Scabraside D Extracted from *Holothuria scabra* Induces Apoptosis and Inhibits Growth of Human Cholangiocarcinoma Xenografts in Mice

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Abstract

Scabraside D, a sulfated triterpene glycoside extract from sea cucumber *Holothuria scabra*, shows various biological activities, but effects on human cholangiocarcinoma cells have not previously been reported. In the present study, we investigated the activity of scabraside D against human cholangiocarcinoma (HuCCA) both *in vitro* and for tumor growth inhibition *in vivo* using a xenograft model in nude mice. Scabraside D (12.5-100 µg/mL) significantly decreased the viability and the migration of the HuCCA cells in a dose-dependent manner, with 50% inhibitory concentration (IC₅₀) of 12.8 ± 0.05 µg/mL at 24 h. It induced signs of apoptotic cells, including shrinkage, pyknosis and karyorrhetic nuclei and DNA fragmentation on agarose gel electrophoresis. Moreover, by quantitative real-time PCR, scabraside D effectively decreased Bcl-2 while increasing Bax and Caspase-3 gene expression levels suggesting that the scabraside D could induce apoptosis in HuCCA cells. *In vivo* study demonstrated that scabraside D (1 mg/kg/day, i.p. for 21 days) significantly reduced growth of the HuCCA xenografts without adverse effects on the nude mice. Conclusively, scabraside D induced apoptosis in HuCCA cells and reduced the growth of HuCCA xenografts model. Therefore, scabraside D may have potential as a new therapeutic agent for cholangiocarcinoma.

Keywords: Scabraside D - *Holothuria scabra* - cholangiocarcinoma - apoptosis - sea cucumber extract

Introduction

Cholangiocarcinoma (CCA), is an incurable cancer of the biliary epithelium, and is an important public health problem in many Asian countries (Sripa and Pairojkul, 2008; Shin et al., 2010; Sripa et al., 2011). High prevalence is found in northeast Thailand, where people eat raw freshwater fish infested with *Opisthorchis viverrini*, the parasitic platyhelminth associated with CCA (Haswell-Elkins et al., 1994). Among the treatment regime of CCA is chemotherapy by 5-fluorouracil (5-FU), which however is rather ineffective (Hejna et al., 1998). The combined treatment of CCA with chemotherapy and radiotherapy causes severe adverse effects without significantly improving the prognosis of a patient (Sirica, 2005). Therefore, for this and other types of incurable cancer, alternative treatments without adverse effects have been widely sought after. Various extracts from natural products, especially from marine plants and animals, appear promising (Adrain, 2007; Rajasekaran et al., 2008). Sea cucumber, as one of marine echinoderms, is a competent source for novel types of biologically active substances with medical utilization. *Holothuria scabra* is widely distributed in the Atlantic and Pacific oceans, and is considered a costly gourmet dish in Asian cuisine, and traditional Chinese medicine uses it in tonics and delicacies (Kerr and Chen, 1995). Extracts from sea cucumbers have been reported as anti-cancer agents. Many species of sea cucumber produce triterpene glycosides, a group of saponin, which are poisonous for other organisms and presumably function as a chemical defense against predation (Caulier et al., 2011; Bahrami et al., 2014). This group of substances shows various biological activities, including antifungal, cytotoxic, hemolytic, cytostatic and immunomodulatory effects (Kitagaw et al., 1989; Stonix et al., 1999; Chludil et al., 2002). The sea cucumber *H. scabra* produces a type of sulfated triterpene glycosides called scabraside D, which inhibits the proliferation of...
mouse leukemic cells and various kinds of human cancer cells including lung, gastric, colorectal and breast cancer cells (Han et al., 2012). The mechanism of action of scabraside D on the inhibition of cancer growth is still unknown. However, effect of scabraside D on human cholangiocarcinoma cells has not previously been determined. In this study, we investigated the effect of scabraside D on human cholangiocarcinoma (HuCCA) cell viability, migration, and apoptosis in vitro and in vivo on tumor growth in xenograft models. To our knowledge, this is the first report of scabraside D on the inhibition of HuCCA cell growth with the studies on mechanisms of its effect, as well as a corresponding study on an in vivo inhibition of HuCCA xenographs in animal model.

