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Research Article

Proteomic investigation of anti-tumor activities exerted by sinularin against A2058 melanoma cells

The extracts from soft corals have been increasingly investigated for biomedical and therapeutic purposes. The aim of this study is to examine and analyze the anti-tumor effects of the genus *Sinularia* extract sinularin on A2058 melanoma cells using MTT assay, cell migration assay, wound healing assay, flow cytometric analysis, and proteomic analysis. Sinularin dose-dependently (1–5 μg/mL) inhibited melanoma cell proliferation while the treatment at identical concentrations suppressed cell migration. Sinularin dose-dependently enhanced apoptotic melanoma cells and caused tumor cell accumulation at G2/M phase, indicating that sinularin exerts apoptosis-induced and cell cycle-delayed activities in A2058 melanoma cells. Comparative proteomic analysis was conducted to investigate the effects of sinularin at the molecular level by comparison between the protein profiling of melanoma cells treated with sinularin and without the treatment. Thirty-five differential proteins (13 upregulated and 22 downregulated) concerning the treatment were identified by liquid chromatography-tandem mass spectrometry. Proteomic data and Western blot displayed the levels of several tumor inhibitory or apoptosis-associated proteins including annexin A1, voltage-dependent anion-selective channel protein 1 and prohibitin (upregulated), heat shock protein 60, heat shock protein beta-1, and peroxiredoxin-2 (downregulated) in A2058 melanoma cells exposed to sinularin. Increased expression of p53, cleaved-caspase-3, cleaved-caspase-8, cleaved-caspase-9, p21, and Bax and decreased expression of Bcl-2 in sinularin-treated melanoma cells suggest that the anti-tumor activities of sinularin against melanoma cells are particularly correlated with these pro-apoptotic factors. These data provide important information for the mechanisms of anti-tumor effects of sinularin on melanoma cells and may be helpful for drug development and progression monitoring of human melanoma.

Keywords:
Melanoma cells / Proteomic analysis / Sinularin

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1 Introduction

The incidence rates of malignant melanoma continue to remarkably rise throughout the world. High incidence of melanoma has been reported in New Zealand and Australia [1, 2]. According to the estimation from American Cancer Society, there were approximately 68, 720 cases of invasive melanoma diagnosed in 2009 in the United States. Metastatic melanoma is mostly incurable in diagnosed people because melanoma does not respond to most systemic treatments [3–6]. The presence of distant metastases is the worse prognosis for melanoma patients. It has been shown that the 5-year survival rate is approximately 97% for patients with a tumor thickness of 1 mm lacking metastases (stage I). The patients with metastases to subcutaneous tissue and distant lymph node had longer survival of 18 months than patients with metastases to lung (12 months), viscera, and other sites (6 months). Surgery is the most effective alternative for patients with early primary lesions whereas survival is dependent on the diagnostic stage of melanoma [7]. For patients with malignant melanoma, surgery is also the most essential treatment [8–10]. Other medical measures such as chemotherapy and cytokine therapy have also been investigated. However, dacarbazine, as the most active single agent against melanoma, accounts for the response rate approximately 15–25% [11, 12]. Cytokine therapy using interleukin-2 (IL-2)

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Abbreviations: **Hsp60**, heat shock protein 60; **HspB1**, heat shock protein beta-1; **Prx2**, peroxiredoxin-2; **VDAC 1**, voltage-dependent anion-selective channel protein 1

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Color Online: See the article online to view Fig. 3 in color.

shows an overall 12.5% response rate but it should be underscored that this response is durable and is with a prolonged disease response [13]. The epidemiological study by Sasse et al. (2007) showed enhanced objective response rates in people treated with chemoimmunotherapy in comparison with those treated with chemotherapy. However, these increased response rates did not reflect higher survival benefit [6].

Therefore, cutaneous malignant melanoma is truly an urgent medical and therapeutic issue. It is important to find new drugs and develop therapies against this highly malignant tumor. Recently, many studies have been carried out to discover new therapeutic drugs for cancer, in particular, using biologically active compounds from natural marine organisms [14]. Soft corals (coelenterata, octocorallia, alcyonaceae) are rich sources of steroids and terpenoids. The majority of the isolated diterpenes are cembranolides. These compounds have been reported to exert various biological activities, such as anti-tumor, anti-microbial, and HIV-inhibitory activity [15–17]. Several compounds including steroids, diterphenoids, diterpenes, and prostanoids, isolated from natural soft coral have been found to induce apoptosis and exert antitumor effects on the cancer cell lines such as prostate cancer cells, breast cancer cells, colon cancer cells, hepatocellular carcinoma, and cervix cancer cells [18–22]. Of importance is the apoptosis-induced activity for anti-cancer therapies and this is also a valuable guide to predict tumor response after anticancer treatment administered. Cell death is likely to be apoptotic, or due to morphological changes like cell shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation with formation of apoptotic bodies. The apoptotic cell death that naturally occurs in cells may benefit cancer therapy development [23, 24]. Sinularin is an active compound isolated from the soft coral *Sinularia flexibilis*. The chemical structure and NMR spectra of sinularin were shown in Fig. 1. The purity of this compound was checked by ^{1}H and ^{13}C NMR spectra (Fig. 1). Herein, we examined the apoptosis-induced and other anti-tumor effects of sinularin on A2058 melanoma cells. The relationship between the effects and the regulation of potential protein markers using proteomic analysis was investigated and the connections between induction of apoptosis and several pro-apoptotic factors after sinularin treatment were also studied. The data in this study may be helpful for therapy development or potential strategies against human melanoma.

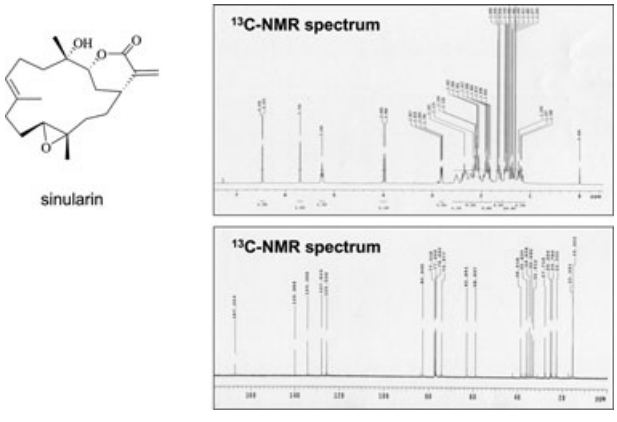

Figure 1. The chemical structure and NMR spectra of sinularin.

2. Materials and Methods

2.1 Materials

Cell Extraction Buffer was obtained from BioSource International (Camarillo, CA, USA). DMEM, MTT and protease inhibitor cocktail were from Sigma (St. Louis, MO, USA). FBS was from Gibco (Carlsbad, CA, USA). The IPG buffer was purchased from GE Healthcare (Buckinghamshire, UK). Rabbit anti-human annexin A1, Prx2, VDAC1, Hsp60, and HspB1 antibodies were obtained from ProteinTech Group (Chicago, IL, USA). Antibodies against prohibitin and p53 were from Epitomics (Burlingame, CA, USA). Antibodies against cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, p21, Bax, and Bcl-2 were from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-human β-actin antibodies were obtained from Sigma. Goat anti-rabbit and horseradish peroxidase conjugated IgG was from Millipore (Bellerica, MA, USA). PVDF (polyvinylidene difluoride) membranes and Chemiluminescent HRP Substrate were from Pierce (Rockford, IL, USA). Chemicals were from Sigma unless otherwise stated.

