Asterosaponins from the Starfish *Astropecten monacanthus* Suppress Growth and Induce Apoptosis in HL-60, PC-3, and SNU-C5 Human Cancer Cell Lines

Nguyen Phuong Thao, a,b Bui Thi Thuy Luyen, a,b Eun-Ji Kim, c Hee-Kyoung Kang, c Sohyun Kim, d Nguyen Xuan Cuong, b Nguyen Hoai Nam, b Phan Van Kiem, b Chau Van Minh, b and Young Ho Kim* a

a College of Pharmacy, Chungnam National University; Daejeon 305–764, Korea; b Institute of Marine Biochemistry, Vietnam Academy of Science and Technology; 18 Hoang Quoc Viet, Caugiay, Hanoi, Vietnam; c Department of Pharmacology, School of Medicine, Institute of Medical Sciences, Jeju National University; and d School of Medicine, Brain Korea 21 Program, and Institute of Medical Sciences, Jeju National University; Jeju 690–756, Korea.

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Using various chromatographic experiments, six asterosaponins (1–6) were isolated from the MeOH extract of the Vietnamese starfish *Astropecten monacanthus*. The cytotoxic activities of the MeOH extract and six asterosaponins were evaluated on three human cancer cell lines, HL-60 (promyelocytic leukemia), PC-3 (prostate cancer), and SNU-C5 (colorectal cancer). Relative to the effects of the positive control mitoxantrone, the MeOH extract (with IC50 values ranging from 0.84±0.03 to 3.96±0.14 µg/mL) and asterosaponide D (5) (with IC50 values ranging from 4.31±0.07 to 5.21±0.15 µM) exhibited potent cytotoxic effects against all three tested human cancer cell lines. In addition, the MeOH extract and asterosaponide D (5) have an effect on leading to apoptosis. Interestingly, the apoptosis of induction was accompanied by down-regulation of phosphatidyl inositol 3-kinase (PI3K)/AKT signaling and extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinase (MAPK) signaling, and decrease of c-myc expression. Further studies are required to establish use of the asterosaponins from *A. monacanthus* as remedial and/or nutraceutical purposes.

Key words starfish; *Astropecten monacanthus*; asterosaponin; cytotoxic; apoptosis

MATERIALS AND METHODS

Cell Culture Promyelocytic leukemia (HL-60), prostate (PC-3), and colorectal (SNU-C5) human cancer cell lines used in this study were obtained from the Korea Cell Line Bank (KCLB) and were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively) at 37°C in a humidified 5% CO2 atmosphere. The exponentially growing cells were used throughout the experiments.

Compounds Asterosaponins, asterosteriosides A–D (1–3 and 5), psilasteroside (4), and marthasteroside B (6), were previously isolated and structure elucidated from the starfish *A. monacanthus*. Their purity (95–98%) was determined by NMR and HPLC analysis. Stock solutions in dimethyl sulfoxide (DMSO) were prepared, kept at −20°C, and diluted to the final concentration in fresh media before each experiment. For not to affect cell growth, the final DMSO concentration did not exceed 0.5% in all experiments.

Cell Viability Assay The effects of the MeOH extract and isolated asterosaponins (1–6) on the growth of human cancer cells were determined by measuring metabolic activity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay was performed as follows: human cancer cell lines (HL-60; 3×10^4 cells/mL, PC-3; 5×10^4 cells/mL, and SNU-C5; 1×10^5 cells/mL) were treated for 3 d with 0.01, 0.1, 1, 10, 50, and 100 µM of the compounds or 0.01, 0.1, 1, 10, 50, and 100 µg/mL of the fraction. After incubation, 0.1 mg (50 µL of a 2 mg/mL solution) MTT (Sigma, Saint Louis, MO, U.S.A.) was added to each well and the plates were incubated at 37°C for 4 h. The plates were
centrifuged at 1000 rpm for 5 min at room temperature and the media was then carefully aspirated. Dimethylsulfoxide (150 µL) was then added to each well to dissolve the formazan crystals. The plates were read immediately at 540 nm on a microplate reader (Amersham Pharmacia Biotech., NY, U.S.A.). All the experiments were performed three times and the mean

![Chemical Structures of Asterosaponins (1–6) from the Starfish *Astropecten monacanthus*](image)

Fig. 1. Chemical Structures of Asterosaponins (1–6) from the Starfish *Astropecten monacanthus*

![Degree of Apoptosis Represented as the DNA Content Measured by Flow Cytometric Analysis in HL-60 (A), PC-3 (B), and SNU-C5 (C) Cells for 24 and 48h with Increase of Percentages of Sub-G1 Hypodiploid Cells](image)