Materials and Methods

Cell lines and cell culture

HuCCA cell line CL6 kindly provided by Associate Professor Dr. Adisak Wongkajornsilp, the Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University. It was used both in vitro and as tumor xenograft in vivo. The cells were cultured in DMEM medium, supplemented with penicillin (100 IU/mL), streptomycin (100 µg/mL) and 15% fetal bovine serum (FBS). They were maintained at 37°C in an incubator with a humidified 95% and 5% (v/v) mixture of air CO₂.

Isolation and purification of scabraside D

Live specimens of H. scabra were caught from Andaman Sea, Krabi Province, southern Thailand. Air-dried and finely powdered body walls (3 kg, dried weight) of sea cucumber H. scabra were percolated with 2.0 L of 70% ethanol for 48 h at room temperature; the ethanol fractions were combined and evaporated in a vacuum chamber to give the crude ethanol extract, which weighed 147.83 g. The extract was dissolved in water and washed twice with cold phosphate-buffered saline (PBS), µg/mL) and control for 24 h. After 24 h, the cells were washed twice with cold phosphate-buffered saline (PBS), and fixed with cold methanol and acetic acid (3/1, v/v) at 4°C overnight. After that, the cells were stained with Hoechst 33342 for 30 min in the dark, washed with PBS, and observed under a Confocal Laser Scanning microscope mounted in a mounting medium (80% glycerol in PBS) and observed under a Confocal Laser Scanning microscope (Olympus FV 1000). In addition, after treatment the cells were processed for Epon resin embedding and semi-thin sections. The cells were fixed with 4.5% glutaraldehyde in phosphate buffer (PB) for 1 h and post-fixed in 1% osmium tetroxide in 0.2 M PB for 1 h at 4°C. After washing, the cells were dehydrated in a series of ethanol (from 30 to 100%) changes of acetone. After that, the cells were incubated in acetone/Epon resin 812 at 1:1 for overnight. This was followed by embedding in 100% Epon resin 812 for 4 h, and incubated at 60°C for 3 days. Semi-thin sections were cut, the stained with toluidine blue and examined under a light microscope (LM).

Cell viability assay

The cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich Co., St. Louis, MO, USA). The MTT assay is based on the ability of succinate dehydrogenase in mitochondria that separates yellow tetrazolium salt into purple insoluble formazan products (Mosmann, 1983). Briefly, HuCCA cells were seeded in 96-well plates at a density 1x10⁴ cells/well. After 24 h, the cells were treated for 24 and 48 h with scabraside D at concentrations from 6.25 to 100 µg/ml, or with 5-FU at similar concentrations as a positive control. In the negative control group, the cells were treated with 0.1% DMSO. After removing the medium, the HuCCA cells were subjected to MTT solution for 4 h to assess their mitochondrial activity (Tada et al., 1986). Absorbance was measured at 490 nm using microplate reader (Bio-Rad, Hercules, CA). Averaged values were derived from at least three independent experiments, and are presented as percentages of cell viability, which is estimated as the ratio of mean absorbance for the treatment and for the negative control group.

Cell migration assay

The cell migration assay was modified from that of Liang and colleagues (Liang et al., 2007). Briefly, HuCCA cells were seeded in 6-well plates at a density 1x10⁴ cells/well and incubated at 37°C overnight. The culture medium was aspirated and then scratch wound was made through the confluent monolayer with a plastic sterile pipette tip. This was followed immediately by addition of various concentrations of scabraside D (6.25-100 µg/mL) into the well plates, which were photographed at 0, 3, 6 and 24 h after scratching. The advance of cell migration to close the wound, with scabraside D treatment or in control plates, was observed under a phase-contrast microscope.

