Anticancer Activity of Hispidin *via* Reactive Oxygen Species-mediated Apoptosis in Colon Cancer Cells

JI-HONG LIM*, YOON-MI LEE*, SA RA PARK, DA HYE KIM and BEONG OU LIM

Department of Applied Biochemistry, College of Biomedical & Health Science, Research Institute of Inflammatory Disease, Konkuk University, Chungju, South Korea

Abstract. *Few studies have been performed on the anticancer activity of hispidin, a phenolic compound produced from the medicinal mushroom Phellinus linteus. Herein, we studied hispidin-induced apoptosis, which is associated with the generation of reactive oxygen species (ROS) in colon cancer cells. Hispidin was found to reduce cell viability both in mouse and human colon cancer cells. Apoptotic cell morphological changes were observed by microscopy, and apoptosis was assessed in hispidin-treated cells using a biochemical method. The results showed accumulation of the sub-G_1 cell population and increase in early apoptosis in a dose-dependent manner. In addition, hispidin induced apoptosis through up-regulation of both intrinsic and extrinsic apoptotic pathways. Although the molecular mechanism underlying hispidin-induced apoptosis is known to involve the generation of ROS, however hispidin did not show any apoptosis in the pre-treatment with a ROS scavenger, N-acetyl-L-cysteine. In conclusion, hispidin induces both intrinsic and extrinsic apoptotic pathways mediated by ROS in colon cancer cells, thereby suggesting that hispidin could be a promising new anticancer agent.*

Colon cancer is regarded as a severe health problem, with a high rate of mortality in patients (1). Patients with colon cancer can be treated by surgery. However, in most cases, surgery cannot be performed since cancer has already metastasized by the time of detection. Therefore, chemotherapy is used as an alternative approach for treating colon cancer. Although, many efforts have been made to develop a chemotherapeutic method for colon cancer, the efficacy of chemotherapy and the defined molecular mechanisms are not yet well-understood (2, 3).

Apoptosis is essential for normal cellular activities. A crucial characteristic of cancer cells is an imbalance between pro- and anti-apoptotic processes that leads to improper cell growth. Therefore, induction of apoptosis could be considered as a therapeutic strategy for the treatment of cancer (4). Many studies have been performed on the treatment of cancer by inducing apoptosis (5, 6). There are two major apoptotic pathways: intrinsic and extrinsic pathways. The intrinsic pathway is triggered in mitochondria by several stimuli such as DNA damage, hypoxia, and oxidative stress. Eventually, proapoptotic proteins increase mitochondrial permeability to promote secretion of cytochrome *c* into the cytoplasm, initiating sequential activation of caspases and poly-(ADP-ribose) polymerase (PARP). The extrinsic pathway is activated upon binding of death ligands to the transmembrane death receptors, forming the death-inducing signaling complex (DISC). This eventually induces the activation of caspases and PARP, which trigger cell death (7, 8).

Due to the undesirable toxicity of chemotherapy, natural products are desirable as medicines for various diseases. Hispidin is a phenolic compound isolated from *Phellinus linteus*, a Basidiomycete fungus belonging to the genus Phellinus. *P. linteus* is a medicinal mushroom that is cultivated in Korea, Japan, and China and is well-known for its anti-oxidant activity. Among the components of *P. linteus*, hispidin has various biological functions such as anti-oxidant and anticancer activities *via* inhibition of protein kinase C (9, 10). However, the anticancer activity of hispidin in colon cancer and its molecular mechanism are not yet clear. To our knowledge, this study is the first to show the anticancer activity of hispidin –involving apoptosis– in colon cancer cells.

Materials and Methods

Reagents and antibodies. Hispidin, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-dichlorofluorescin diacetate (DCF-DA), NAC (*N*-acetyl-L-cysteine) and propidium iodide (PI) were obtained from Sigma (St.

*These Authors contributed equally to this study.

Correspondence to: Beong Ou Lim, Sang-Huh Research Building #304, Konkuk University, Chungwondaero 268, Chungju, Chungbuk, Korea, 380-701. Tel: +82 438403570, Fax: +82 438563572, e-mail: beongou@kku.ac.kr

Key Words: Apoptosis, colon cancer, p53, death receptor 3, reactive oxygen species (ROS) hispidin.

Louis, MO, USA). Hoechst 33342 was purchased from BD Biosciences (San Jose, CA, USA), and Annexin V-FITC (fluorescein isothiocyanate) was purchased from Enzo Life Sciences (New York, NY, USA). All antibodies (p53, BCL2, β-Actin, DR3 and caspases) were purchased from Santa Cruz (Santa Cruz, CA, USA).