Fig. 2. The Degree of Apoptosis Represented as the DNA Content Measured by Flow Cytometric Analysis in HL-60 (A), PC-3 (B), and SNU-C5 (C) Cells for 24 and 48h with Increase of Percentages of Sub-G1 Hypodiploid Cells
Table 1. Cytotoxic Effects of the MeOH Extract and Asterosaponins (1–6) from *Astropecten monacanthus*

<table>
<thead>
<tr>
<th>Compounds</th>
<th>HL-60 (leukemia)</th>
<th>PC-3 (prostate)</th>
<th>SNU-C5 (colorectal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrosterioside A (1)</td>
<td>50.50±2.27</td>
<td>73.17±1.89</td>
<td>57.90±0.27</td>
</tr>
<tr>
<td>Astrosterioside B (2)</td>
<td>25.28±0.93</td>
<td>26.10±0.93</td>
<td>22.61±1.23</td>
</tr>
<tr>
<td>Astrosterioside C (3)</td>
<td>24.73±0.66</td>
<td>32.45±0.88</td>
<td>27.45±0.84</td>
</tr>
<tr>
<td>Psilasteroside (4)</td>
<td>20.32±1.66</td>
<td>30.85±1.32</td>
<td>28.54±0.42</td>
</tr>
<tr>
<td>Astrosterioside D (5)</td>
<td>4.31±0.07</td>
<td>5.16±0.07</td>
<td>5.21±0.15</td>
</tr>
<tr>
<td>Marthasteroside B (6)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>MeOH extract&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.84±0.03</td>
<td>3.86±0.09</td>
<td>3.96±0.14</td>
</tr>
<tr>
<td>Mitoxantrone&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.80±0.09</td>
<td>5.17±0.34</td>
<td>22.01±1.19</td>
</tr>
</tbody>
</table>

<sup>a</sup>) µg/mL,  <sup>b</sup>) Positive control. NI: No inhibition (values <100 µM are considered to be active). Results are the means±S.D. of three independent experiments in triplicate.

Fig. 3. The Degree of Apoptosis Represented as the Fluorescent Image of Nuclei in HL-60 (A), PC-3 (B), and SNU-C5 (C) Cells by Hoechst 33342 Staining for 24 and 48 h

The MeOH extract and compound 5 induced morphological changes including formation of apoptotic bodies.
absorbance values were calculated. The results are expressed as the percentage of inhibition that produced a reduction in the absorbance by the treatment of the compounds compared to the untreated controls. A dose–response curve was generated and the inhibitory concentration of 50% (IC50) was determined for each sample as well as each cell line.

**Morphological Analysis of Apoptosis by Hoechst 33342 Staining**

HL-60 (3 × 10^5 cells/mL), PC-3 (5 × 10^4 cells/mL), and SNU-C5 (1 × 10^5 cells/mL) cells were treated with the IC50 values of MeOH extract and astrosterioside D (5) for 12, 24, and 48 h. The cells were incubated in a Hoechst 33342 (culture medium at a final concentration of 10 µg/mL) DNA-specific fluorescent staining solution at 37°C for 20 min. The stained cells were observed with an inverted fluorescent microscope equipped with an IX-71 Olympus camera and photographed (magnification ×200).

**Flow Cytometric Analysis of Apoptosis**

HL-60 (3 × 10^5 cells/mL), PC-3 (5 × 10^4 cells/mL), and SNU-C5 (1 × 10^5 cells/mL) cells were treated with the IC50 values of MeOH extract and astrosterioside D (5) for 24 and 48 h. After treatment, the cells were harvested and washed two times with cold PBS. The cells were lysed with lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol bis(2-aminoethyl ether)-N,N′,N′,N′ ‑tetraacetic acid (EGTA), 1 mM NaVO₄, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 25 µg/mL aprotinin, 25 µg/mL leupeptin, 1% Nonidet P-40) and kept on ice for 30 min at 4°C. The lysates were centrifuged at 15000 rpm at 4°C for 15 min. The supernatants were stored at −20°C until use. Protein content was determined by the Bradford assay. The same amount of lysates were separated on 8–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, U.S.A.) by glycin buffer (192 mM glycine, 25 mM Tris–HCl [pH 8.8], and 20% MeOH [v/v]) at 200 mA for 2 h. After blocking with 5% nonfat dried milk, the membrane was incubated with primary antibody against Bcl-2 (1 : 500), Bax (1 : 1000), poly(ADP-ribose) polymerase (PARP) (1 : 1000), cleaved caspase-9 (1 : 1000), cleaved caspase-3 (1 : 1000), extracellular signal-regulated kinase (ERK) 1/2 (1 : 1000), phospho-ERK1/2 (1 : 1000), C-myc (1 : 1000), AKT (1 : 1000), phospho-AKT (1 : 1000), and β-actin (1 : 5000) antibodies and incubated with a secondary horseradish peroxidase (HRP) antibody (1 : 5000; Vector Laboratories, Burlingame, VT, U.S.A.) at room temperature. The membrane was exposed on X-ray films (AGFA, Belgium), and protein bands were detected using a WEST-ZOL® plus Western Blot Detection System (iNtRON, <br>Fig. 4. Effects of MeOH Extract and Compound 5 on the Levels of Bcl-2, Bax, Cleaved Caspase-9, Cleaved Caspase-3, Cleaved PARP, and on the Activation of ERK1/ERK2 and AKT in HL-60 Cells Protein expressions were detected by Western blotting.
Gyeonggi-do, Korea).

