Cy3-labeled Annexin V. Cy3-positive cells (apoptotic cells) were analyzed by flow cytometry. (B) Capan-1 cells were treated with 20 μM HymnPro for the indicated time, and DNA was extracted. DNA laddering was visualized by UV light illumination after 1% agarose gel electrophoresis. (C) Capan-1 cells grown on coverslips were treated with 20 μM HymnPro for 48 h and stained by the TUNEL method using Br-dUTP and anti-BrdU-FITC antibody. Propidium iodide (PI) was used as a counterstain. TUNEL-positive cells (apoptotic cells) were analyzed under an EVOSf1 fluorescence microscope.

spherically arranged chromosomes following HymnPro treatment (Figure 7C). These data demonstrate that HymnPro-induced apoptosis is mediated through a caspase-dependent pathway.

Involvement of MAPK Pathways in HymnPro-Induced Apoptosis. Microtube-interfering agents stimulate mitogen-activated protein kinases (MAPKs), which play an important role in mediating apoptosis.[16] To address the possible involvement of MAPK signalings, the phosphorylation status of Erk1/2, JNK1/2, and p38 MAPK was assessed by Western blotting. Following HymnPro treatment, phosphorylation of JNK1/2 and p38 MAPK was detected at 1 h, reached a broad plateau from 2 through 12 h, and decreased thereafter (Figure 7D). In contrast, the basal level of Erk1/2 phosphorylation was high but increased slightly in response to HymnPro treatment. These data suggest that JNK, Erk, and p38 MAPK pathways are activated by HymnPro. To further investigate the role of MAPK signalings in HymnPro-induced apoptosis, we used pharmacological inhibitors of MAPKs. Pretreatment of Capan-1 cells with SP600125 (JNK inhibitor) or U0126 (MEK1 inhibitor) substantially blocked HymnPro-induced cleavage of caspase-3, caspase-7, and PARP (Figure 7E). In contrast, pretreatment with SB203580 (p38 kinase inhibitor) slightly affected the DK-48-induced apoptotic response. These results suggest that MAPK pathways may play an important role in mediating HymnPro-induced apoptosis.

DISCUSSION

Natural and synthetic chalcones display broad bioactivity, including antitumor activity. However, the molecular actions of benzochalcone derivatives have not been studied. The purpose of this study was to characterize the antitumor activity of benzochalcone derivatives and to identify the molecular target(s) involved in the mechanism(s) underlying their antitumor activity. Here, we found that the HymnPro compound, 2-hydroxy-4-methoxy-2′,3′-benzochalcone, strongly suppressed the clonogenicity of Capan-1 pancreatic cancer cells and induced antiproliferative activity in pancreatic cancer cells, including Capan-1 and Mia-PaCa2 as well as HCT116 colon cancer cells. We confirmed the efficacy of HymnPro for inhibiting tumor growth in vivo. This antiproliferative activity was related to inhibition of tubulin polymerization, which led to mitotic arrest followed by triggering of apoptotic cell death.

Microtubules are essential components of the cytoskeleton and play a critical role in many cellular processes, including cell division. Mitotic arrest induced by tubulin-binding agents, such as taxanes (paclitaxel, docetaxel) and vinca alkaloids (vinblastine, vincristine), are usually associated with activation of Cdk1/cyclin B1 activity in many cancer cell lines.[17] Here, we found that treatment with HymnPro reduced cell proliferation in a dose- and time-dependent manner and that this reduction probably resulted from cell cycle arrest at mitosis and apoptotic cell death. Within 18 h of HymnPro treatment, the G_2/M phase cell population had increased at the expense of a decreased population of G_1 phase cells. After 46 h of HymnPro treatment, a slight increase was observed in the sub-G_1 cell population, a