Cell culture. Mouse colon cancer cells (CMT-93) and human colon cancer cells (HCT 116) were obtained from the Korean cell line bank (Seoul, Korea). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 (Gibco BRL, Life technologies, Grand Island, NY, USA), containing 10% heat-inactivated fetal bovine serum with 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified incubator with 5% CO_2.

MTT assay. Cells were seeded in 96-well plates at a density of 1×10^4 cells per well. The cells were treated with hispidin (0-200 μg/ml) on the next day for 24 h. MTT solution (final concentration, 0.5 mg/ml in media) was then added to each well, and the plate was incubated at 37˚C for an additional 4 h. After removing all medium in each well, 100 μl of DMSO was added to each well and the optical density (O.D.) was measured at 570 nm.

Analysis of cell morphology. Cells were seeded in 6-well plates at a density of 1×10^4 per well. On the next day, the cells were treated with hispidin (0-200 μg/ml) for 18 h. Cell morphologies were then observed and analyzed by phase-contrast microscopy.

Hoechst 33342 staining of cells. Cells were seeded in 96-well plates at a density of 1×10^4 per well. The cells were treated with hispidin (0-200 μg/ml) for 18 h. Then, 100 μl of 2 μg/ml Hoechst 33342 staining solution was added to each well for 15 min. Results were observed using florescence microscopy.

Measurement of cell-cycle distribution. Cells were treated with hispidin (0-200 μg/ml) for 18 h. The cells were then harvested, fixed with ice-chilled 70% ethanol, and stored at 4˚C overnight. On the next day, they were centrifuged at 16,000 ×g for 5 min with 1 ml of cold PBS. After discarding the supernatant, the cell pellets were stained with PI (500 μg/ml) in the presence of RNase A (10 μg/ml) and incubated at 37˚C for 30 min. Cell cycle distribution was analyzed by using flow cytometry (Cellquest 3.1 software; Becton-Dickinson, San Jose, CA, USA.).

Annexin V/FITC staining. Cells were treated with hispidin (0-200 μg/ml) for 18 h. The cells were then harvested and suspended with 1 × binding buffer (according to the supplier's instructions). Afterwards, the cells were stained with Annexin V and PI, followed by incubation on ice for 10 min in the dark. Results were analyzed using flow cytometry (Cellquest 3.1 software; Becton-Dickinson).

Western blotting. Cells were treated with hispidin (0-200 μg/ml) for 18 h. The cells were then harvested and lysed with RIPA buffer [25 mM Tris-Cl (pH 7.4), 120 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, and 1% sodium deoxycholate with protease inhibitors and phosphatase inhibitors]. The protein concentration of each sample was measured using the Bio-Rad BCA protein assay reagents. Equal amounts of protein samples were loaded onto SDS/polyacrylamide gels and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Afterwards, the membranes were incubated with 5% skim milk (blocking solution) at room temperature for 1 h, followed by incubation with primary antibodies (p53, BCL2, β-Actin, DR3 and caspases) at 4˚C overnight. On the next day, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h. Enhanced chemiluminescence (ECL, SurModics, MN, USA) was used to detect proteins immobilized on membranes.

Measurement of intracellular ROS levels. Cells were seeded in 96-well plates at a density of 1×10^4 cells per well and treated with hispidin (0-200 μg/ml) for 18 h in the absence or presence of NAC. The cells were then harvested, washed with cold PBS, and stained with 10 μM DCF-DA for 30 min in dark. Values were analyzed by reading Ex/Em=485/530nm (Wallac Victor2 Multi-label Counter).

Statistical analysis. All experiments were performed in triplicates; data are presented as mean±standard deviation (SD). Statistical analysis was performed using Graph Pad Prism (La Jolla, CA, USA) and Microsoft Office Excel 2007 (for Student's *t*-test). Graphical evaluations were performed by Sigma plot 10.0. Values were considered significant when $p<0.05$.

Results

Hispidin affects the viability of colon cancer cells. The viability of colon cancer cells, treated with hispidin, was measured by the MTT assay. Hispidin significantly decreased the viability of mouse colon cancer cells (CMT-93) (Figure 1A) and human colon cancer cells (HCT 116) (Figure 1B) in a dose-dependent manner. These results showed that hispidin had an inhibitory effect on cell viabilities both in mouse and human colon cancer cells.

