Imperatorin exhibits anticancer activities in human colon cancer cells via the caspase cascade

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Abstract. Despite advances in medical treatments for colon cancer, it remains one of the leading causes of cancer-related mortality among men. Thus, more efficacious treatment strategies for colon cancer are needed. Imperatorin is one of the major ingredients present in the root of Angelica dahurica, and has been used in herbal formulations for the treatment of hypertension and cardiovascular diseases. However, the medical properties of imperatorin remain unclear. In the present study, the anti-proliferative activities of imperatorin were investigated in the HT-29 colon cancer cell line. The results showed that imperatorin significantly inhibited HT-29 colon cancer cell growth with an IC50 value of 78 µM. Imperatorin induced the apoptosis of colon cancer cells through upregulation of p53 and the caspase cascade. Our findings revealed that imperatorin induced cell cycle arrest in the G1 phase. The apoptotic index showed a steady increment when the imperatorin concentration was increased. The results suggest that imperatorin exerts considerable anti-proliferative activities in HT-29 colon cancer cells and highlight the potential of imperatorin as an anticancer agent for colon cancer.

Introduction

Colon cancer is one of the leading causes of cancer-related mortality. Colorectal cancer requires time to develop. Prognosis and removal of early-stage polyps can prevent the development of cancer. Early colorectal cancer exhibits no symptoms. Moreover, a cure is not possible for most patients with metastatic colorectal cancer. Chemotherapy is the most common treatment option, which can alleviate symptoms and prolong patient life. Yet, alternative medicines are required. Oxidative stress can be caused by the accumulation of intracellular reactive oxygen species (ROS) through metabolism of toxic substances. Cancer cells demand high ROS concentrations to maintain their high proliferation rate due to a higher metabolic rate. However, ROS levels can be used as a checkpoint with which to monitor cell damage. The implications for ROS regulation are highly significant for cancer therapy as commonly used radiotherapy and chemotherapeutic drugs influence tumor proliferation through ROS modulation. Anticancer phytochemicals are being used to modulate the ROS level in cancer cells which is the principal mechanism by which most anticancer agents function to elicit inhibition of tumor proliferation. Imperatorin is one of the major active coumarins found in the root of Imperata cylindrica Beauv. with less than 4% in the herbal extract (1). It was reported that imperatorin exhibits many pharmacological activities including anti-inflammatory, anti-coagulant and anti-proliferative (1-3). Previous studies showed that imperatorin inhibited SNU 449 liver cancer cell growth (1). Other studies have shown that imperatorin mediates oncogene expression and induces cell cycle arrest (4-8). A recent study showed that imperatorin suppressed voltage-gate sodium channels and neuronal excitability through inhibition of the voltage-gate sodium channels (9). In the present study, we investigated the anti-proliferative activities of imperatorin in HT-29 cancer cells and its effects on signaling pathways involved in apoptosis as well as its antioxidant effects. We investigated the expression of marker proteins involved in these processes and the potential of imperatorin as an anticancer agent.

Materials and methods

Cells, chemicals and reagents. The HT-29 colon cancer cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Acetonitrile (ACN) (E. Merck, Darmstadt Germany), methanol (RCI, Labscan, Thailand) and trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO, USA) were of HPLC grade. Distilled and deionized water (ddH2O) was prepared using the MilliPore water purification system (Millipore, Milford, MA, USA). All other reagents used were of analytical reagent grade purchased from Sigma-Aldrich unless stated otherwise. All antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) except anti-caspase-8, -Bax, -p21 and -MDM2 which were obtained...
from Santa Cruz (Dallas, TX, USA). Imperatorin was purchased from Sigma-Aldrich.