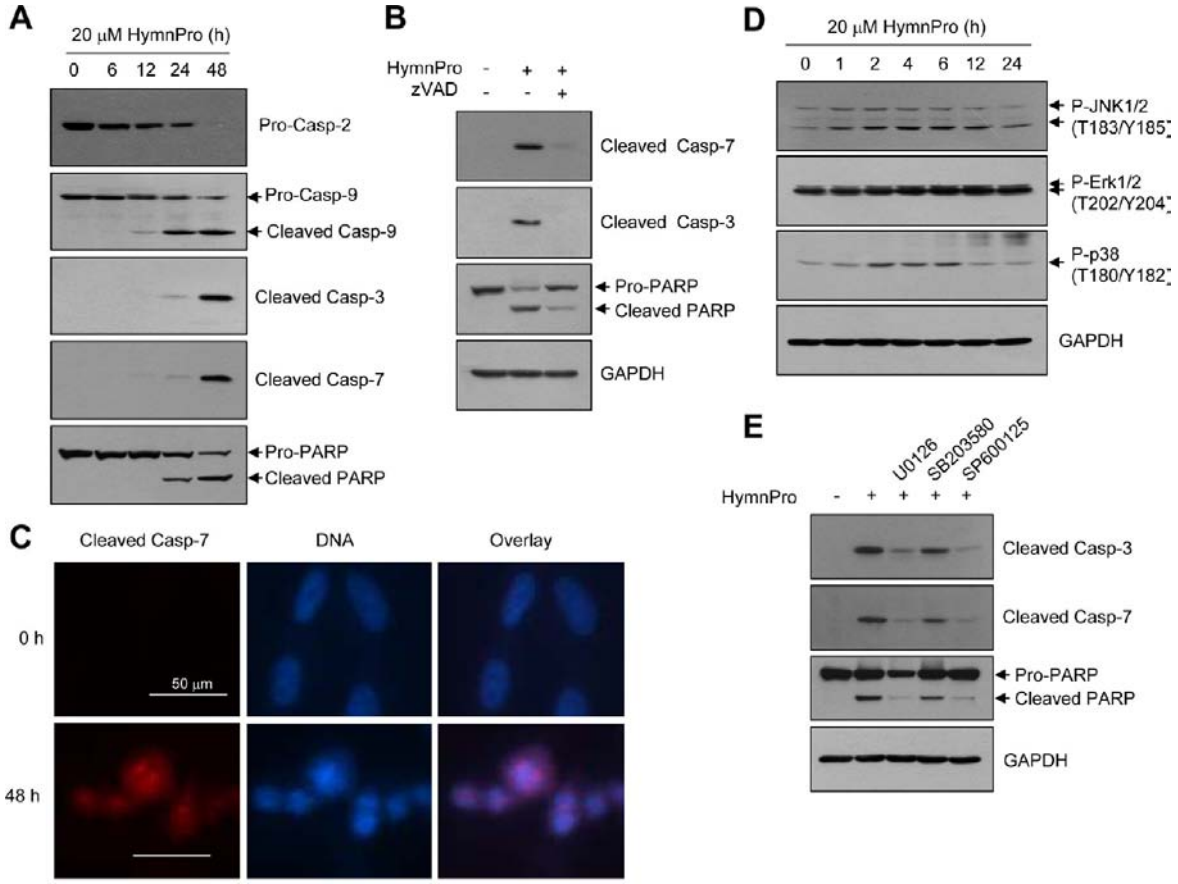

Figure 7. Effect of HymnPro on activation of the caspase cascade. (A, B) Capan-1 cells were treated with 20 μM HymnPro for the indicated time (A) or for 48 h in the absence or presence of the pan-caspase inhibitor zVAD-fmk (B). Whole-cell lysates were then subjected to Western blotting using antibodies against the indicated proteins. GAPDH antibody was used as an internal control to show equal protein loading. (C) Capan-1 cells grown on coverslips were either untreated or treated with 20 μM HymnPro for 48 h. The cells were fixed and incubated with an antibody against cleaved caspase-7 for 2 h, followed by incubation with an AlexaFluor 555-conjugated secondary antibody (red signal) for 30 min. Nuclear DNA was stained with 1 μg/mL Hoechst 33258 for 10 min (blue signal). Overlay images are shown on the right. Fluorescence-positive cells were examined under an EVOSf1 fluorescence microscope. (D) Capan-1 cells were treated with 20 μM HymnPro for the indicated lengths of time, and whole-cell lysates were then subjected to Western blotting using antibodies against the indicated proteins. GAPDH antibody was used as an internal control to show equal protein loading. (E) Capan-1 cells were pretreated with 5 μM U0126, 10 μM SB203580, or 10 μM SP600125 for 30 min and were then treated with 20 μM HymnPro for 48 h. Total protein extracts were analyzed by Western blotting using antibody against cleaved caspase-3 or caspase-7 or PARP.