2.2 Cell culture and treatment with sinularin

A2058 cells were grown in DMEM with 4 mM L-glutamine adjusted to contain 1.5g/L sodium bicarbonate and 4.5g/L glucose, supplemented with 10% (v/v) FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1 mM sodium pyruvate in a humidified atmosphere with 5% CO_2 in air at 37°C. When cells reached above 70% confluency, subculture was conducted at a split ratio of 1:6. A2058 cells were cultured in a 10-cm dish for each assay. Sinularin (Fig. 1) was isolated from the soft coral *S. flexibilis*. Control cultures were made by adding dimethyl sulfoxide (DMSO) at the same final concentration as in the treated samples (0.01% v/v). Cells were added with different concentrations of sinularin (1 μg/mL, 2 μg/mL, 3 μg/mL, and 5 μg/mL) and harvested after 24-h incubation. All the experiments were repeated three times to confirm reproducibility.

2.3 MTT assay

The anti-proliferative effect of sinularin against A2058 cells was determined by MTT assay. A2058 cells ($1 \times 10^5/cm^2$) were incubated in 96-well plates. After the treatment with various concentrations of sinularin for 24 h, each well was added with 50-μl MTT solution (1 mg/mL in PBS) and the plates were incubated at 37°C for 4 h. To achieve solubility of purple-blue MTT formazan crystals in viable cells, 200 μl of DMSO was added to each well. The absorbance was measured

at 595 nm on a microtiter plate ELISA reader while DMSO was used as blank.

2.4 Cell migration assay and wound healing assay

For cell migration assay, A2058 melanoma cells in serum-free media were seeded onto polycarbonate membranes (8.0 μm, BD Biosciences, CA, USA) in the culture inserts. The inserts were then placed in a culture well with DMEM containing 10% FBS. Melanoma cells with or without sinularin treatment were allowed to migrate for 24 h. After removing nonmigrating cells on the upper site, migrated cells on the lower site were fixed and stained by 100% methanol and Giemsa (Merck, Germany), respectively. Migrated cells were observed and counted at 100× magnification [25]. For wound healing assay, melanoma cells were seeded in 6-well plates. After the cells grew to confluence, an artificial scratch/wound was made with a pipette tip in each of the wells. Unattached tumor cells were removed by the wash with PBS. Images of control and experimental groups (0, 1, 2, and 3 μg/mL sinularin) were acquired at 0 h, 12 h, and 24 h after the treatments.

2.5 Assessment of cell cycle distribution and apoptosis

A2058 cells were treated with solvent vehicle or indicated concentrations of sinularin for 24 h. To examine the phase distribution of the cell's DNA content, propidium iodide (PI, Sigma) staining was performed. After treatment, the cells were collected, washed twice with PBS, and fixed in 70% ethanol overnight. After centrifugation at 700 rpm for 5 min at 4°C, the cell pellet was stained with 10 μg/ml PI and 10 μg/mL RNase A in PBS buffer for 15 min at room temperature in the dark. To determine the apoptosis induced by sinularin in A2058 melanoma cells, the annexin V staining (Strong Biotech Corporation, Taipei, Taiwan) was performed [26]. A total of 1×10^6 cells per 100-mm Petri dish were treated with vehicle or sinularin for 24 h and subsequently labeled with 10 μg/mL of annexin V-FITC. Apoptosis and cell cycle distribution of sinularin-treated A2058 melanoma cells were assessed using a FACScan flow cytometer (Becton-Dickinson, Mansfield, MA, USA) and Cell-Quest software (Becton-Dickinson).

2.6 Protein extraction and estimation

A2058 cells were untreated or treated with different concentrations of sinularin (0, 1, 2 , and 3 μg /mL) for 24 h and then lysed with Cell Extraction Buffer (BioSource International, Camarillo, CA, USA) and protease inhibitor cocktail (Sigma). The total protein in the supernatant was then precipitated out for overnight (−20°C) by triple the volume of 10% TCA/acetone solution containing 20 mM DTT. After centrifugation at 8000 rpm for 30 min at 4°C, the supernatant was discarded. The pellet was suspended in a rehydration buffer (6 M urea, 2 M thiourea, 0.5% CHAPS, 0.5% IPG buffer, 20 mM DTT, and 0.002% bromophenol blue) at 4°C for overnight. The IPG buffer was purchased from GE Healthcare. The protein contents were determined using 2-D Quant Kit (GE Healthcare).

2.7 2DE

The first dimension electrophoresis (isoelectric focusing) was performed on GE Healthcare Ettan IPGphor 3 at 20°C with a current limit of 30 A per strip [27]. A sample was dissolved in a rehydration buffer as described above and applied on an IPG strip in a strip holder. Every 11-cm IPG strip (p*I* 3–10, Immobiline DryStrip) was rehydrated at 30 V for 12 h and then focused according to the preset program: 200 V (2 h), 500 V (2 h), 1000 V (2 h), 4000 V (1 h), 8000 V (4 h), until the total Vh reached 39400. The equilibrated strip was placed on the top of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (12.5%), sealed with 0.5% agarose, then the second-dimension electrophoresis was run at 150 V for 6.5 h. Electrophoretic unit used for the second-dimension electrophoresis was SE 600 Ruby (Hoeffer). 2DE images were made in triplicate for each sample and normalized prior to statistical analysis.

2.8 Protein identification by LC-MS/MS

2.8.1 In-gel digestion

A protein spot of interest was excised into a piece of 1 mm × 1 mm, then placed in a microcentrifuge tube. A 100 μL of 50 mM DTT in 25 mM ammonium bicarbonate (pH 8.5) was added to the tube which was shaken at 37°C for 1h. After removing excess DTT in supernatant, a 100 μL of 100 mM iodoacetamide (IAA) in 25 mM ammonium bicarbonate (pH 8.5) was added to the tube which was then shaken for 30 min at RT in dark. The excess IAA in supernatant was removed. A 100 μL of 50% acetonitrile in 25 mM ammonium bicarbonate buffer (pH 8.5) was added and then the gel piece was soaked for 30 min followed by complete removal of the buffer. A 0.1 μg of trypsin in 10 μL 25 mM ammonium bicarbonate (pH 8.5) was added to the gel piece. Digestion was run for 16 h at 37°C. The peptide solution was then concentrated for the LC-MS/MS analysis.

2.8.2 LC-MS/MS analysis

A peptide mixture was separated by nanoflow reversed phase C18 chromatography on nano LC using the Agilent 1200 System and PepMap100 C18, 75 μm × 15 cm (3 μm) nanoLC column or HPLC (high-performance liquid chromatography) using the Agilent NanoLC 1200 System and Agilent Zobax 2.1 mm × 150 mm C18 column. LC-MS/MS analysis employed a 10-min online trapping and desalting step followed by a 60 min 5–40% mobile B gradient at nano flow and a 15 min 5–40% mobile B gradient