Morphological examinations

In order to observe the nuclear characteristics of HuCCA cells after scabraside D treatment, nuclear staining was performed using Hoechst 33342 (Sigma-Aldrich Co., St. Louis, MO, USA). The HuCCA cells 5x10⁵ cells/ml were grown on cover slips placed in 6-well plates overnight, then treated with scabraside D (6.25 to 100 µg/mL) and control for 24 h. After 24 h, the cells were washed twice with cold phosphate-buffered saline (PBS), and fixed with cold methanol and acetic acid (3/1, v/v) at 4°C overnight. After that, the cells were stained with Hoechst 33342 for 30 min in the dark, washed with PBS, mounted in a mounting medium (80% glycerol in PBS) and observed under a Confocal Laser Scanning microscope (Olympus FV 1000). In addition, after treatment the cells were processed for Epon resin embedding and semi-thin sections. The cells were fixed with 4.5% glutaraldehyde in phosphate buffer (PB) for 1 h and post-fixed in 1% osmium tetroxide in 0.2 M PB for 1 h at 4°C. After washing, the cells were dehydrated in a series of ethanol (from 30 to 100%) changes of acetone. After that, the cells were incubated in acetone/Epon resin 812 at 1:1 for overnight. This was followed by embedding in 100% Epon resin 812 for 4 h, and incubated at 60°C for 3 days. Semi-thin sections were cut, the stained with toluidine blue and examined under a light microscope (LM).

DNA fragmentation

The HuCCA cells (1x10⁵ cells) were grown in 6-well plates overnight, followed by treatments with various concentrations of scabraside D (6.25 to 100 µg/ml) for 24
h. After that, the cells were harvested washed twice with ice-cold PBS, resuspended in Tris-buffered saline-EDTA (10 mM Tris-HCl at pH 7.6, 140 mM NaCl, and 1 mM EDTA) and lysed in the extraction buffer (0.1 M NaCl, 10 mM Tris-HCl, 25 mM EDTA, pH 8.0; 100 µL per 1x10^6 cells). The lysates were incubated with 25 µL of 10% sodium dodecyl sulfate (SDS) (25 µL, 10%) and 5 µL of proteinase K (20 mg/mL) for 1 h at 50°C. One µL of RNase (1 mg/µL) was added and incubated for 1 h at 37°C. Then, 100 µL of 5 M NaCl was added and DNA was extracted twice with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) solution. The extracted DNA was precipitated by adding 500 µL of cold absolute ethanol at 1 h. Then, it was centrifuged at 12,000× g for 10 min at 4°C and rinsed with 500 µL of 70% ethanol. This was followed by 12,000× g centrifugation for 10 min at 4°C and the DNA pellets were allowed to dry for 20 min at room temperature. The DNA pellets were then dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0); the solution was loaded on 2% agarose gel, separated by electrophoresis (110 V, 35 min) and visualized by ethidium bromide staining.

RNA extraction
HuCCA cells were collected at 24 h after treatment and washed twice with PBS. Total RNA was extracted using 1 mL of TRIzol® Reagent (Invitrogen, Paisley, UK). The sample was added to one-fifth volume of chloroform (0.2 mL), shaken for 15 s, incubated for 5 min at room temperature. The mixture was centrifuged at 15,000× g for 10 min at 4°C, and the aqueous phase was transferred to a new tube. Isopropanol (0.5 mL) was added into the solution, which was kept at room temperature for 15 min to precipitate the RNA. The sample was centrifuged at 15,000× g for 10 min at 4°C and the supernatant was discarded. The pellet was washed twice with 1 mL of 75% ethanol, centrifuged at 7,500× g for 10 min at 4°C, and dried for 10 min at 55°C. The dried pellets were re-suspended in de-ionized distilled water (30 µL) and stored at -80°C until use. The total RNA was converted to cDNA using the SuperScript™ III reverse transcription kit following the instruction manual (Invitrogen, Paisley, UK).