hallmark of apoptotic cell death. Between 46 and 72 h after HymnPro exposure, the population of sub-G_1 cells had dramatically increased, accompanied by a decrease in the fraction of G_2/M phase cells. These results suggest that apoptotic cell death might result from the G_2/M arrest. The induction of apoptosis by HymnPro was confirmed by internucleosomal DNA laddering, translocation of PS by Annexin V staining, and in situ nick-end labeling by TUNEL staining.

Cell cycle progression is tightly regulated by Cdk/cyclin complexes and other regulatory factors. The G_2/M transition and mitosis are controlled by Cdk1/cyclin B1 complex activity.[18] Cdk1/cyclin B1 activity depends on several factors, including the expression level of cyclin B1 and the phosphorylation status of Cdk1 on different residues. Cdk1 is maintained in an inactive form by Wee1 kinase-mediated phosphorylation at Thr14 and Tyr15.[10c,d] This inhibition is reversed by Cdc25C phosphatase at the late G_2 phase and the beginning of mitosis, which catalyzes the dephosphorylation of these two residues.[10e,f] Cdc25C activity decreases upon exit from mitosis. When Cdc25C is phosphorylated at Ser216, it binds to 14-3-3 proteins, which causes their sequestration in the cytoplasm and results in the inhibition of Cdk1/cyclin B1 complex activity. Our data demonstrated that HymnPro increased cyclin B1 levels and decreased the phosphorylation of Cdk1 on Tyr15, and that of Cdc25C on Ser216, for 48 h after HymnPro treatment. Furthermore, dephosphorylation of Cdk1 on Tyr15 was accompanied by increased phosphorylation of aurora kinases and PLK1. PLK1 is expressed in early G_2 phase and maintained until late M phase;[19] it stimulates the catalytic activity of Cdk1, leading to mitosis entry as well as regulation of several mitotic events, such as mitotic spindle formation. Considering that >85% of cells accumulated at the G_2/M phase after 24 h of HymnPro treatment, it seems likely that HymnPro promotes the cell cycle progression through activation of Cdk1/cyclin B1 until late M Phase. Indeed, well-characterized antimitotic drugs, such as taxanes and vinca alkaloids, up-regulate Cdk1/cyclin B1 activity and induce mitosis arrest.[17,20] Thus, we suggest that HymnPro-induced G_2/M arrest is not associated with defects in G_2/M regulatory proteins or premature mitotic entry, but rather is closely linked with acceleration of entry into mitosis.

To test whether HymnPro disrupts tubulin polymerization to induce mitotic arrest in a manner similar to those of taxanes or vinca alkaloids, we analyzed the effect of HymnPro on tubulin polymerization in vitro and in vivo. Our data clearly showed that HymnPro directly inhibited tubulin polymerization. In addition, HymnPro-exposed cells displayed an altered microtubule network, as revealed by α-tubulin staining using immunofluorescence microscopic analysis. We therefore concluded that HymnPro induces mitotic arrest through targeting the microtubule network. The most remarkable feature of mitotic arrest by HymnPro was the formation of aberrant mono- or multipolar spindles with a spherical arrangement of multinucleated chromosomes. We also observed that HymnPro caused asymmetric microtubules with uncongressed chromosomes that remained at the spindle pole, suggesting that HymnPro might lead to mitotic arrest specifically at the metaphase/anaphase transition. Therefore, we propose that the ability of HymnPro to bind tubulin could cause attenuation of microtubule dynamics, leading to chromosome segregation error or chromosomal structural abnormality during mitosis, which results in apoptotic cell death.