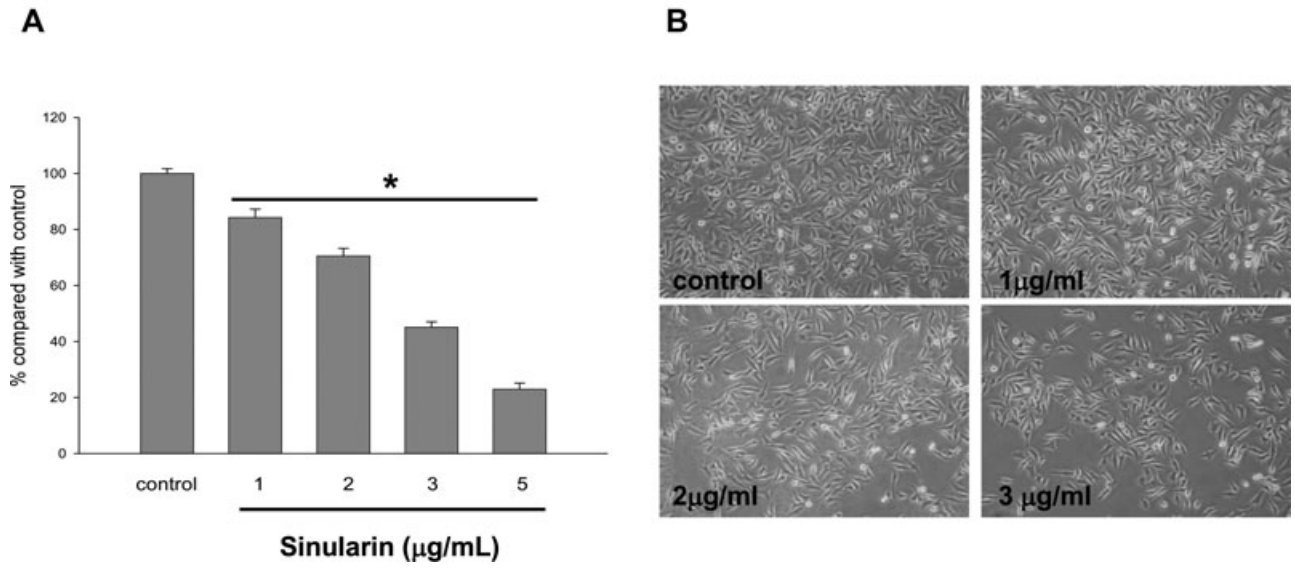

Figure 2. (A) The viability of A2058 melanoma cells was dose-dependently inhibited by treatment with 1–5 μg/mL sinularin for 24 h. Inhibition of cell proliferation was assessed by MTT assay (*$p < 0.001$). (B) Reduced population and morphological changes of A2058 melanoma cells exposed to different concentrations (1, 2, and 3 μg/mL) of sinularin for 24 h.

at higher flow (mobile B = 98% ACN, 0.1% formic acid). Samples were analyzed on the AB SCIEX QTRAP® 5500 Q mass spectrometer (Applied Biosystems, CA, USA). The scan range was from m/z 100–1000 for MS. The raw data was processed into a text file format of WIFF with Analyst 1.5.1.

2.9 Western blotting analysis

After SDS-PAGE analysis of the treated samples and the controls under reducing conditions, the proteins on gel were transferred to a PVDF membrane (Millipore) for 1.5 h at 400 mA using Transphor TE 62 (Hoeffer). The membranes were then incubated with human annexin A1, VDAC1, prohibitin, Prx2, Hsp60, HspB1, p53, cleaved-caspase-3, cleaved-caspase-8, cleaved-caspase-9, p21, Bax, Bcl-2, and β-actin antibodies at 4°C for 2 h or overnight. The membranes were washed three times in PBST (10 mM NaH_2PO_4, 130 mM NaCl, 0.05% Tween 20), then probed with the second antibodies (goat anti-rabbit and horseradish peroxidase conjugate, 1:5000 in blocking solution) for 1 h. After washing with PBST for three times, the enzyme activity on the blot was visualized through chemiluminescence by adding ECL Western Blotting Reagents (Pierce).

2.10 Statistical analysis

Results were pooled from three to four independent experiments. Data of MTT assay, cell migration assay, and flow cytometric analysis are expressed as mean ± standard error of mean (SEM) and analysis of variance (ANOVA) followed by the Tukey–Kramer test performed on GraphPad InStat 3 (San Diego, CA, USA) to determine significant differences ($p \leq 0.05$) between experimental groups [28].

3. Results

3.1 The anti-tumor effects of sinularin on A2058 cells

A2058 cells were treated with sinularin of various concentrations (1, 2, 3, 5 μg/mL) for 24 h. Cell viabilities were determined by MTT assay and migratory capacity of cells was assessed by wound healing assay and cell migration assay. Sinularin from 1 μg/mL to 5 μg/mL dose-dependently inhibited the proliferation of A2058 melanoma cells. When the A2058 cells were exposed to 3 μg/mL sinularin, cell viability was reduced to the level approximately 45% of control. The half maximal inhibitory concentration (IC50) of sinularin is 3.1 μg/mL. Morphological changes of sinularin-treated cells were observed by inverted light microscopy. A2058 cells treated with 2 and 3 μg/mL sinularin showed shrinkage as well as apparently decreased cell population compared with the control (Fig. 2). After 12-h and 24-h treatment with 2 and 3 μg/mL sinularin, suppression of A2058 cell migration was observed by wound healing assay (Fig. 3). Data of cell migration assay showed 1–5 μg/mL sinularin dose-dependently suppressed A2058 melanoma cell migration as the suppression rates were approximately 45%, 70%, and 95% for 2, 3, and 5 μg/mL sinularin treatment, respectively (Fig. 3).

3.2 Apoptosis of A2058 cells treated with sinularin

To investigate apoptosis-induced effects of sinularin, A2058 cells exposed to sinularin were analyzed using annexin

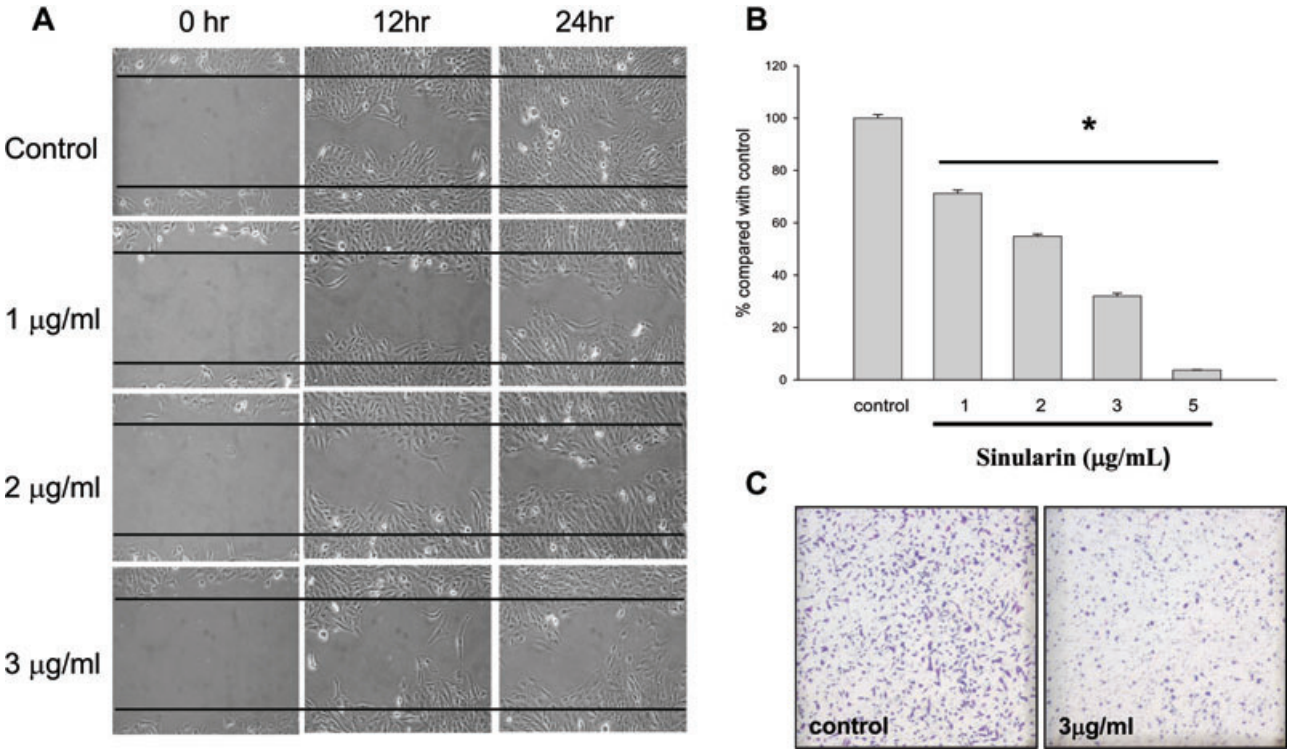

Figure 3. Anti-migratory effects of sinularin at different concentrations against A2058 melanoma cells examined by wound healing assay and cell migration assay. Wound healing assay: (A) A2058 cells in the areas between two solid lines were migrated cells in the gaps during the indicated time periods. The images represented apparent reduction in A2058 cell migration when 1, 2, and 3 μg/mL sinularin were treated for both 12 and 24 h. Cell migration assay: (B) Sinularin from 1 to 5 μg/mL dose-dependently suppressed A2058 cell migration (*$p < 0.001$). (C) Migrated A2058 cells were clearly reduced (3 μg/mL sinularin treated) compared with control at 100× magnification.

