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Synthesis of apoptotic chalcone analogues in HepG2 human hepatocellular carcinoma cells

Cheon-Soo Park [a,†], Yongchel Ahn [b,†], Dahae Lee [c], Sung Won Moon [d,e], Ki Hyun Kim [f], Noriko Yamabe [c], Gwi Seo Hwang [c], Hyuk Jai Jang [a], Heesu Lee [g], Ki Sung Kang [c,*], Jae Wook Lee [e,h,*]

[a] Department of Surgery, Gangneung Asan Hospital, University of Ulsan College of Medicine, Gangneung 210-711, Republic of Korea
[b] Department of Hematology and Oncology, Gangneung Asan Hospital, University of Ulsan College of Medicine, Gangneung 210-711, Republic of Korea
[c] College of Korean Medicine, Gachon University, Seongnam 461-701, Republic of Korea
[d] Department of Chemistry, Gangneung Wonju National University, Gangneung 210-340, Republic of Korea
[e] Natural Product Research Center, Korea Institute of Science and Technology, Gangneung 210-340, Republic of Korea
[f] Natural Product Research Laboratory, School of Pharmacy, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[g] Department of Oral Anatomy, College of Dentistry, Gangneung Wonju National University, 201-340, Republic of Korea
[h] Department of Biological Chemistry, University of Science and Technology (UST), Daejeon 305-333, Republic of Korea

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ABSTRACT

Eight chalcone analogues were prepared and evaluated for their cytotoxic effects in human hepatoma HepG2 cells. Compound **5** had a potent cytotoxic effect. The percentage of apoptotic cells was significantly higher in compound **5**-treated cells than in control cells. Exposure to compound **5** for 24 h induced cleavage of caspase-8 and -3, and poly (ADP-ribose) polymerase (PARP). Our findings suggest that compound **5** is the active chalcone analogue that contributes to cell death in HepG2 cells via the extrinsic apoptotic pathway.

Hepatocellular carcinoma (HCC) is a highly lethal tumor that commonly occurs in patients with chronic liver disease and cirrhosis.[1] HCC has a poor prognosis due to its high recurrence rate and resistance to chemotherapy.[2] To date, liver transplantation is the best form of treatment because it removes the tumor as well as damaged hepatic tissues that may provoke chronic liver disorders.[1] Several experimental and clinical investigations have occurred, with the goal of preventing recurrence and secondary tumors and improve the clinical outcome of HCC patients.[3]

Chalcone is an aromatic ketone or enone structure that forms the central core and exhibits a wide range of biological activities, including anticancer effects.[4] Isoliquiritigenin is a natural pigment with the simple chalcone structure 4,2′,4′-trihydroxychalcone. Isoliquiritigenin inhibits the proliferation of human hepatoma cells (HepG2) by G2/M phase arrest and programmed cell death.[5] Xanthohumol, the major prenylated chalcone found in hops, induces apoptosis in two HCC cell lines (HepG2 and Huh7).[6] In addition, chalcone derivatives from the fern *Cyclosorus parasiticus* exhibit potent cytotoxicity against six cancer cell lines (e.g., lung cancer A549, HepG2, breast cancer MCF-7 and MDA-MB-231, leukemia ALL-SIL, and pancreatic cancer SW1990).[7] Based on these findings, in this study we synthesized eight chalcone analogues and evaluated their cytotoxic effects on human hepatoma HepG2 cells (see Fig. 1).

We synthesized chalcone analogues (1–8) with methoxy or halogenated groups that have electron-donating or electron-accepting properties. For synthesis, we used Claisen–Schmidt condensation between substituted acetophenone and benzaldehyde in the presence of aqueous NaOH solution.[8] A reaction mixture of benzaldehyde and acetophenone in EtOH treated with NaOH solution was refluxed overnight. The synthesized chalcone derivatives were purified by either silica gel column chromatography or recrystallization with EtOH. All products were characterized using ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, and liquid chromatography–mass spectrometry (LC–MS).