Hispidin induces apoptotic changes in cell morphology. Changes in cell morphology after hispidin (0-200 μg/ml) treatment were analyzed by phase-contrast microscopy (Figure 2A). On treatment with hispidin (0-200 μg/ml), a wide range of morphological changes were observed in cells, such as detachment or shrinkage, consistent with previous reports on the morphological features of apoptotic cells (11). These changes in cellular architecture were dose-dependent. Furthermore, Hoechst 33342 staining was performed to examine apoptotic nuclear condensation (Figure 2B). We observed a normal nuclear appearance in vehicle-treated cells, whereas the nucleus became condensed with an increase in the dose of hispidin. Thus, we found that hispidin induces apoptotic changes in the morphology of colon cancer cells.

Analysis of hispidin-induced apoptosis by flow cytometry. As cell growth inhibition was observed in hispidin-treated cells, effects of hispidin on cell cycle were analyzed by examining cell-cycle distribution with flow cytometry (Figure 3A). Upon hispidin treatment, cell population in the sub-G_1 phase in CMT-93 cells increased from 25.23% to 40.4% in a dose-dependent manner. These results indicated that hispidin induced apoptosis of the cells. We further examined hispidin-induced apoptosis in CMT-93 cells by Annexin V and PI

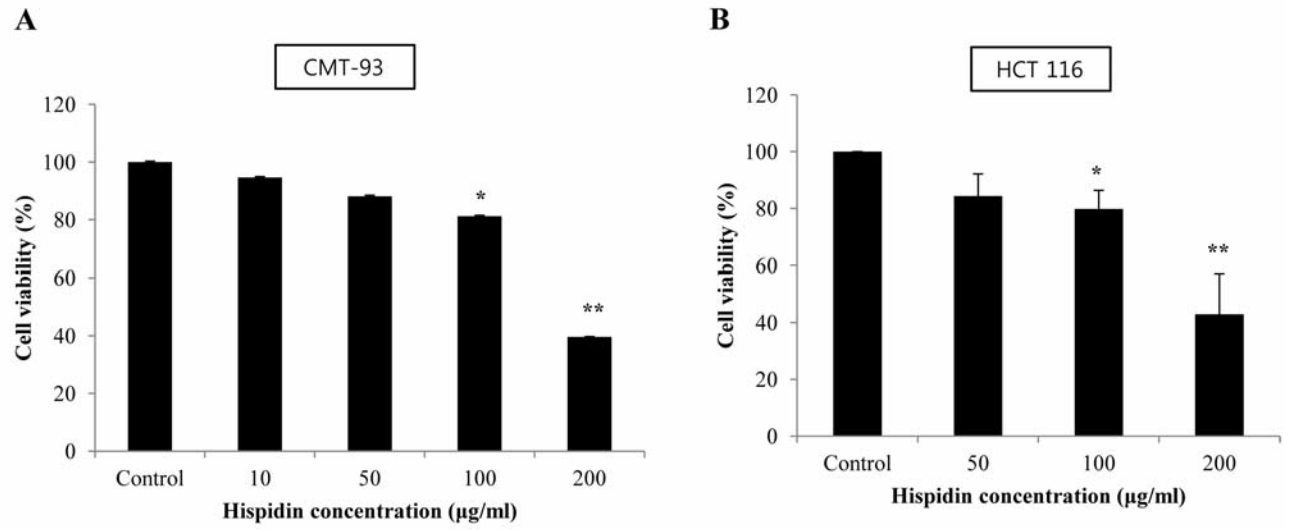

Figure 1. *Hispidin affects the viability of colon cancer cells. Mouse colon cancer cells (CMT-93) (A) and human colon cancer cells (HCT 116) (B) were treated with hispidin (0-200 μg/ml) for 24 h. Results were analyzed by the MTT assay and expressed as the mean±S.D. (n=4). *p<0.05, **p<0.01 vs. vehicle-treated cells.*

staining (Figure 3B). When cells were treated with hispidin, early apoptotic changes (lower right panel) were observed and increased gradually in a dose-dependent manner compared to the extent seen in vehicle-treated cells. Thus, it can be assumed that hispidin induced apoptosis in colon cancer cells.