V-FITC & PI staining on a flow cytometer (Becton-Dickinson). A2058 cells at sub-G1 phase were increased after treatment with 1, 2, and 3 μg/mL sinularin. The percentage of tumor cells of 2 μg/mL sinularin-treated group was enhanced to 12.06% compared with 6.87% of control. (Fig. 4 and Table 1). Sinularin (2 and 3 μg/mL) significantly increased the cell population at G2/M phase, suggesting that treatments with both 2 and 3 μg/mL sinularin are able to cause G2/M arrest (Fig. 4 and Table 1). The induction of apoptosis in A2058 melanoma cells was determined by the flow cytometer based-annexin V staining. Sinularin from 1 to 3 μg/mL dose-dependently enhanced the percentage of apoptotic melanoma cells (Fig. 5A and B). These results indicated that treatment with sinularin is capable of inducing apoptosis and delaying cell cycle at G2/M phase in A2058 melanoma cells.

3.3 Proteomic analysis of A2058 cells treated with sinularin

The A2058 cells were harvested after being treated with a 2 μg/mL solution of sinularin. The proteins were extracted from cultured cells with lysis buffer. The supernatants were collected and the proteins were precipitated by TCA/acetone. The 2DE maps of the A2058 cells treated with sinularin were compared with those of the control samples to examine the effect of sinularin on A2058. The 2DE were run with a loading of 50-μg protein (p*I* 3–10) and visualized by silver staining (Fig. 6 and 7). PDQuest image analysis software (Bio-Rad) was employed for detecting the differential protein spots which were defined as the proteins showing a more than 1.5-fold intensity difference in 2DE maps between the treated A2058 cells and the control samples. The protein identification was carried out by LC-MS/MS analysis after in-gel digestion. MASCOT protein identification search software was used for the identification of the differential protein spots. A total of 35 differential protein spots were successfully identified. A list of the identified proteins with their MASCOT score, MS/MS-matched sequences, apparent and theoretical MW, p*I*, coverage, and fold of change in expression level (upregulation or downregulation) were shown in Table 2.

The varied expression of proteins distributed throughout the entire gels indicated that multiple clusters of proteins were involved in the effects of sinularin on A2058 cell. A total of 13 differential proteins were upregulated after sinularin treatment. They were nucleophosmin (NPM), proteasome subunit beta type 7 precursor, peroxiredoxin-6, fructose-bisphosphate aldolase A , elongation factor Tu, mitochondrial precursor (EF-Tu) (P43), phosphoglycerate mutase 1, phosphoserine aminotransferase,

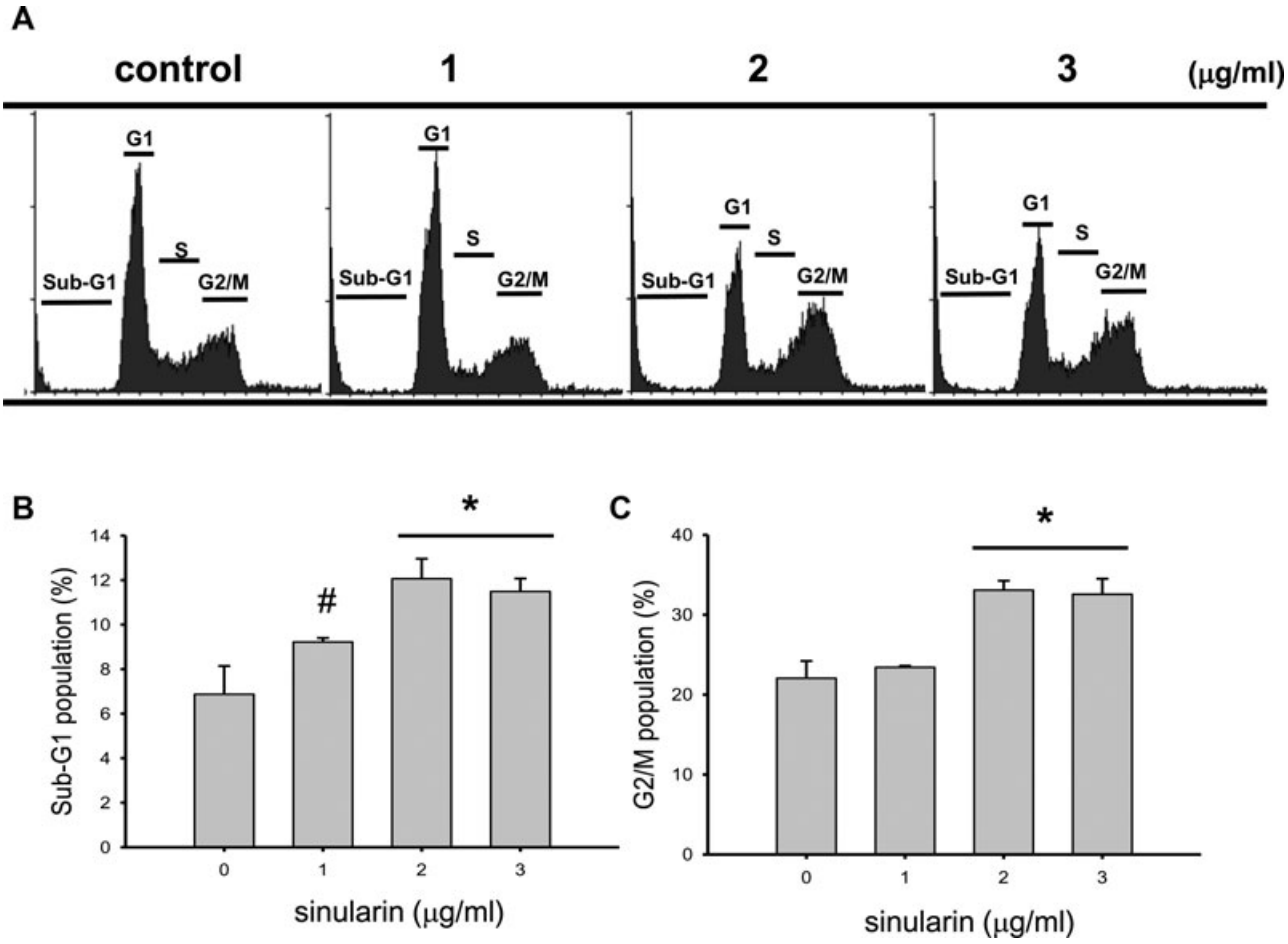

Figure 4. Accumulation at G2 phase induced by sinularin. (A) Modulation of cell cycle distribution by sinularin. (B) Sinularin increased the accumulation of sub-G1 population (#$p < 0.05$; *$p < 0.01$) and (C) caused G2/M phase arrest compared with that of the control. (*$p < 0.01$).

FK506-binding protein 3, prohibitin, elongation factor 2 (EF-2), annexin A1, LIM, and SH3 domain protein 1 (LASP-1) and VDAC 1. A total of 22 differential proteins were downregulated after sinularin treatment. They were splicing factor, arginine/serine-rich 1, 60 kDa heat shock protein, heat shock protein beta-1, adenyl cyclase-associated protein 1, transketolase, pyruvate kinase isozymes M1/M2, peroxiredoxin-1, glyceraldehyde-3-phosphate dehydrogenase, succinyl-CoA:3-ketoacid-coenzyme A transferase 1, proteasome subunit beta type 3, protein disulfide-isomerase precursor, 78 kDa glucose-regulated protein precursor, fascin (55 kDa actin-bundling protein), uromodulin precursor, interleukin-23 receptor precursor (IL-23R), heat shock cognate 71 kDa protein, alpha-enolase, 60S acidic ribosomal protein P0 (L10E), 26S proteasome nonATPase regulatory subunit 14, and proliferating cell nuclear antigen.