Aberrant mitosis would be characterized by mitotic catastrophe. Mitotic catastrophe is a type of cell death that can occur during mitosis, as a result of DNA damage or abnormal spindle formation with uncondensed chromosomes.[14d] Apoptosis occurring during the metaphase/anaphase transition is characterized by the activation of caspase-2[14a−c] and the release of mitochondrial cytochrome *c*, which leads to sequential activation of caspase-9 and caspase-3. Previous studies have demonstrated that microtube-interfering agents activate ASK1/JNK MAPK signaling,[16a] which acts upstream of caspase-3 activation[21] and plays an important role in apoptosis.[21,22] In line with these findings, we observed that activation of JNK1/2 by HymnPro is followed by sequential activation of caspase-2, caspase-9, caspase-3, and caspase-7. Furthermore, inhibition of JNK by SP600125, a selective JNK inhibitor, abrogated HymnPro-induced activation of caspase-7 and cleavage of PARP, raising the possibility that inhibition of tubulin polymerization by HymnPro may constitute a general strategy to activate the mitotic catastrophe-mediated cell death program.

In conclusion, HymnPro is able to induce mitotic arrest followed by apoptosis through activation of caspase-2, caspase-9, caspase-3, and caspase-7, resulting in the inhibition of tumor growth. These novel effects of HymnPro may provide new insights into the molecular mechanisms responsible for the antitumor effects of benzochalcone derivatives. Overall, our results suggest that the HymnPro compound may be a promising antitumoral agent and may encourage further application of benzochalcone derivatives for cancer treatment and prevention.

AUTHOR INFORMATION

Corresponding Authors

*(Y. Lim) Phone: +82-2-450-3760. Fax: +82-2-444-6176. E-mail: yoongho@konkuk.ac.kr.

*(Y. H. Lee) Phone: +82-2-2049-6115. Fax: +82-2-3437-9781. E-mail: yhlee58@konkuk.ac.kr.

Funding

This study was supported by the Korea Healthcare Technology R&D Project, the Ministry for Health and Welfare (Grant A111778), and the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (NRF-2012R1A2A2A01). This paper was supported by the KU Research Professor Program of Konkuk University.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

HymnPro, (*E*)-1-(2-hydroxy-4-methoxyphenyl)-3-(naphthalen-1-yl)prop-2-en-1-one; MAPK, mitogen-activated protein kinases; PI3K, phosphatidylinositol 3-kinase; PLK, polo-like kinase; PARP, poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling

REFERENCES

(1) (a) Pietta, P. G. Flavonoids as antioxidants. *J. Nat. Prod.* **2000**, *63* (7), 1035−1042. (b) Yao, L. H.; Jiang, Y. M.; Shi, J.; Tomas-Barberan, F. A.; Datta, N.; Singanusong, R.; Chen, S. S. Flavonoids in food and their health benefits. *Plant Foods Hum. Nutr.* **2004**, *59* (3), 113−122.

(2) Orlikova, B.; Tasdemir, D.; Golais, F.; Dicato, M.; Diederich, M. Dietary chalcones with chemopreventive and chemotherapeutic potential. *Genes Nutr.* **2011**, *6* (2), 125−147.

(3) Mollinedo, F.; Gajate, C. Microtubules, microtubule-interfering agents and apoptosis. *Apoptosis* **2003**, *8* (5), 413−450.

(4) Pasquier, E.; Kavallaris, M. Microtubules: a dynamic target in cancer therapy. *IUBMB Life* **2008**, *60* (3), 165−170.

(5) Carlson, R. O. New tubulin targeting agents currently in clinical development. *Expert Opin. Invest. Drugs* **2008**, *17* (5), 707−722.

(6) Hwang, D.; Hyun, J.; Jo, G.; Koh, D.; Lim, Y. Synthesis and complete assignment of NMR data of 20 chalcones. *Magn. Reson. Chem.* **2011**, *49* (1), 41−45.

(7) Hwang, D.; Shin, S. Y.; Lee, Y.; Hyun, J.; Yong, Y.; Park, J. C.; Lee, Y. H.; Lim, Y. A compound isolated from *Schisandra chinensis* induces apoptosis. *Bioorg. Med. Chem. Lett.* **2011**, *21* (20), 6054−6057.