**Statistical Analysis** Data are presented as the means±S.D. of at least three independent experiments performed in triplicate. Statistically significant differences were determined by the ANOVA using SPSS statistical software (SPSS, IL, U.S.A.).

**RESULTS AND DISCUSSION**

In recent years, significant efforts have been directed towards studying marine organisms to isolate potential compounds for the development of new anticancer agents.\(^{14}\) Starfish has been reported to possess saponin analogues with known cytotoxic activities but the specific mechanisms that mediate this have not been well studied. In this study, the cytotoxic effects of the MeOH extract of the edible starfish *A. monacanthus* were examined against three human cancer cell lines, HL-60 (leukemia), PC-3 (prostate), and SNU-C5 (colorectal), using a MTT assay. Treatment of these cell lines with the MeOH extract for 72 h yielded IC\(_{50}\) values of 0.84±0.03, 3.86±0.09, and 3.96±0.14 µg/mL, respectively. Subsequently, all isolated astrosteriosides (1–6) from *A. monacanthus* were also evaluated on the same cell lines using a MTT assay for 72 h (Table 1).

As a result, astrosterioside D (5) exhibited the most potent cytotoxic activity on all three tested cell lines with IC\(_{50}\) values of 4.31±0.07, 5.16±0.07, and 5.21±0.15 µM, respectively. For comparison, the positive control, mitoxantrone had IC\(_{50}\) values of 6.80±0.09, 5.17±0.34, and 22.01±1.19 µM, respectively. Mitoxantrone, an anthracene compound, disrupts DNA synthesis and DNA repair through it intercalates into and crosslinks DNA.\(^{15}\) It is clinically used in the treatment of cancer, mostly leukemia, non-Hodgkin's lymphoma, and various advanced solid tumors including colorectal cancer and prostate cancer.\(^{15–17}\) Moderate cytotoxic activities were observed with astrosteriosides 2–4 and weak effects of 1 were evident on all three cancer cell lines. Marthasteroside B (6) showed no cytotoxic activity (Table 1). The structures of compounds 1–6 suggested that the presence of two ketone groups in the side chain might play an important role in their cytotoxic effects. Interestingly, the cytotoxicity of the methanol extract was more potent than those of the isolated saponins. This evidence suggested that the cytotoxic activity of MeOH extract might arise from the synergy of many compounds but not of each individual.

Due to the observed cytotoxic activity, we next investigated whether the inhibitory effects of the MeOH extract and astrosterioside D (5) on HL-60, PC-3, and SNU-C5 cells might be due to induction of apoptosis (see Materials and Methods). Apoptotic characteristics were examined after following treatment with the MeOH extract and astrosterioside D (5) at IC\(_{50}\) levels after 24 and 48 h. Flow cytometric analysis showed that the percentages of sub-G1 hypodiploid cells exposed to the MeOH extract and astrosterioside D (5) were significantly increased after 48 h (Fig. 2). These results indicate the induction of apoptosis, which was further supported by an increase of...
in the number of apoptotic bodies found in sample-treated cells that had been stained with the cell-permeable DNA dye Hoechst 33342 and visualized by fluorescence microscopy (Fig. 3).