Quantitative real-time PCR
Quantitative real-time PCR was performed to determine the expression levels of BCL-2, Bax, caspase-3 and GAPDH. A 20 µL PCR reaction mixture contained 2 µL of first-strand cDNA of each primer and 18 µL of KAPA SYBR® FAST qPCR kit Master Mix (2x) (KAPA Biosystems, Woburn, MA), and was processed in an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA). The specific primers were designed according to the full cDNA sequences of Bax (GeneBank accession no. AY217036), Bcl-2 (GeneBank accession no. BC027258), caspase-3 (GeneBank accession no. NM_004346), and GAPDH (GeneBank accession no. NM_002046) using Primer Blast Program (Ye et al., 2012). The following primer sets (Pacific Science Co., Ltd., Thailand) were used: Bax, 5’-ATGCCGTTCTACACTGTCGTC-3’ (sense) and 5’-GAAGTGTTGCTCAGGAGGAG-3’ (antisense) 400 bp product; Bcl-2, 5’-TGGAAGACGCGTACGATTA-3’ (sense) and 5’-CATGCCACCCCGAACCCTCACTGACCA-3’ (antisense) 460 bp product; caspase-3, 5’-GTTGCGTGCCCTTGAATCTG-3’ (sense) and 5’-TGAGTTTGGCTGATCAGGACA-3’ (antisense) 349 bp product; and GAPDH, 5’-CTCTGGTTGCACTGACGGC-3’ (sense) and 5’-TCCCCGTTCTCCGGTGCACGAC-3’ (antisense) 262 bp product. The PCR conditions were as follows: the amplification primary incubation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 min, annealing at 60°C for 1 min, and extension at 60°C for 1 min. The sequences of these PCR products were obtained by direct sequencing. Each amplicon was cloned into PGEM®-t Vector (Promega, Madison, WI, USA) in order to create standard curves for target cDNA. The mRNA levels were reported as ratios of the copy numbers of target cDNAs to GAPDH cDNA.

Effect on xenograft model
Six-week-old female BALB/cMlac-nu mice were purchased from the National Laboratory Animal Center, Salaya District, Nakorn Prathom Province, Thailand. The experimental protocol was conducted by National Research Council (NRC), Thailand, and was approved by the Faculty of Veterinary Science, Mahidol University. HuCCA cells, 2.5×10^6 in number, were used for xenografting all mice. The cells were injected subcutaneously into the right flanks of 10 nude mice. At day 14 after the injection, the tumor size reached approximately 50 mm³. At that time, the mice were randomly divided into two groups, five each, and treated with scarabaside D (1 mg/kg/day) or vehicle control [100 µL normal saline solution (NSS)] by intraperitoneal (i.p) injections. The body weights of the mice were recorded every 7 days, and tumor size was recorded every 3 days. The tumor size was measured in two dimensions (maximum longitudinal and transverse diameters) using a caliper and the volume estimated as: (length) × (width)^2/2 (Plengsuriyakarn et al., 2012). After 21 days of treatment, all the mice were euthanized and the tumors were dissected and weighed.

Statistical analysis
Data were expressed as mean ± SD from three or more independent experiments. Statistical significance was accessed by analysis of variance (ANOVA) followed by Bonferroni post-test in GraphPad Prism program version 5 (GraphPad software, San Diego, CA). Difference with p-values < 0.05 was considered statistically significant.

Results
Inhibition of HuCCA cell viability and migration
HuCCA cell viability decreased in a dose-dependent manner with scarabaside D treatments, as observed at 24 and 48 h, and with 5-FU treatments for observation at 48 h (Figure 2A). Statistically significant inhibition was detected with doses from 12.5 to 100 µg/ml for both scarabaside D and 5-FU treatments, compared to the respective control groups (0 µg/mL dose). There was no statistically significant difference in inhibition between scarabaside D (for 24 h and 48 h treatments)
and 5-FU treatment (48 h treatment), on comparing equal concentrations. The averaged 50% inhibitory concentration (IC50) value of scabraside D on HuCCA cell growth was approximately 12.80 ± 0.05 µg/ml. In addition, the effect of scabraside D on HuCCA cell viability was compared with the treatment with 5-FU. The result showed that scabraside D could decrease the cell viability as effectively as 5-FU at the same concentration. The inhibition of HuCCA cell migration in a plate culture is shown in figure 2B. In the control group (no treatment), after the layer of the cultured cells was scratched, the dividing cells along the sides of the scratch migrated into the scratch area and almost filled it up within 24 h. Under scabraside D treatment, the rate by which the migratory cells filled the scratch was slower than in the control group, and clearly slowed down with increasing doses of scabraside D. The result indicates that the substance retarded HuCCA cell migration in a dose-dependent manner.