(8) Shin, S. Y.; Kim, J. H.; Lee, J. H.; Lim, Y.; Lee, Y. H. 2′-Hydroxyflavanone induces apoptosis through Egr-1 involving expression of Bax, p21, and NAG-1 in colon cancer cells. *Mol. Nutr. Food Res.* **2012**, *56* (5), 761−774.

(9) D'Arcy, P.; Ryan, B. A.; Brodin, B. Reactivation of p53 function in synovial sarcoma cells by inhibition of p53-HDM2 interaction. *Cancer Lett.* **2009**, *275* (2), 285−292.

(10) (a) Smits, V. A.; Medema, R. H. Checking out the G(2)/M transition. *Biochim. Biophys. Acta* **2001**, *1519* (1−2), 1−12. (b) Donzelli, M.; Draetta, G. F. Regulating mammalian checkpoints through Cdc25 inactivation. *EMBO Rep.* **2003**, *4* (7), 671−677. (c) Liu, F.; Stanton, J. J.; Wu, Z.; Piwnica-Worms, H. The human Myt1 kinase preferentially phosphorylates Cdc2 on threonine 14 and localizes to the endoplasmic reticulum and Golgi complex. *Mol. Cell. Biol.* **1997**, *17* (2), 571−583. (d) McGowan, C. H.; Russell, P. Human Wee1 kinase inhibits cell division by phosphorylating p34cdc2 exclusively on Tyr15. *EMBO J.* **1993**, *12* (1), 75−85. (e) Parker, L. L.; Piwnica-Worms, H. Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase. *Science* **1992**, *257* (5078), 1955−1957. (f) Nilsson, I.; Hoffmann, I. Cell cycle regulation by the Cdc25 phosphatase family. *Prog. Cell Cycle Res.* **1998**, *4*, 107−114.

(11) (a) Tu, H. Y.; Huang, A. M.; Hour, T. C.; Yang, S. C.; Pu, Y. S.; Lin, C. N. Synthesis and biological evaluation of 2′,5′-dimethoxychalcone derivatives as microtubule-targeted anticancer agents. *Bioorg. Med. Chem.* **2010**, *18* (6), 2089−2098. (b) Zhang, H.; Liu, J. J.; Sun, J.; Yang, X. H.; Zhao, T. T.; Lu, X.; Gong, H. B.; Zhu, H. L. Design, synthesis and biological evaluation of novel chalcone derivatives as antitubulin agents. *Bioorg. Med. Chem.* **2012**, *20* (10), 3212−3218. (c) Kim do, Y.; Kim, K. H.; Kim, N. D.; Lee, K. Y.; Han, C. K.; Yoon, J. H.; Moon, S. K.; Lee, S. S.; Seong, B. L. Design and biological evaluation of novel tubulin inhibitors as antimitotic agents using a pharmacophore binding model with tubulin. *J. Med. Chem.* **2006**, *49* (19), 5664−5670.