Hispidin affects the expression of apoptotic genes. For evaluation of hispidin-induced apoptosis, we investigated the expression of a representative tumor-suppressor protein, p53. Interestingly, p53 protein levels were increased by hispidin in a dose-dependent manner. In addition, the levels of the Bax (BCL2 associated X) protein increased. Bax induces apoptosis as a downstream signaling molecule of p53, whereas the protein expression of the anti-apoptotic Bcl2 was decreased by hispidin (Figure 4A). Furthermore, we determined the effect of hispidin on death receptor 3, which is an integral part of the extrinsic apoptotic pathway. Treatment of CMT-93 cells with hispidin increased the expression of death receptor 3 protein and its downstream proteins: cleaved caspase-1, cleaved caspase-8, and cleaved Parp (Figure 4B). Overall, the results suggest that hispidin treatment resulted in induction of both intrinsic and extrinsic apoptosis.

Hispidin induces apoptosis of colon cancer cells by increasing intracellular ROS levels. The molecular mechanism underlying the induction of apoptosis by hispidin was examined in colon cancer cells. As increased production of ROS was found to induce cell death, we examined whether hispidin affected ROS levels in colon cancer cells (Figure 5). It was found that hispidin significantly induced ROS generation in a dose-dependent manner. These results indicate that hispidin induced apoptosis by generating ROS.

Hispidin does not affect cell viability in the presence of NAC. Hispidin treatment inhibited cell viability of both mouse and human colon cancer cells. However, in cells pre-treated with NAC, a ROS scavenger, hispidin did not show any significant inhibitory effect on cell viability (Figure 6). Thus, we confirmed that hispidin-induced apoptosis is regulated by ROS.

Discussion

Colon cancer is a malignant tumor that occurs in developed countries and is responsible for a high rate of mortality in patients (12). Although a large number of chemotherapeutic agents have been developed, they failed in many cases due to various adverse effects and resistance. Therefore, natural products with low toxicity were thought to be potential options for the treatment of cancer (13, 14). Previously, we reported the anticancer activities of cordycepin isolated from *Cordyceps militaris* through induction of apoptosis in human HT-29 colon cancer cells (5). Our present study showed that hispidin is a novel natural product that can be used in the treatment of colon cancer.

The anticancer activity of hispidin has not been well studied. In the present study, we demonstrated that hispidin, a natural product produced from *P. linteus*, has certain anticancer activity against colon cancer. In mouse and human

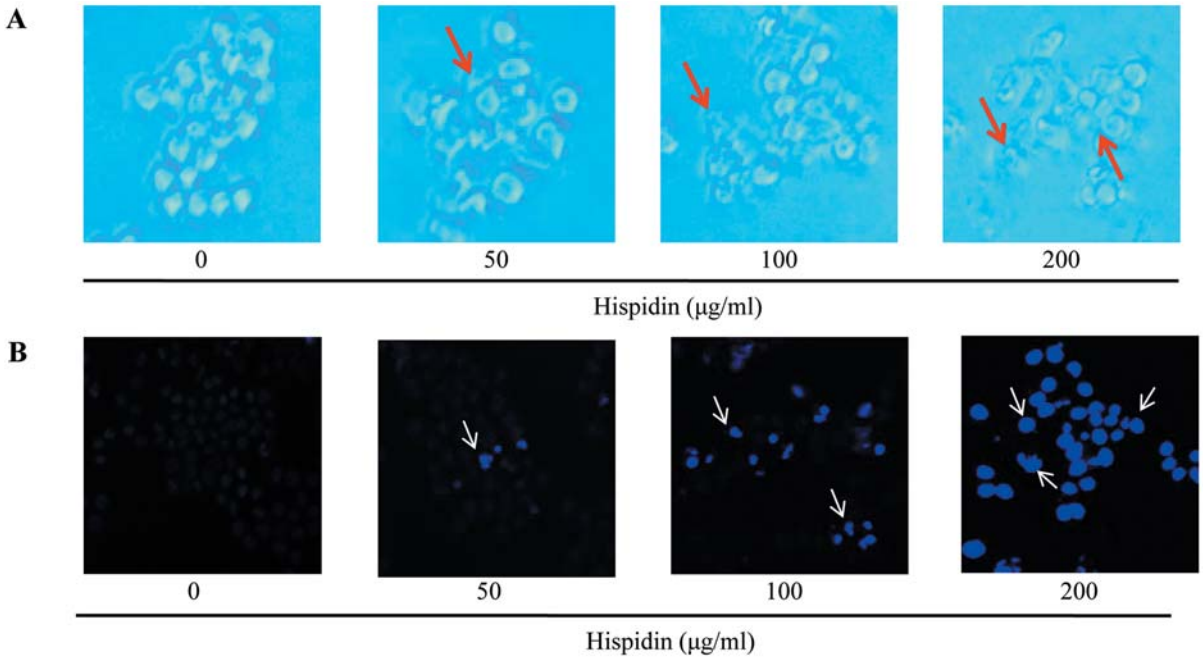

Figure 2. *Hispidin induces apoptotic changes in cell morphology. CMT-93 cells were treated with hispidin (0-200 μg/ml) for 18 h. Results were assessed by phase-contrast microscopy (×200; arrows indicate apoptotic changes in cell morphology) (A) and by fluorescent microscopy (×200; arrows indicate chromosome condensation) (B).*