The B cell lymphoma-2 (Bcl-2) family is separated into two subfamilies that either inhibit (anti-apoptotic proteins such as Bcl-2 and Bcl-xL) or promote apoptosis (pro-apoptotic proteins such as Bax, Bid, and Bak). Pro-apoptotic members of the Bcl-2 family induce apoptosis through the release of cytochrome c from mitochondria, which results in the cleavage and activation of cysteine-aspartic acid protease-9 (caspase-9). Upon activation, caspase-9 initiates a protease cascade leading to the rapid activation of caspase-3, an effector caspase in cells undergoing apoptosis.\(^{18-20}\) Therefore, to determine the possible mechanism underlying the induction of apoptosis, we monitored the expression of apoptosis-related proteins such as Bcl-2, Bax, cleaved-caspase-3, caspase-9 and cleavage (PARP) in HL-60, PC-3, and SNU-C5 cells. When treated with IC\(_{50}\) values of the MeOH extract and astrosterioside D (\(5\)), we could observe the alteration of apoptosis-related protein levels such as increase of Bax, decrease of Bcl-2, cleavage of caspase-9, cleavage of caspase-3, and the cleavage of PARP in a time-dependent manner (Figs. 4–6). Caspase execute apoptosis by cleaving a variety of substrates such as nuclear lamins, ICAD/DEF45, and PARP.\(^{21}\) The obtained results indicated that the MeOH extract and astrosterioside D (\(5\)) induced apoptosis in HL-60, PC-3, and SNU-C5 cells via activation of caspase-3 which resulted from increase of Bax and/or decrease of Bcl-2.

The MAPK pathway and the PI3K/AKT signaling pathway regulate cell survival, cell growth, and apoptosis.\(^{22,23}\) To establish the MAPK and PI3K/AKT mechanism of apoptosis induced by the MeOH extract and compound \(5\), we examined the expression of ERK 1/2 MAPK and AKT in HL-60, PC-3, and SNU-C5 cells. Treatment with either the MeOH extract or compound \(5\) significantly decreased phospho-ERK1/2 and phospho-AKT levels (Figs. 4–6). Several studies have stated that activation of the ERK 1/2 MAPK pathway contributes to the stabilization of C-myc, which is one of oncoproteins.\(^{24}\) In addition, AKT activation promotes the transcription of C-myc.\(^{23}\) Interestingly, treatment with the MeOH extract or compound \(5\) resulted in a decrease in C-myc levels along with downregulation of phospho-ERK 1/2 and phospho-AKT (Figs. 4–6). These results suggested that the induction of apoptosis by the MeOH extract and astrosterioside D (\(5\)) was also accompanied by the inactivation of PI3K/AKT and ERK 1/2 MAPK pathways and the downregulation of C-myc in HL-60, PC-3, and SNU-C5 cells. In mammals, six distinct groups of MAPKs have been characterized including extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK), and the p38 isoforms. The ERK pathway provides a rich target area, of which potential targets downstream of ERK play key roles in angiogenesis, cell migration, invasion, and metastasis. One important mechanism whereby ERK signalling may promote a more malignant phenotype is by disrupting Rho signalling pathways. Additionally, ERK also can phosphorylate a number of proteins involved in cell migration as well as activate C-myc.

\[\text{Fig. 6. Effects of MeOH Extract and Compound 5 on the Levels of Bel-2, Bax, Cleaved Caspase-9, Cleaved Caspase-3, Cleaved PARP, and on the Activation of ERK1/ERK2 and AKT in SNU-C5 Cells} \]

Protein expressions were detected by Western blotting.
as regulate the expression of proteases involved in basement membrane degradation.\textsuperscript{23} Moreover, one of the major functions of AKT/PKB is to promote growth factor-mediated cell survival and block apoptosis was suggested by a large amount of experimental evidence. AKT/PKB has been an attractive therapeutic target. Recently, specific AKT/PKB inhibitor has been identified, which proved a useful tool for cancer therapy.\textsuperscript{25} Therefore, pharmaceutical compounds that specifically inactivate AKT/PKB will be potential candidates for further investigations regarding cancer remedy.

In conclusion, the MeOH extract and astrosterioside D (5) from \textit{A. monacanthus} exhibited potent \textit{in vitro} cytotoxic activity against HL-60, PC-3, and SNU-C5 human cancer cell lines. Moreover, both the MeOH extract and compound 5 induced apoptosis of these three cancer cells \textit{via} the inactivation of PI3K/AKT and ERK 1/2 MAPK pathways and the downregulation of C-myc. Starfish are an important ingredient in traditional Vietnamese medicine and medicinal foods. To date, knowledge of the mechanistic basis of cytotoxic activity of asterosaponins is limited. The obtained results in this paper demonstrated potent cytotoxic activity of the MeOH extract and astrosterioside D (5) from \textit{A. monacanthus}, which might be resulted from the induction of apoptosis \textit{via} inactivation of PI3K/AKT and ERK 1/2 MAPK pathways. Thus, further studies are required to establish use of the asterosaponins from \textit{A. monacanthus} as remedial and/or nutraceutical purposes.

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REFERENCES