Table 1. Statistical significance acquired between control and sinularin-treated A2058 cells at sub-G1 phase and G2/M phase

Sinularin (μg/mL)	Control	1	2	3
Sub-G1	6.87 ± 1.27	$9.22 \pm 0.18^{a)}$	$12.06 \pm 0.90^{b)}$	$11.49 \pm 0.58^{b)}$
G0/G1	50.63 ± 1.93	$51.6 \pm 0.38^{c)}$	$31.93 \pm 0.12^{b)}$	$35.65 \pm 0.27^{b)}$
S	20.46 ± 0.7	$15.76 \pm 0.37^{b)}$	$21.92 \pm 0.53^{c)}$	$20.29 \pm 1.09^{c)}$
G2/M	22.05 ± 2.16	$23.42 \pm 0.19^{c)}$	$33.09 \pm 1.17^{b)}$	$32.57 \pm 1.94^{b)}$

a) $p < 0.05$.
b) $p < 0.01$.
c) $p > 0.05$, vs. control.

3.4 Validation by Western blotting analysis

The identities of annexin A1, VDAC1, prohibitin, Prx2, Hsp60, and HspB1 were further validated by Western blotting (Fig. 8). Modulation of these proteins was in agreement with the 2DE data. Upregulation of p53, cleaved-caspase-3, cleaved-caspase-8, cleaved-caspase-9, p21, and Bax as well as downregulation of Bcl-2 in sinularin-treated A2058 melanoma cells were represented by Western blot (Fig. 9).

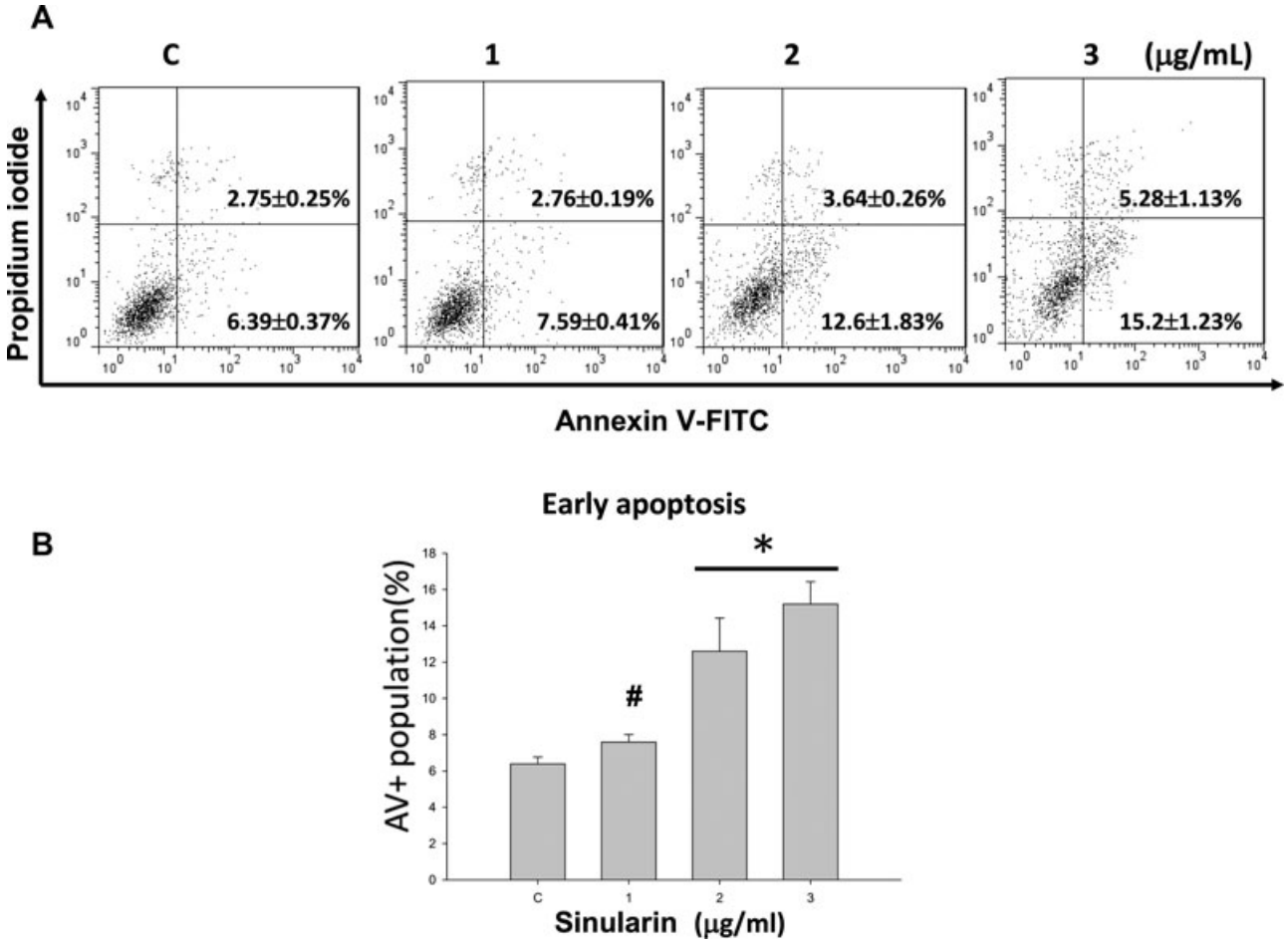

Figure 5. Sinularin-induced apoptosis of A2058 melanoma cells. (A) Cells were cultured with sinularin at different concentrations (1–3 μg/mL) for 24 h and stained with annexin V/PI to detect externalization of PS from cell membrane. (B) Quantitative analysis of early stage of apoptosis (annexin V-positive and PI-negative) displayed that sinularin enhanced apoptosis of A2058 melanoma cells in a dose-dependent manner (#$p < 0.05$ and *$p < 0.01$).

4 Discussion

Comparative proteomic analysis is able to reveal the changes of protein expression of a proteome with a specific treatment by comparison with the control sample. The proteomic data could provide clues for the investigation of the effects of the specific treatment and the further understanding of the mechanisms at molecular level. We have previously shown that chemical compound 11-dehydrosinulariolide from soft coral Sinularia *leptoclados* exerts anti-cancer activities against oral squamous cell carcinoma [29]. In this study, the anti-tumor and apoptosis-induced effects of sinularin isolated from soft coral *S. flexibilis* on A2058 melanoma cells were investigated. Results in our study indicated that sinularin exerts anti-proliferative, anti-migratory, apoptosis-induced, and cell cycle delayed activities against melanoma cells. The proteins related with the effects of sinularin were identified by comparative proteomic analysis. We found that the changes of some crucial proteins such as annexin A1, VDAC1, prohibitin, Prx2, Hsp60, and HspB1 are associated with apoptosis or the inhibitory activities against melanoma cells. Upregulation of p53, cleaved-caspase-3, cleaved-caspase-8, cleaved-caspase-9, p21, and Bax as well as downregulation of Bcl-2 shown by Western blot connected these pro-apoptotic factors with the sinularin-1-induced apoptosis in A2058 melanoma cells.