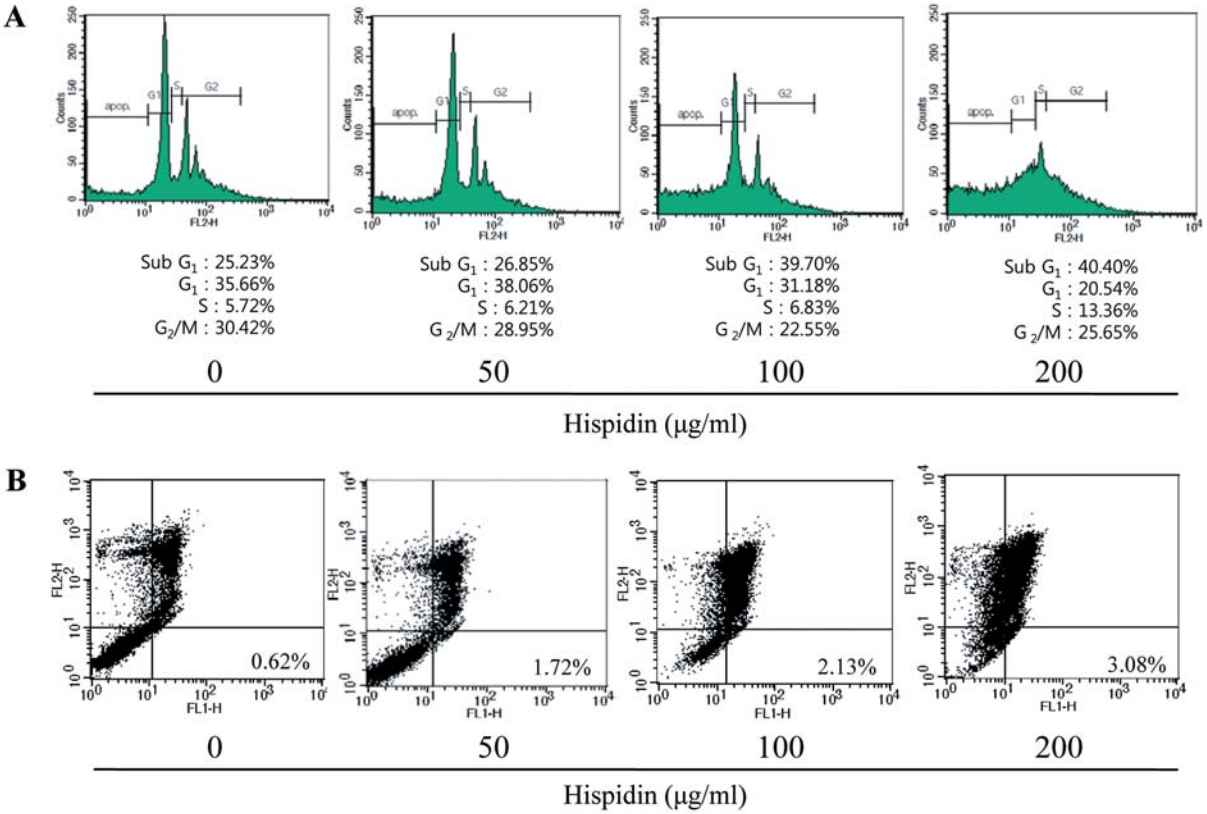

Figure 3. *Analysis of hispidin-induced apoptosis by flow cytometry. CMT-93 cells were treated with hispidin (0-200 μg/ml) for 18 h. Cells were then stained with PI (propidium iodide) for cell-cycle distribution analysis (A) or with Annexin V-FITC (fluorescein isothiocyanate) (B), and analyzed using flow cytometry.*