**MTT assay.** The growth inhibitory effect of imperatorin was evaluated in colon cancer HT-29 cells. All cells were seeded onto 96-well microtiter plates, at a density of 6x10^3 cells per well and allowed to incubate overnight. The cells were treated with either 0.5% DMSO [as solvent control (Ctrl)] or various concentrations of imperatorin (as treatment group) and incubated for 24, 48, and 72 h at 37°C, respectively. After incubation, MTT solution (50 µl) was added to each well, and the plates were incubated at 37°C for 3 h. Then the solution in each well was replaced by DMSO (150 µl). The absorbance was measured at 540 nm by TECAN Infinite M200 (Tecan Group Ltd., Männedorf, Switzerland). Each treatment or control was performed in replicates of 4-6 times. Mean absorbance readings from solvent control groups were defined as having 100% viability, and the results from the treatment groups were shown as the percentage of viability relative to the controls.

**Lactate dehydrogenase (LDH) activity assay.** Cells that undergo necrosis are lysed to release cytosolic LDH into culture medium. The CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI, USA) was used to evaluate the cytotoxic effect of imperatorin on HT-29 cells by assessing the activity of LDH present in the culture medium. After a 48-h treatment of the HT-29 cells with imperatorin, the culture medium was transferred to a 96-well plate, followed by addition of an equal volume of CytoTox 96 reagent in each well. The plate was maintained in the dark at room temperature for 30 min. The enzymatic reaction was stopped by adding an equal volume of stop solution in each well. The absorbance was measured at 490 nm by TECAN infinite M200. Maximum LDH activity was achieved by addition of lysis buffer and normalized by a corresponding volume correction control. Background values (medium only) were subtracted from the sample readings, and the percentage of necrotic cell death was calculated in relation to the maximum LDH release.

**TUNEL-based detection of DNA fragmentation.** ApoDIRECT In Situ DNA Fragmentation Assay kit was used to detect DNA fragmentation in apoptotic cells though terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. In brief, HT-29 cells were seeded onto a 6-well plate, at a density of 2x10^6 cells per well and allowed to incubate overnight. Then the cells were treated with various concentrations of imperatorin for 48 h. After a 48-h treatment, the cells were harvested and fixed with 1% (w/v) paraformaldehyde (PFA) in physiological buffer (PBS). The cells were washed with PBS twice and permeabilized with ice-cold 70% ethanol in PBS for ≥30 min. After fixation, the cells were centrifuged at 500 x g for 5 min and the supernatant was decanted. Cell pellets were re-suspended in wash buffer and washed twice prior to a 1-h incubation in staining buffer. After incubation, the stained cells were rinsed twice by adding rinsing buffer, followed by a 30-min incubation in propidium iodide (PI)/RNase A solution in the dark at room temperature. The amount of fluorescein-labeled samples was observed using the FACSVerse system (BD Biosciences, San Jose, CA, USA). The samples presenting a FITC+/PI signal were considered as having fragmented DNA.

**Flow cytometric analysis of the cell cycle.** HT-29 cells were seeded onto a 6-well plate, at a density of 1x10^6 cells per well and allowed to incubate overnight. The cells were treated with imperatorin for 48 h. After a 48-h treatment, the cells were washed with PBS once and harvested with trypsin. The harvested cells were washed with cold PBS twice and fixed with 70% ethanol for ≥1 h. The cells were centrifuged at 500 x g for 5 min and washed twice with cold PBS, followed by addition of PI (final concentration, 40 µg/ml). The cells were incubated in the dark at 37°C for 15 min. Fluorescence-activated cells in each sample were recorded with 10,000 events using the FACSVerse system (BD Biosciences). The cell cycle distribution was analyzed with ModFit (10).

**Casparase-3/7 assay.** The Apo-ONE Homogeneous Caspase-3/7 Assay kit was used to evaluate the activities of apoptosis by assessing the caspase-3/7 enzymatic activities in the imperatorin-treated HT-29 cells. HT-29 cells were seeded onto a 96-well black plate (with clear bottom), at a density of 6x10^3 cells per well and allowed to incubate overnight. Then the cells were treated by either 0.5% DMSO [as negative control group (Ctrl)] or various concentrations of imperatorin and incubated for 48 h. After incubation, an equal volume of Apo-ONE caspase-3/7 reagent was added to each well. The plate was incubated for 3 h at room temperature prior to recording the rate of fluorescence (RFU) of each well at excitation 485 nm and emission 530 nm using a TECAN infinite M200.