4.1 Annexin A1

Sinularin-treated A2058 melanoma cells exhibited a 2.2-fold increase in annexin A1 compared with those without the treatment in our study. Annexin A1 is known as macrocortin, renocortin, lipomodulin, lipocortin-1 [30–34]. This 37 kDa endogenous anti-inflammatory intracellular polypeptide was found to be capable of binding calcium and phospholipids and functioning as a second messenger involved in the inhibition of eicosanoid synthesis and phospholipase 2 (PLA2) [30, 34]. Releasing of annexin A1 is considered to bind to its

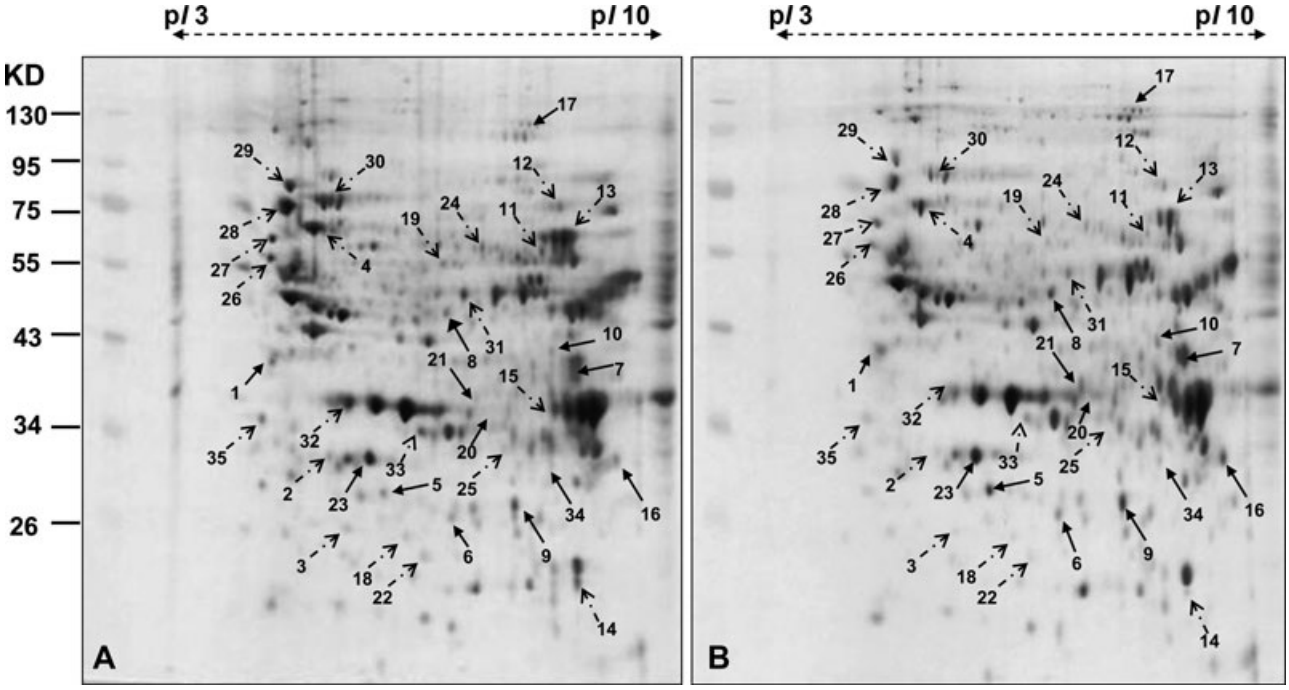

Figure 6. The 2-DE maps of A2058 melanoma cells treated with 2 μg/mL sinularin for 24 h (B) compared to the control (A). Protein spots marked on the maps were considered differential expression and identified by LC-MS/MS. The results are represented from three independent runs.

receptor, which is associated with cell detachment, inhibition of leukocyte migration, and thereby suppressing the inflammatory response [32, 33]. In addition, the inhibition of PLA2 activity and decrease of arachadonic acid production, was considered to be a consequence of binding of annexin A1 to the substrate rather than directly inhibiting the enzyme [35]. Annexin A1 has been indicated to have connection with the modulation of cell proliferation and apoptosis. Earlier study by Croxtall et al. (1993) suggested that annexin A1 plays inhibitory roles in lung cancer cell growth and proliferation [36], and the inhibitory effect on lung cancer cell proliferation mediated by the receptor was also reported [37]. Interestingly, it has been shown that exogenous annexin A1 enhances cellular hydrogen peroxide-induced apoptosis in rat thymocytes whereas it protects the cells from necrotic death. Anti-annexin A1 antibodies inhibited hydrogen peroxide-induced apoptosis, resulting in an increase in necrosis in rat thymocytes [38]. Moreover, annexin A1 has been shown by Arur et al. (2003) as a caspase-dependent engulfment ligand in the apoptotic cells ensuring phagocytosis. They found that annexin A1 is recruited to phosphatidylserine-rich regions of surfaces on apoptotic cells via a caspase-mediated mechanism which the release of intracellular calcium is involved in the resolution phase of inflammation [39]. These findings were further supported by the defective engulfment of apoptotic cells after annexin A1 siRNA knock-down. Therefore, annexin A1 might serve as an endogenous phosphatidylserine ligand, mediating engulfment of apoptotic cells [39]. In the current study, an increase of annexin A1 and upregulation of cleaved-caspase-3, cleaved-caspase-8 as well as cleaved-caspase-9 displayed by 2DE and Western blot in sinularin-treated melanoma cells suggest that the enhancement of annexin A1 is correlated with elevated cellular stress or apoptosis brought by treatment with sinularin. Furthermore, annexin A1 may function as an apoptosis-enhanced protein in the melanoma cells. Hence, combined these data we propose that annexin A1 may be associated with the promotion of caspase-mediated apoptosis and the inhibition of the growth of A2058 melanoma cells exerted by sinularin.

4.2 VDAC 1

VDAC of the outer mitochondrial membrane has been shown to be associated with type 2 diabetes and cell metabolism [40, 41]. There are three types of VDAC while in humans VDAC1 particularly is able to control adenine nucleotide and Ca^{2+} transit. Recently VDAC, together with Bax and apoptosis-inducing factor (AIF) have been indicated to be involved in the control of apoptosis [42–45]. It is noteworthy that VDAC, p53 protein and Bax, as pro-apoptotic factors, have been shown to induce mitochodrial apoptosis in several cancer cells such as gastric epithelial carcinoma cells, colorectal carcinoma cells, and cervical cancer cells [46–48]. In the current study, flow cytometric data indicated sinularin is able to enhance apoptosis in A2058 melanoma cells. VDAC1 was upregulated after sinularin treatment in melanoma cells while Western blot represented the increased expression of