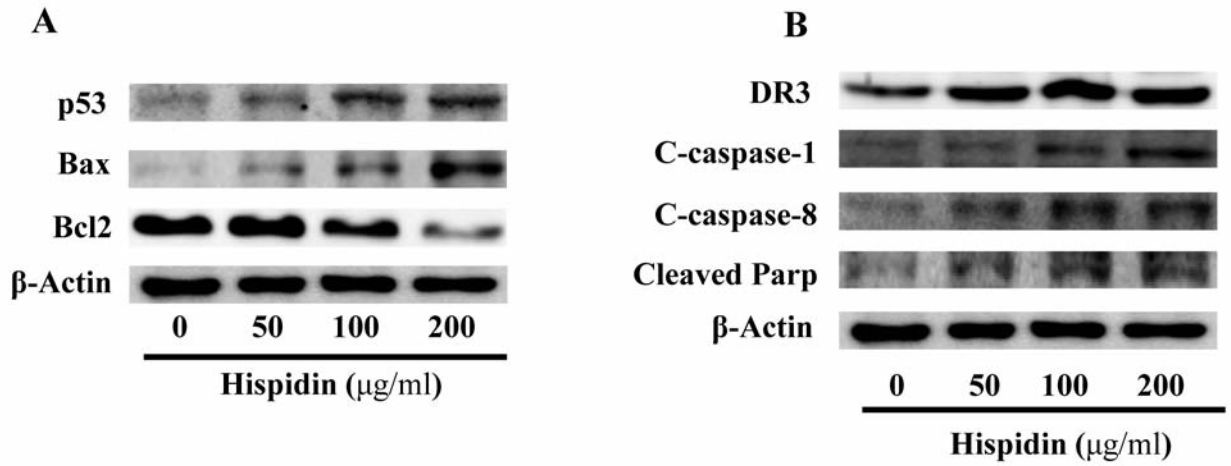

Figure 4. *Hispidin affects the expression of apoptotic genes. CMT-93 cells were treated with hispidin (0-200 μg/ml) for 18 h. p53, BAX (BCL2-associated X), BCL2 (B cell lymphoma-2), β-actin (A) and death receptor 3 (DR3), cleaved caspase-8, cleaved caspase-1, cleaved PARP (poly-(ADP-ribose) polymerase), and β-actin (B) protein levels were then analyzed by western blotting.*

colon cancer cells, that is, CMT-93 and HCT 116, respectively, we found that hispidin inhibited cell viability in a dose-dependent manner. The cells manifested many features of apoptosis, both in the nucleus and cytoplasm (4). Apoptotic morphological changes such as detached, shrunken, or round-shaped cells were observed by phase-contrast microscopy in the presence of hispidin. Moreover, we observed chromatin condensation and nuclear fragmentation in hispidin-treated cells by Hoechst 33342 staining. These apoptotic morphological changes in cells were more remarkable with increase in the dose of hispidin.

Normally, cell-cycle checkpoints control cell-cycle progression in response to stress signals. De-regulation of checkpoints in the cell cycle contributes to the accumulation of genetic material and chromosomal mutation, leading to tumorigenesis. Therefore, cell-cycle disruption is one of the hallmarks of cancer cells (15). The results of analysis of the cell cycle distribution in hispidin-treated cells indicated that hispidin induced apoptosis through accumulation of a sub-G_1 cell population. Apoptosis can be detected by several biochemical methods. We assessed hispidin-induced apoptosis by Annexin V-FITC staining. Our results showed that early apoptosis was gradually enhanced with an increase in the concentration of hispidin.

Apoptotic pathways are classified into two major categories: intrinsic and extrinsic pathways. Intrinsic apoptosis is mediated through the ratio of pro-apoptotic gene (*BAX*) and anti-apoptotic gene (*BCL2*) (16). *p53* is a representative tumor-suppressor gene that is up-regulated under specific signals such as DNA damage, UV, and other gene alterations. p53 also acts as a transcription factor to promote the expression of various downstream genes that

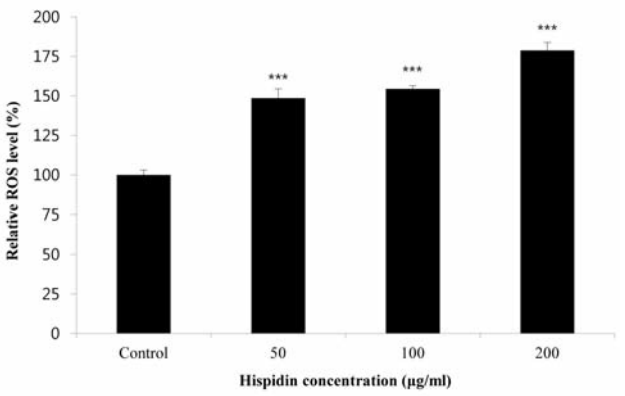

Figure 5. *Hispidin induces apoptosis of colon cancer cells by increasing intracellular ROS levels. CMT-93 cells were treated with hispidin (0-200 μg/ml) for 18 h. Cells were stained with 2',7'-dichlorofluorescein diacetate, and analyzed by reading excitation and emission wavelengths of 485 and 530 nm, respectively. Results are expressed as mean±S.D. (n=4). ***p<0.001 vs. vehicle-treated cells.*