Cytotoxic effects of the chalcone analogues were assessed in HepG2 cells using a MTT assay.[9] As shown in Figure 2, two compounds (**3** and **5**), each possessing a 3,5-dimethoxy-4-bromo phenyl group, inhibited HepG2 cell growth by more than 50%. Of

* Corresponding authors. Tel.: +82 33 750 5402; fax: +82 33 750 5416 (K.K.S.); tel./fax: +82 33 650 3502 (L.J.W.).
E-mail addresses: kkang@gachon.ac.kr (K.S. Kang), jwlee5@kist.re.kr (J.W. Lee).
† These authors contributed equally to this work.

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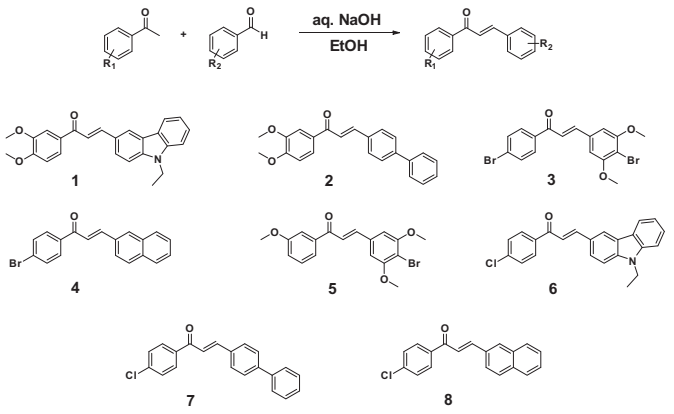

Figure 1. Synthesis of chalcone analogues and their structures.

these, compound **5** inhibited HepG2 cell growth up to 91% and 98% of the control growth at 50 and 100 μM, respectively. However, for the other chalcone analogues (1, 2, 4, 6–8), we observed weak to no inhibition in cell growth when compared to the control. Consequently, we concluded that, of the eight synthesized chalcone derivatives, compound **5** was the most cytotoxic to HepG2 cells (IC_{50}: 26 μM) and less cytotoxic activity against normal porcine kidney proximal tubular epithelial LLC-PK1 cells (IC_{50}: 83 μM). Therefore, further mechanistic studies were conducted with compound **5**.

To determine whether compound **5** induced apoptosis in HepG2 cells, we performed flow cytometry using annexin V as a fluorescein isothiocyanate (FITC) conjugate in combination with propidium iodide (PI) as an exclusion dye to determine cell viability.[10]

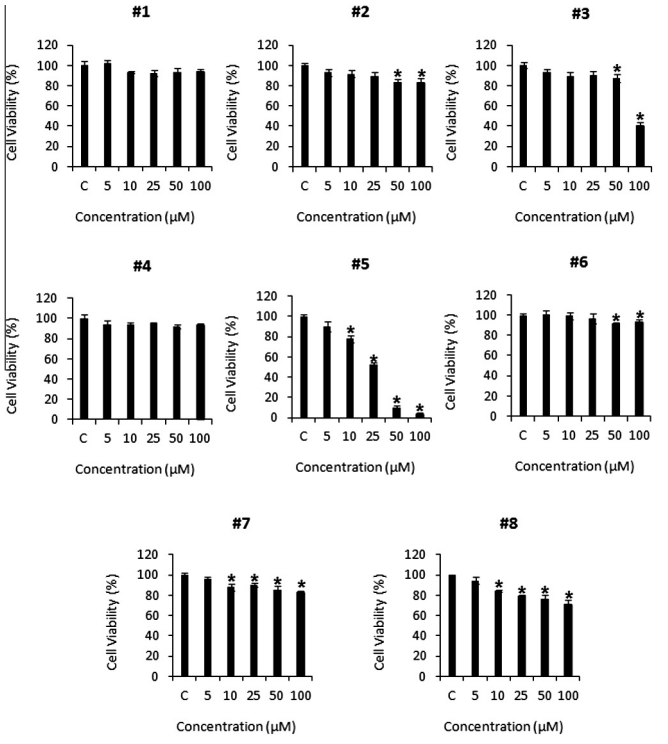

Figure 2. Comparison of the cytotoxic effects of all chalcone derivatives on HepG2 cells. HepG2 cells were pretreated with various concentrations (up to 100 μM) of chalcone derivatives for 24 h. Cell viability was assessed using a MTT assay. $^{*}p < 0.05$ compared to the control.