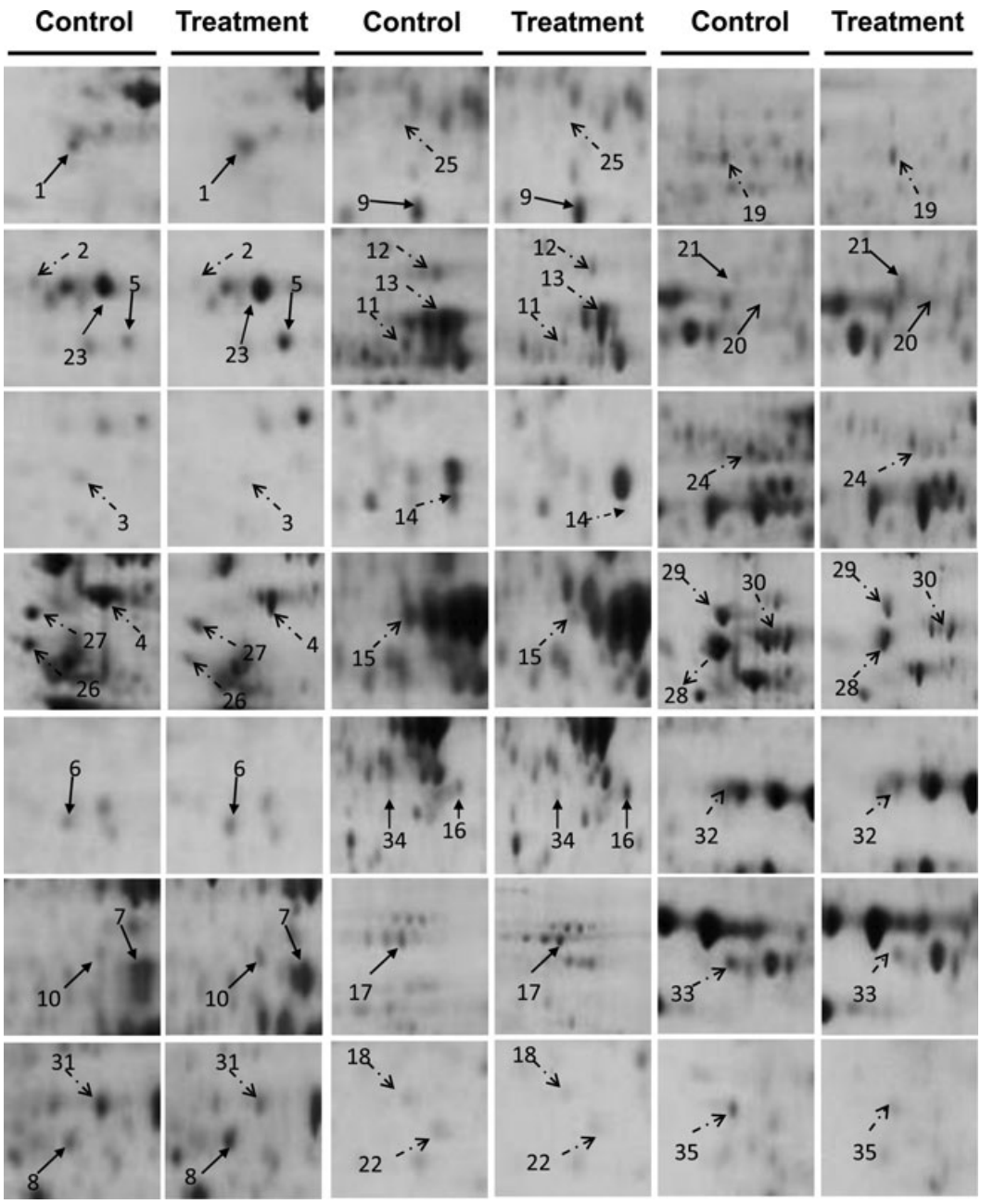

Figure 7. Enlarged 2-DE map of the 35 differential proteins in A2058 melanoma cells treated with sinularin. Solid arrows indicate upregulated proteins and dashed arrows indicate downregulated proteins.

Bax and p53 in A2058 melanoma cells exposed to sinularin, suggesting that Bax and p53 may be the pro-apoptotic proteins associated with enhancement of VDAC1 and the capacity of sinularin on apoptosis induction in melanoma cells. In addition, our results implicate that the upregulations of proteomic analytic proteins including Hsp60, HspB1, prohibitin, and VDAC1 are reasonably linked with the induction of p53 export and the subsequent apoptosis and the changes of both HspB1 and VDAC1 may be correlated with increased expression of Bax after sinularin treatment in A2058 melanoma cells.

4.3 Prohibitin

Prohibitins are ubiquitous and evolutionary conserved proteins that localize to mitochondria [49]. This protein has been indicated to work together with chromatin remodeling molecules in transcriptional regulation [50–53] and be involved in p53-associated apoptosis [54–56]. It was shown that prohibitin regulates cellular signaling, cell proliferation, cell migration, and stabilizes mitochondrial proteins [57–59]. The translocation of prohibitin to mitochondria couples with a simultaneous translocation of p53, which was highly correlated

Table 2. Identities of the differential proteins in A2058 melanoma cells treated with 2 μg/mL sinularin analyzed by LC-MS/MS

Spot number	Protein name	Accession number	Calculate Mr/p*I*	Peptide matched	Sequence covered Percentage	MASCOT score	Regulation[a)] (fold- change)[a)]
1	Nucleophosmin (NPM)	P06748	32.55/4.64	25	33	219	+2.8
2	Splicing factor, arginine/serine-rich 1	Q07955	27.72/10.37	1	4	73	−3.0
3	Heat shock protein beta-1 (HspB1)	P04792	22.76/5.98	13	38	176	−3.0
4	60 kDa heat shock protein	P10809	61.01/5.7	35	46	601	−2.5
5	Proteasome subunit beta type 7 precursor	Q99436	29.94/7.57	10	36	111	+3.3
6	Peroxiredoxin-6	P30041	25.02/6.0	14	52	242	+2.4
7	Fructose-bisphosphate aldolase A	P04075	39.39/8.3	34	31	299	+3.1
8	Elongation factor Tu, mitochondrial precursor (EF-Tu) (P43)	P49411	49.51/7.26	4	7	93	+2.4
9	Phosphoglycerate mutase 1	P18669	28.78/6.67	8	31	79	+2.4
10	Phosphoserine aminotransferase	Q9Y617	40.39/7.56	13	22	210	+2.1
11	Adenylyl cyclase-associated protein 1 (CAP 1)	Q01518	51.82/8.27	9	22	46	−3.0
12	Transketolase	P29401	67.83/7.58	19	27	258	−2.0
13	Pyruvate kinase isozymes M1/M2	P14618	57.9/7.96	52	49	796	−2.6
14	Peroxiredoxin-1	Q06830	22.09/8.27	49	49	457	−2.6
15	Glyceraldehyde-3-phosphate dehydrogenase	P04406	36.03/8.57	16	29	164	−2.4
16	FK506-binding protein 3	Q00688	25.16/9.29	13	36	113	+2.5
17	Elongation factor 2 (EF-2)	P13639	95.27/6.41	35	34	427	+2.7
18	Heat shock protein beta-1 (HspB1)	P04792	22.76/5.98	21	50	168	−2.2
19	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1	P55809	56.12/7.14	21	38	237	−2.3
20	LIM and SH3 domain protein 1 (LASP-1)	Q14847	29.69/6.61	12	31	125	+3.2
21	Annexin A1	P04083	38.69/6.57	12	34	180	+2.2
22	Proteasome subunit beta type 3	P49720	22.93/6.14	5	26	149	−2.1
23	Prohibitin	P35232	29.78/5.57	24	68	499	+2.3
24	Fascin (55 kDa actin-bundling protein)	Q16658	54.49/6.84	18	23	198	−3.2
25	Peroxiredoxin-2	P32119	21.87/5.66	4	14	58	−2.7
26	Uromodulin precursor	P07911	69.74/5.05	7	13	89	−2.8
27	Protein disulfide-isomerase precursor	P07237	57.08/4.76	58	54	446	−2.1
28	Interleukin-23 receptor precursor (IL-23R)	Q5VWK5	71.66/5.32	2	1	39	−2.9
29	78 kDa glucose-regulated protein precursor	P11021	72.28/5.07	88	47	873	−3.1
30	Heat shock cognate 71 kDa protein	P11142	70.85/5.37	85	51	674	−2.8
31	Alpha-enolase	P06733	47.13/7.01	16	22	134	−2.7
32	60S acidic ribosomal protein P0 (L10E)	P05388	34.25/5.71	40	48	301	−3.5
33	26S proteasome non-ATPase regulatory subunit 14	O00487	34.55/6.06	3	8	59	−2.2
34	Voltage-dependent anion-selective channel protein 1	P21796	30.75/8.62	17	38	217	+2.6
35	Proliferating cell nuclear antigen	P12004	28.75/4.57	18	46	164	−2.9

a) Regulations (fold changes) of differentially expression proteins are expressed at 24-h treatment of 2 μg/mL sinularin.

with inhibition of cancer growth. It has been revealed that p53 translocates to the mitochondria in response to apoptosis [60–62]. Our previous proteomic data showed an increase of prohibitin in oral squamous cell carcinoma treated with another soft coral-isolated compound 11-dehydro- sinulariolide [29]. In the current study, 2DE and Western blot further represented that both prohibitin and p53 were increased after sinularin treatment. These proteomic and Western blotting data of sinularin in this study suggest that the greater expression of prohibitin was correlated with the p53-involved apoptosis induced by sinularin in A2058 melanoma cells.