trigger DNA repair, cell-cycle arrest, and apoptosis (17). Therefore, p53 can be considered an important factor for cancer treatment. When cells were treated with hispidin, we found that p53 protein levels were increased significantly, promoting expression of its downstream Bax proteins. Alternatively, a decrease in the level of anti-apoptotic gene *BCL2* contributed to intrinsic apoptotic pathway by releasing cytochrome *c* from mitochondria (18). The extrinsic apoptotic pathway is mediated through transmembrane death receptors and their death ligands (19). Generally, in most human tumors, *p53* tumor-suppressor gene is deleted or

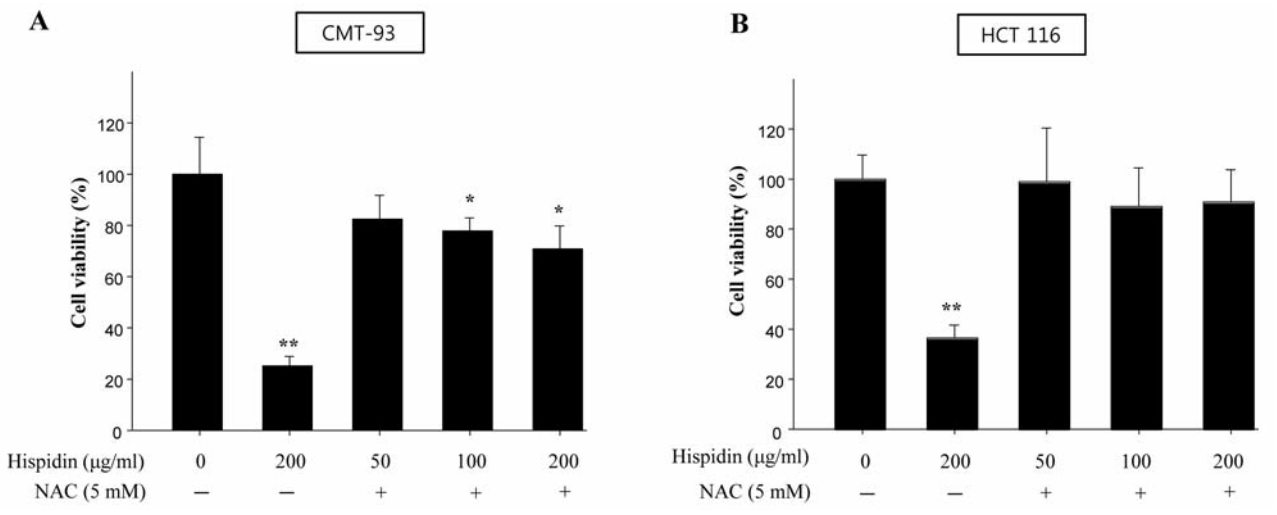

Figure 6. *Hispidin does not affect cell viability in the presence of NAC (N-acetyl-L-cysteine). CMT-93 cells (A) and HCT 116 cells (B) were treated with hispidin (0-200 μg/ml) in the presence of NAC (5 mM). Cell viability was analyzed by the MTT assay. *p<0.05, **p<0.01 vs. vehicle-treated cells.*

mutated (20). In the case of patients bearing the *p53*-inactivated gene, lignads-stimulated death receptors, followed by a cascade of caspase activities could be of great importance in cancer treatment. As shown in Figure 4B, expression of death receptor 3, cleaved caspase-8, cleaved caspase-1, and cleaved Parp proteins was induced by hispidin. Our results suggest that hispidin induces apoptosis through both the intrinsic and extrinsic pathways in colon cancer cells.

ROS are generated during normal cell metabolism, and homeostatic regulation of ROS is required for normal cell growth and survival. Several signals initiate apoptosis in cells by inducing excessive amounts of ROS. An imbalance in ROS levels induces damage to DNA, lipid, and proteins to disrupt the normal function of cells. High levels of ROS may be beneficial for cancer treatment (21). To elucidate the molecular mechanism underlying the induction of apoptosis by hispidin, we determined intracellular ROS levels in hispidin-treated cells. Surprisingly, ROS accumulated in a dose-dependent manner in hispidin-treated cells. We further confirmed that hispidin-induced apoptotic conditions could be reversed by pre-treating the cells with a ROS scavenger, NAC. We did not observe any intracellular accumulation of ROS in the case of combined treatment with hispidin and NAC (data not shown).