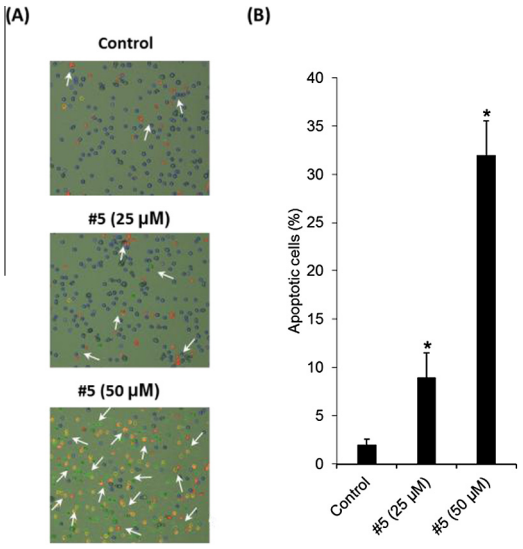

Figure 3. Effects of compound **5** on HepG2 cell apoptosis. (A) Representative images for the detection of apoptosis and (B) percentage of annexin V^{+} apoptotic cells. Dead cells and apoptotic cells were stained red and green, respectively. Apoptosis was determined using a Tali image-based cytometer. $^{*}p < 0.05$ compared to the control.

Representative data of the annexin V/PI flow cytometry results are shown in Figure 3. Exposure to compound **5** resulted in significant apoptosis. Compound **5**, at 25 and 50 μM, resulted in 8.5 ± 2.2% and 31.2 ± 2.9% early apoptotic cell populations, respectively, compared to the control (1.9 ± 0.5%) (Fig. 3).

The control HepG2 cells had a normal appearing morphology (Fig. 4(A)). However, a large proportion cells shrank, and

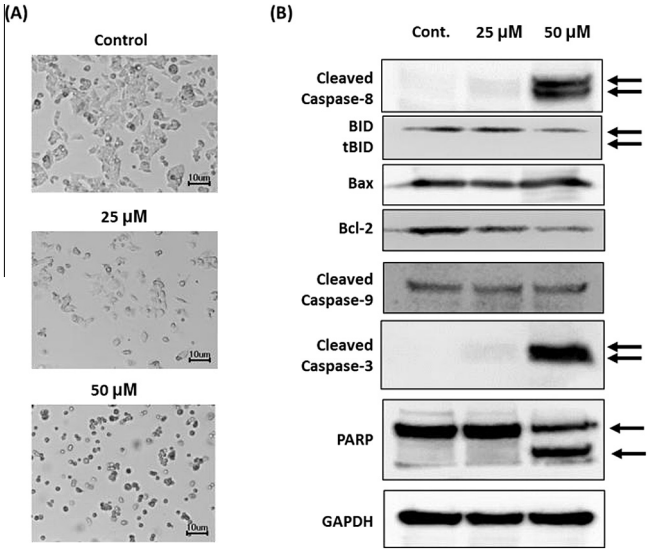

Figure 4. Compound **5** activates the extrinsic apoptotic pathway in HepG2 cells. (A) Morphological changes were confirmed using phase-contrast microscopy. (B) Protein expression levels of cleaved caspase-8, BID, Bax, Bcl-2, cleaved caspase-9, cleaved caspase-3, PARP, and GAPDH in HepG2 cells. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h at 37 °C. The cells were treated with different concentrations of compound **5**. Results from Western blot analysis shows the levels of cleaved caspase-8, BID, Bax, Bcl-2, cleaved caspase-9, cleaved caspase-3, PARP, and GAPDH in HepG2 cells treated with compound **5** for 24 h. Whole-cell lysates (20 μg) were separated by SDS–PAGE, transferred onto PVDF membranes, and probed with the indicated antibodies. Proteins were visualized using an ECL detection system.