**Western blot analysis.** Western blot analysis was carried out following the recommended protocol from Abcam (Cambridge, UK). In brief, HT-29 cells were seeded onto 100-mm culture dishes, at a density of 2x10^6 cells per dish and incubated for 24 h prior to a 48-h treatment with various concentrations of imperatorin. After a 48-h treatment, the cells were harvested and lysed using Nonidet-P40 lysis buffer (150 mM sodium chloride, 1% Triton X-100, 50 mM Tris, pH 8.0, supplemented with protease inhibitor cocktails (Roche)). The protein concentration was determined using the DC protein assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocols using bovine serum albumin (BSA) as the
standard. Normalized protein samples were denatured in a boiling water bath for 10 min and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate the proteins using 80-120 V for 2 h. Proteins were transferred from the gel to a 0.25-µm PVDF membrane at 12 V for 30 min using the Novex Semi-dry blotter (Invitrogen Life Technologies, Carlsbad, CA, USA). Then the membrane was blocked with non-fat dry milk (5% w/v) in TBST for 1 h at room temperature before incubation with the specific primary antibody (at appropriate dilution suggested by the product data sheet) in 4 ml of 5% non-fat milk at 4˚C overnight. After washing with TBST, the membrane was incubated with the species appropriate HRP-conjugated secondary antibody (at 1:2,000 dilution) in 4 ml of 5% non-fat milk at room temperature for 1 h. Signals were developed using ECL chemiluminescence detection reagent (GE, Fairfield, CT, USA) and blots were visualized after exposure to Fuji Super RX film. The membrane was re‑probed with β‑tubulin as an internal control. The intensity of the blots was quantified by ImageJ image processing program.

ROS assay. Image-iT LIVE Green Reactive Oxygen Species Detection kit is used to detect the production of ROS in live cells. In brief, HT-29 cells (1x10^6 per well) were seeded on a 6-well plate and allowed to incubate overnight. Then the cells were treated with various concentrations of imperatorin and incubated for 24 h. After a 24-h treatment, the cells were washed with warm Hank's balanced salt solution (HBSS) once before a 25-min incubation with 25 µM carboxy-H_2DCFDA and an additional 5-min incubation with 5 µM Hoechst 33342. The coverslips were gently washed three times with warm HBSS and mounted for fluorescent imaging at excitation 485 nm and emission 530 nm by Nikon E80i (Nikon, Tokyo, Japan).

Data analysis. In all viability assays, the viable cells were measured as a percentage relative to the solvent control groups and expressed as means ± SD (n=3). Nonlinear regression test was applied to the viability assays to obtain a fit curve (R^2>0.98). Statistical analysis of data was carried out by one-way ANOVA (coupled with post-test, Dunnett's test) to test for differences between the control group and treatment groups. Values of P<0.05, P<0.01, P<0.005 and P<0.001 were considered to indicate statistically significant differences and are indicated by asterisks in the figures.

Results and Discussion

In the present study, different concentrations of imperatorin were used to treat HT-29 cells. An in vitro study on HT-29 colon cancer cells revealed that imperatorin inhibited cell viability with an IC_{50} value of 239 µM after 24 h, 101 µM after 48 h and 78 µM after 72 h of incubation (Fig. 1). The percentage of necrotic cell death remained unaffected with an increase in imperatorin concentration (Fig. 2). The results suggest that imperatorin did not cause necrosis of HT-29 cells. In contrast, the apoptotic index which is used to determine cancer cell growth and is inversely related to necrosis showed...
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Figure 4. The induction of cell cycle arrest in HT-29 cells by imperatorin. HT-29 cells were treated with different concentrations of imperatorin for 48 h. The cells in the solvent control group were treated with 0.5% DMSO. The percentage of cells in the G1, S and G2/M phases of the cell cycle was determined by propidium iodide (PI) staining and observed by flow cytometry (A-C). Typical distribution of the cell populations in cells treated with 150 µM imperatorin and control cells is shown (D and E, respectively). Data are presented as mean ± SD (n=3); *P<0.05; **P<0.005; ***P<0.001 vs. the control group.