In the present study, we examined the anticancer activities of hispidin in colon cancer cells. It will be of further interest to study the effects of this compound on other cancer cell lines as well as *in vivo*. In addition, further investigation is required to understand the molecular mechanism underlying ROS-induced apoptosis.

In conclusion, hispidin might induce apoptotic signals in colon cancer cells through both intrinsic and extrinsic pathways by generating ROS. Therefore, we suggest that hispidin could be considered a potential therapeutic agent for colon cancer.

Acknowledgements

This work was supported by the Konkuk University in 2014.

References

1 Watson AJM: Apoptosis and colorectal cancer. Gut *53*: 1701-1709, 2004.
2 Yang SY, Sales KM, Fuller BJ, Seifalian AM and Winslet MC: Inducing apoptosis of human colon cancer cells by an IGF-1 D domain analogue peptide. Mol Cancer *8*: 7-17, 2008.
3 Labianca R, Pessi MA and Zamparelli G: Treatment of colorectal cancer. Current guidelines and future prospects for drug therapy. Drugs *53*: 593-607, 1997.
4 Wong RS: Apoptosis in cancer: from pathogenesis to treatment. J Exp Clin Cancer Res *30*: 87, 2011.
5 Lee SY, Debnath T, Kim SK and Lim BO: Anticancer effect and apoptosis induction of cordycepin through DR3 pathway in the human colonic cancer cell HT-29. Food Chem Toxicol *60*: 439-447, 2013.
6 Park KW, Kundu J, Chae IG, Kim DH, Yu MH, Kundu JK and Chun KS: Carnosol induces apoptosis through generation of ROS and inactivation of STAT3 signaling in human colon cancer HCT 116 cells. Int J Oncol *44*: 1309-1315, 2014.
7 Burz C, Berindan-Neagoe I, Balacescu O and Irimie A: Apoptosis in cancer: key molecular signaling pathways and therapy targets. Acta Onco *48*: 811-821, 2009.
8 Martin SJ and Green DR: Protease activation during apoptosis: death by a thousand cuts? Cell *82*: 349-352, 1995.

9 Gonindard C, Bergonzi C, Denier C, Sergheraert C, Klaebe A, Chavant L and Hollande E: Synthetic hispidin, a PKC inhibitor, is more cytotoxic toward cancer cells than normal cells *in vitro*. Cell Biol Toxicol *13*: 141-153, 1997.
10 Park IH, Chung SK, Lee KB, Yoo YC, Kim SK, Kim GS and Song KS: An antioxidant hispidin from the mycelial cultures of *Phellinus linteus*. Arch Pharm Res *27*: 615-618, 2004.
11 Saraste A and Pulkki K: Morphologic and biochemical hallmarks of apoptosis. Cardiovasc Res *45*: 528-537, 2000.
12 Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin *61*: 69-90, 2011.
13 Gorlick R and Bertino JR: Drug resistance in colon cancer. Semin Oncol *26*: 606-611, 1999.
14 Gordaliza M: Natural products as leads to anticancer drugs. Clin Transl Oncol *9*: 767-776, 2007.
15 Pietenpol JA and Stewart ZA: Cell cycle checkpoint signaling: cell cycle arrest *versus* apoptosis. Toxicology *181-182*: 475-481, 2002.
16 Reed JC: Bcl-2 family proteins: regulators of apoptosis and chemoresistance in haematologic malignancies. Semin Hematol *34*: 9-19, 1997.
17 Riley T, Sontag E, Chen P and Levine A: Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Bio *9*: 402-412, 2008.
18 Haupt S, Berger M, Goldberg Z and Haupt Y: Apoptosis – the p53 network. J Cell Sci *116*: 4077-4085, 2003.
19 Ashkenazi A and Dixit VM: Death receptors: signaling and modulation. Science *281*: 1305-1308, 1998.
20 Petitjean A, Achatz MI, Borresen-Dale AL, Hainaut P and Olivier M: TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Oncogene *26*: 2157-2165, 2007.
21 Mates JM and Sanchez-Jimenez FM: Role of reactive oxygen species in apoptosis: implications for cancer therapy. Int J Biochem Cell Biol *32*: 157-170, 2000.

Received May 8, 2014
Revised June 16, 2014
Accepted June 17, 2014