(12) Wu, R.; Ding, W.; Liu, T.; Zhu, H.; Hu, Y.; Yang, B.; He, Q. XN05, a novel synthesized microtubule inhibitor, exhibits potent activity against human carcinoma cells in vitro. *Cancer Lett.* **2009**, *285* (1), 13−22.
(13) Chen, H.; Gos, A.; Morris, M. A.; Antonarakis, S. E. Localization of a human homolog of the mouse pericentrin gene (PCNT) to chromosome 21qter. *Genomics* **1996**, *35* (3), 620−624.
(14) (a) Andersen, J. L.; Johnson, C. E.; Freel, C. D.; Parrish, A. B.; Day, J. L.; Buchakjian, M. R.; Nutt, L. K.; Thompson, J. W.; Moseley, M. A.; Kornbluth, S. Restraint of apoptosis during mitosis through interdomain phosphorylation of caspase-2. *EMBO J.* **2009**, *28* (20), 3216−3227. (b) Fabbri, F.; Amadori, D.; Carloni, S.; Brigliadori, G.; Tesei, A.; Ulivi, P.; Rosetti, M.; Vannini, I.; Arienti, C.; Zoli, W.; Silvestrini, R. Mitotic catastrophe and apoptosis induced by docetaxel in hormone-refractory prostate cancer cells. *J. Cell Physiol.* **2008**, *217* (2), 494−501. (c) Mhaidat, N. M.; Wang, Y.; Kiejda, K. A.; Zhang, X. D.; Hersey, P. Docetaxel-induced apoptosis in melanoma cells is dependent on activation of caspase-2. *Mol. Cancer Ther.* **2007**, *6* (2), 752−761. (d) Castedo, M.; Perfettini, J. L.; Roumier, T.; Andreau, K.; Medema, R.; Kroemer, G. Cell death by mitotic catastrophe: a molecular definition. *Oncogene* **2004**, *23* (16), 2825−2837.
(15) Erener, S.; Petrilli, V.; Kassner, I.; Minotti, R.; Castillo, R.; Santoro, R.; Hassa, P. O.; Tschopp, J.; Hottiger, M. O. Inflammasome-activated caspase 7 cleaves PARP1 to enhance the expression of a subset of NF-kappaB target genes. *Mol. Cell* **2012**, *46* (2), 200−211.
(16) (a) Wang, T. H.; Wang, H. S.; Ichijo, H.; Giannakakou, P.; Foster, J. S.; Fojo, T.; Wimalasena, J. Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. *J. Biol. Chem.* **1998**, *273* (9), 4928−4936. (b) Xiao, D.; Pinto, J. T.; Soh, J. W.; Deguchi, A.; Gundersen, G. G.; Palazzo, A. F.; Yoon, J. T.; Shirin, H.; Weinstein, I. B. Induction of apoptosis by the garlic-derived compound S-allylmercaptocysteine (SAMC) is associated with microtubule depolymerization and c-Jun NH(2)-terminal kinase 1 activation. *Cancer Res.* **2003**, *63* (20), 6825−6837.
(17) Donaldson, K. L.; Goolsby, G. L.; Kiener, P. A.; Wahl, A. F. Activation of p34cdc2 coincident with taxol-induced apoptosis. *Cell Growth Differ.* **1994**, *5* (10), 1041−1050.
(18) Nurse, P. Universal control mechanism regulating onset of M-phase. *Nature* **1990**, *344* (6266), 503−508.
(19) Hamanaka, R.; Smith, M. R.; O'Connor, P. M.; Maloid, S.; Mihalic, K.; Spivak, J. L.; Longo, D. L.; Ferris, D. K. Polo-like kinase is a cell cycle-regulated kinase activated during mitosis. *J. Biol. Chem.* **1995**, *270* (36), 21086−21091.
(20) Ling, Y. H.; Consoli, U.; Tornos, C.; Andreeff, M.; Perez-Soler, R. Accumulation of cyclin B1, activation of cyclin B1-dependent kinase and induction of programmed cell death in human epidermoid carcinoma KB cells treated with taxol. *Int. J. Cancer* **1998**, *75* (6), 925−932.
(21) Wang, T. H.; Popp, D. M.; Wang, H. S.; Saitoh, M.; Mural, J. G.; Henley, D. C.; Ichijo, H.; Wimalasena, J. Microtubule dysfunction induced by paclitaxel initiates apoptosis through both c-Jun N-terminal kinase (JNK)-dependent and -independent pathways in ovarian cancer cells. *J. Biol. Chem.* **1999**, *274* (12), 8208−8216.
(22) (a) Maundrell, K.; Antonsson, B.; Magnenat, E.; Camps, M.; Muda, M.; Chabert, C.; Gillieron, C.; Boschert, U.; Vial-Knecht, E.; Martinou, J. C.; Arkinstall, S. Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. *J. Biol. Chem.* **1997**, *272* (40), 25238−25242. (b) Blagosklonny, M. V.; Giannakakou, P.; el-Deiry, W. S.; Kingston, D. G.; Higgs, P. I.; Neckers, L.; Fojo, T. Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death. *Cancer Res.* **1997**, *57* (1), 130−135. (c) Poruchynsky, M. S.; Wang, E. E.; Rudin, C. M.; Blagosklonny, M. V.; Fojo, T. Bcl-xL is phosphorylated in malignant cells following microtubule disruption. *Cancer Res.* **1998**, *58* (15), 3331−3338.