Figure 5. The induction of apoptosis in the HT-29 cells by imperatorin. HT-29 cells were treated with different concentrations of imperatorin for 48 h. The cells in the solvent control group were treated with 0.5% DMSO. The percentage of apoptotic cells was determined by Annexin V-FITC/PI staining and observed by flow cytometry. The cells generating an FITC+/PI- (LR) signal were in the early apoptotic stage while the cells generating an FITC+/PI+ (UR) signal were dead cells. Typical distribution of cell population following the treatment of 0, 50, 100 and 150 µM imperatorin is shown (A-D, respectively). The percentage of early apoptotic cells and total dead cells (LR + UR) is shown (E and F, respectively). Data are presented as mean ± SD (n=3); **P<0.005 vs. control group.
a concentration-dependent increase in the HT-29 cells (Fig. 3). The results indicate that imperatorin induced programmed cell death and provide supportive evidence for an imperatorin-induced apoptotic mechanism that could be mediated through different apoptotic activities (11). The duration of the apoptotic activity was largely dependent on the concentration of the anti-proliferative agent. The common technique that is adopted for measuring the apoptotic index is flow cytometry. Cell cycle analysis showed that imperatorin induced cell cycle arrest at the G1 phase (Figs. 4 and 5). Apoptotic cell death can result from activation of interrelated molecular cascades. The extrinsic pathway transduces extracellular stimulus through the plasmatic membrane. In contrast, the intrinsic pathway modulates the intracellular environment that controls the survival or death of cells. Mitochondria play an important role in controlling the signaling pathways leading to death or survival of cells; thus, anti-proliferative agents can induce oxidative stress and alterations in the mitochondrial membrane that leads to apoptosis activation. These two pathways translate into a convergent point where caspases downregulate the apoptotic activities. The intrinsic apoptotic pathway involves the mitochondria and activation of the caspase cascade which was triggered by imperatorin (Fig. 6). The caspase-3/7 activities in the HT-29 cells after treatment with imperatorin showed a considerable increase. Stimulation of the intrinsic apoptotic pathway by imperatorin triggered a signaling cascade that resulted in the increase of the apoptotic process with caspase -3/7, which were activated with the increasing concentration of imperatorin. These data demonstrate the anti-proliferative activity of imperatorin in HT-29 cells. The caspase-3/7 activities in the HT-29 cells after treatment with imperatorin were evaluated by the caspase -3/7 assay. Data are presented as mean ± SD (n=3); **P<0.01; ***P<0.001 vs. control group.

Figure 6. Caspase-3/7 activities in the HT-29 cells after treatment with imperatorin. HT-29 cells were treated with different concentrations of imperatorin for 48 h. The cells in the solvent control group were treated with 0.5% DMSO. The caspase-3/7 activities in HT-29 cells were evaluated by the caspase-3/7 assay. Data are presented as mean ± SD (n=3); **P<0.01; ***P<0.001 vs. control group.

Figure 7. Expression of apoptosis-related proteins in HT-29 cells after treatment of imperatorin. HT-29 cells were treated with different concentrations of imperatorin for 48 h. The cells in the solvent control group were treated with 0.5% DMSO. Western blot analysis was conducted with antibodies against mitochondrial-mediated apoptotic proteins: Bax and Bcl-2; death-receptor-mediated apoptotic proteins: p53, p21 and MDM2; proteins in caspase-dependent apoptotic signaling pathways: pro-caspase-8, cleaved caspase-8, pro-caspase-9, cleaved caspase-9, pro-caspase-3, cleaved caspase-3, pro-caspase-7, cleaved caspase-7, PARP and cleaved PARP.