Induction of apoptosis

In comparison to the control group (Fig. 3A), the scabraside D-treated HuCCA cells showed higher degrees of cell shrinkage under a phase contrast microscope (Fig. 3B). The treated cells also showed some degree of nuclear fragmentation and condensation under Hoechst fluorescent staining (Figure 3D, compared to Figure 3C), and revealed nuclear shrinkage with pyknosis and karyorrhexis in semi-thin sections (Figure 3F, compared to Figure 3E). Furthermore, by agarose gel electrophoresis, the DNA extracts from the scabraside D-treated cells displayed cleaved DNA fragments observed as DNA ladder on
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The expression levels of Bcl-2, Bax and caspase-3 in the scabraside D-treated cells were determined by Quantitative real-time PCR analysis. In comparison to the control group, scabraside D treatment increased Bax but decreased Bcl-2 levels in HuCCA cells in a dose-dependent manner (Figure 5A-B). These resulted in an increasing ratio of Bax/Bcl-2 mRNA in the scabraside D-treated group in a dose-dependent manner as well (Figure 5C). The expression of caspase-3 increased in the scabraside D-treated group, more or less in a dose-dependent manner (Figure 5D). In all the values obtained, significant differences to the untreated group (p < 0.05) were observed when the dose of scabraside D was at 25, 50 and 100 µg/mL. These changes in gene expressions induced by scabraside D confirm the apoptosis-induced action as a mechanism suppressing the viability and migration of HuCCA cells.

Inhibition of tumor growth in xenograft model

To confirm the pharmacological relevance of the in vitro data, effects of the scabraside D on human CCA tumor growth were investigated in xenograft model. The anti-cancer activity of scabraside D was evaluated in *vivo* in BALB/cMac-nu mice inoculated with HuCCA cells. The growth of the HuCCA tumor xenografts was recorded every third day for 21 contiguous days after intraperitoneal injection of scabraside D at 1 mg/kg/day. Figure 6A shows a representative HuCCA tumor from a xenograft model. The sizes of HuCCA xenografts in the animals injected with scabraside D were significantly smaller than in the control group from day 9 of the treatment onwards, tumor size with treatment reduced 41.12% of that of the control
substrates result in DNA fragmentation (Woo et al., 1998; Zheng et al., 1998; Tilli et al., 2002; Bras et al., 2005). As the growth and development of cancer are relating to unbalanced cell growth and apoptosis (Piotr et al., 2004), thus, one of the anti-cancer mechanisms is to induce apoptosis to the cancer cells.

Another anti-cancer agent with similar chemistry and similar mechanism of action is fomitoside-K, a lanostane triterpen glycoside isolated from the mushroom Fomitopsis nigra. This substance has been found to induce apoptosis of human oral squamous cell carcinoma via down-regulation of Bcl-2 and up-regulation of Bax, which leads to the activation of caspase-3 (Bhattarai et al., 2012). In the current study, scabraside D down-regulated Bcl-2 while up-regulating Bax, thus increasing Bax/Bcl-2 ratio and causing apoptosis of HuCCA cells. This in vitro study was corroborated by an in vivo study, in which scabraside D suppressed the growth of HuCCA xenograft in nude mice, the tumor size and tumor weight of treatment group was smaller than that of the control group. In addition, the animals survived throughout the cause of the experiment without apparent abnormalities at necropsy, or any other visible signs of toxicity. This result suggested the absence of untoward side effects of scabraside D. Although we only demonstrated apoptosis as a mechanism by scabraside D, other mechanisms of action inhibiting the viability, migration and growth of the cancer cells may exist. For instance, inhibition of neoangiogenesis and preventing in this way the vascularization of a tumor would slow down tumor growth and metastasis. It has been shown that a sulfated saponin isolated from the sea cucumber Pentacta quadrangularis inhibit neoangiogenesis in the choioallantoic membrane of chicken (Tian et al., 2005). As the CCA is highly malignant, it remains to find out whether scabraside D could also prevent its metastasis. The potential application of scabraside D against other cancer types also awaits further research.

In conclusion, our study showed that the scabraside D could induce HuCCA cell apoptosis, which likely caused the decrease in HuCCA cell viability and migration. Furthermore, we demonstrated that the scabraside D could reduce HuCCA tumor growth in the xenograft models. Based on these results, the scabraside D could be a promising candidate for the cholangiocarcinoma therapy.

Acknowledgements

This research was financially supported by the Royal Golden Jubilee Ph.D. Program (RGJPHD), PHD0012/2552.

References