progressive condensation and break up (karyorrhexis) of the nucleus were detected in most cells after co-treatment with the compound **5** (Fig. 4(A)). Western blotting was performed to evaluate the expression of proteins involved in the apoptotic response to determine if apoptosis occurs via the intrinsic (mitochondrial) or extrinsic pathway.[11] Results from Western blot analysis are shown in Figure 4. Exposure to compound **5** for 24 h induced the cleavage of caspase-8 and -3, and poly (ADP-ribose) polymerase (PARP). In addition, we observed a significant increase in the ratio of Bax/Bcl-2 (Fig. 4(B)). Caspase-9 is activated through the mitochondria-dependent intrinsic pathway.[12] Based on no changes in cleaved capase-9, compound **5** induced apoptosis via the extrinsic apoptotic pathway.

Cesar et al. reported a relationship between the structural characteristics of synthetic chalcones and their anti-tumor activities.[4] Treatment of HepG2 cells with synthetic 2′-hydroxychalcones for 24 h induced apoptosis, which was mediated via the activation of caspase-9 and subsequently nuclear apoptosis.[4] Zhang et al. reported that chalcone with a *p*-substituted methyl group at the B ring and thiosemicarbazide had the greatest inhibitory effect on HepG2 cells compared to other synthesized chalcone-type thiosemicarbazide compounds.[13] In the present study, we determined that 3,5-dimethoxy-4-bromo phenyl structure of compounds (**3** and **5**) is important for inducing apoptosis in HepG2 cells via the extrinsic pathway.

In summary, compound **5** showed strong cytotoxicity and promoted apoptosis in human hepatoma HepG2 cells. Exposure to compound **5** for 24 h induced the cleavage of caspase-8 and -3, and PARP. The percentage of apoptotic cells was significantly higher in compound **5**-treated cells than in control cells. These findings suggest that compound **5** is the active chalcone analogue that contributes to apoptotic cell death in human hepatoma cells. Future studies will focus on the effect of compound **5** on unexplored areas like tumor invasion and metastasis.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.10.093.

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9. Human hepatoma HepG2 cells were purchased from the American Type Culture Collection (ATCC, Rockville, USA). HepG2 cells were maintained in DMEM (Gibco, Gaithersburg, USA) supplemented with 10% fetal bovine serum (Gibco), 100 units/mL penicillin, and 100 μg/mL streptomycin, and incubated at 37 °C in a humidified incubator with 5% CO_2. The MTT assay was conducted according to a previously published study: Eom et al *BMB Rep.* **2015**, *48*, 461.
10. Apoptotic cells were analyzed by annexin V and PI staining using an Annexin-V-FLUOS Staining Kit (Roche, Indianapolis, USA) according to the manufacturer's recommendations. Briefly, whole cells were collected, rinsed with DPBS, incubated with 100 μL annexin-V binding buffer containing 2 μL annexin-V and 2 μL PI at rt for 15 min in the dark, and analyzed by a FACSCalibur flow cytometer. At least 20,000 events were evaluated in these experiments: Lee et al *Bioorg. Med. Chem. Lett.* **2015**, *25*, 1929.
11. Human hepatoma cells were grown in 60 mm dishes and treated with 25 and 50 μM compound **5** for 24 h. Whole-cell extracts were prepared as reported previously using RIPA buffer (Cell Signaling, Beverly, USA) supplemented with 1× protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride: Park et al *J. Ginseng Res.* **2014**, *38*, 22. For Western blot analysis, proteins (whole-cell extracts: 30 μg/lane) were separated by electrophoresis through a NuPAGE 4–12% Bis-tris gel (Invitrogen, Carlsbad, USA), transferred onto a PVDF membrane, and analyzed with the epitope-specific primary and secondary antibodies. Bound antibodies were visualized using ECL Advance Western Blotting Detection Reagents (GE Healthcare, Waukesha, USA) and an ImageQuant LAS-4000 Imager (Fujifilm, Tokyo, Japan).
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