The Bcl-2 family members exhibit a regulatory role in determining cell viability. These members exert checkpoints to modulate apoptotic activities. The ratio between pro-apoptotic factors including Bax, Bad, Bak, Bid, MDM2 and anti-apoptotic members is a useful marker for measuring apoptosis. Changes in expression of Bcl-2 is indicative of the alteration of mitochondrial membrane potential. Fig. 7 shows that imperatorin modulated the alteration of the expression of these key apoptotic members in the HT-29 cells. Other key molecules involved in apoptosis regulation is alteration of the transcription factor p53 (Fig. 8). The main role of p53 is to mediate tumorigenesis (12). Cancer involves complex cellular changes which are associated with uncontrollable replication and resistance to growth-controlled mechanisms.

Malignant tumors may metastasize through the circulatory system. Consequently, metastasis causes significant tumor burden. At present, strategies of cancer treatment using phytochemicals in combination with targeted therapies are considered promising, and may lead to greater efficacy and enhanced patient survival rates (13). Malignant cells may find alternative survival mechanisms. Indeed, imperatorin is capable of modulating cell conditions that may cause alteration in expression profile of oncopgenes and cell cycle mechanisms of cancer cells. Anti-proliferative agents can consequently be used to suppress cancer cell survival or death based on the modulation of the intrinsic pathway. Imperatorin was shown to
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Figure 8. (A-J) Expression levels of proteins were quantified by ImageJ image processing program. Particularly, the Bax/Bcl-2 expression ratio is shown in C. β-tubulin was used as an internal control to normalize data. Data are presented as mean ± SD (n=3); *P<0.05; **P<0.01; ***P<0.005; ****P<0.001 vs. the control group.
Figure 8. (Continued). (K-N) The expression levels of the proteins were quantified by ImageJ image processing program. β-tubulin was used as an internal control to normalize data. Data are presented as mean ± SD (n=3); *P<0.05; **P<0.01; ***P<0.005; ****P<0.001 vs. control group.

Figure 9. The production of reactive oxygen species (ROS) in HT-29 cells after treatment with imperatorin. HT-29 cells were treated with different concentrations of imperatorin for 48 h. The cells in the solvent control group were treated with 0.5% DMSO. The generation of ROS in imperatorin-treated cells was detected by an ROS detection kit. Imperatorin-treated cells were imaged under a bright-field microscope (A-D) as well as a fluorescence microscope (E-H). Intracellular ROS are indicated in green.
modulate the intrinsic pathway involving Bcl-2 members, p53, p21, MDM2 and the caspase cascade (Fig. 8), and triggered considerable changes in Bax, caspase-8, MDM2, p53 and p21 expression (Fig. 8).

The maintenance of homeostasis is known to be important in normal tissues that require a balance between cell proliferation and cell death. Bcl-2 family members play a significant role in the regulation of cell death. Bcl-2 is involved in programmed cell death. Overexpression of Bcl-2 in cells results in extended cell survival. Bcl-2 does not appear to affect the generation of reactive oxygen radicals but may prevent oxidative damage to cellular constituents. A pre-set ratio of Bcl-2/Bax appears to modulate the intrinsic pathway involving Bcl-2 members, p53, p21, MDM2 and the caspase cascade (Fig. 8), and triggered considerable changes in Bax, caspase-8, MDM2, p53 and p21 expression (Fig. 8).

The results were validated by individual images. Together, these results provide experimental evidence for apoptotic signaling after treatment of HT-29 cells with imperatorin. The anti-proliferative activities of imperatorin in HT-29 cells were demonstrated in the present study.

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References