4.4 Prx2

Peroxidoxins (Prx or Prxd) are an anti-oxidant enzyme group capable of decreasing the level of H_2O_2 and hydroperoxides. Formation of H_2O_2 based on its intensity and duration, is able to outline different signals from cell proliferation to apoptosis. Thus, induction of the last reaction may be essential for development of anti-cancer therapies [63]. Except Prx6 isoform, other members of this enzyme family are considered as thioredoxin peroxidases, which are able to utilize thioredoxin as an electron donor [64, 65]. Human Prx1 and Prx2 have over 90% amino acid homology and these two proteins have been shown to influence functions or processes like proliferation, cell survival, and apoptosis in a number of tumor cells [66]. Prx2, a 25 kDa protein, is abundant in the cytoplasma and has high affinity to H_2O_2. This enzyme has been indicated to be functionally similar to glutathione peroxidase. In particular, Prx2 has been indicated that this protein protects cardiomyocytes from the oxidative stress-induced cell death and apoptosis [65]. Deficiency in Prx2 resulted in an increase of H_2O_2 level and Prx2 knockout mice displayed hemolytic anemia, suggesting that Prx2 plays an essential role in oxidative-stress protection in erythrocytes

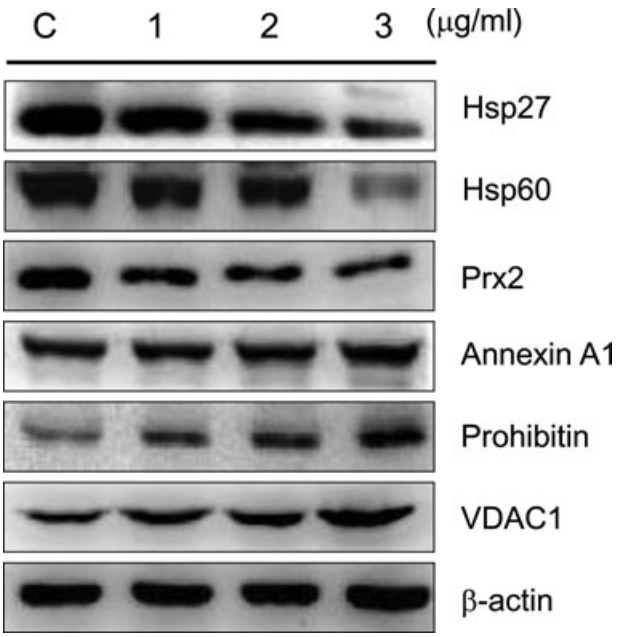

Figure 8. Western blotting analysis for the changes of Hsp60, HspB1, Prx2, prohibitin, annexin A1, and VDAC1 expression in A2058 melanoma cells treated with increasing concentrations of sinularin for 24 h. β-actin was used as the internal control.

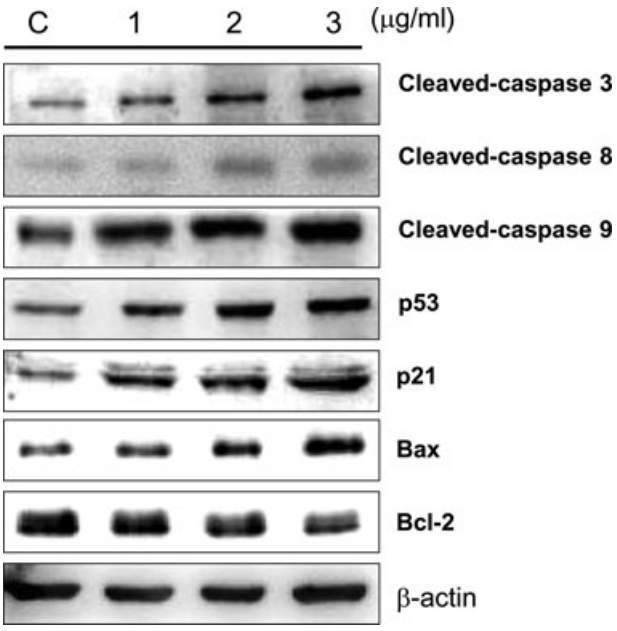

Figure 9. Western blotting analysis for the changes of p53, cleaved-caspase-3, cleaved-caspase-8, cleaved-caspase-9, p21, Bax, Bcl-2 expression in A2058 melanoma cells treated with increasing concentrations of sinularin for 24 h. β-actin was used as the internal control.

[67]. The importance of Prx2 has been highlighted in tumor-related studies. The prognostic and diagnostic values of Prx2 have also been demonstrated in cervical cancer and bladder cancer whereas Prx1 was underlined in nonsmall cell lung cancer cells [66, 68, 69]. In our study, we found that Prx2 was reduced (2.7-fold) after the treatment with sinularin-1 in A2058 melanoma cells as this compound also exerted anti-proliferation, anti-migration, and apoptosis-induced effects in A2058 melanoma cells. These findings not only imply that a decrease of Prx2 resulted from sinularin treatment may contribute to the diminished ability in apoptosis and oxidative-stress protection in A2058 melanoma cells, but also suggest that Prx2 could be a valuable marker in the therapeutics on melanoma. On the other hand, we found that Prx6 displayed 2.4-fold enhancement in A2058 melanoma cells after exposure to sinularin. Previous studies on oncology have shown quite diverse data of the regulation of Prx6. It was shown that Prx6 is upregulated in patients with esophageal squamous cell carcinoma [70]. In contrast, previous proteomic analysis using malignant transformative cell models exhibited the downregulation of Prx6 [71]. Our data are similar to those on esophageal squamous cell carcinoma. However, further studies on the roles of Prx6 in tumorigenesis are required.

4.5 Hsp60 and HspB1

Heat shock proteins are a housekeeping protein family and they are considered as chaperones to recognize proteins with abnormal structures against stress conditions [72]. They have been believed to protect cells against stress-correlated damage and are overexpressed in several malignant tumors [74]. In the current study, downregulation of Hsp60 and HspB1 was identified by 2DE in A2058 melanoma cells exposed to sinularin. Hsp60 has been indicated to widely appear in nature and play a role as chaperones in enhancing cell survival under stress circumstances [74]. The connections between Hsp60 level and anti-tumor effects or drug resistance in cancer cells have been studied. Our previous work showed downregulation of Hsp60 in CAL-27 oral squamous cell carcinoma treated with soft coral-isolated compound 11-dehydrosinulariolide, which exerts anti-tumor effects in vitro [29]. The basal levels of Hsp60 were increased in cisplatin-resistant cervix squamous cell carcinoma cell subline A431/Pt compared with those in nonresistant A431 cells [75]. Acute ablation of Hsp60 by small interfering RNA-induced mitochondrial dysfunction, destabilized mitochondrial pool of survivin, and activated both p53-dependent and mitochondrial apoptosis [76]. In our study, treatment with sinularin significantly enhanced apoptosis in A2058 melanoma cells and the 2.5-fold reduction of Hsp60 in melanoma cells after the treatment is reasonably consistent with the findings in previous studies. Downregulation of Hsp60 and increased expression of p53 protein in sinularin-treated cells were verified by Western blot. Supported by previous findings as well as current Western blotting data, herein flow cytometric data and downregulation of Hsp60 suggest that sinularin possesses apoptosis-induced capacity against A2058 melanoma cells and p53 is very likely involved in the pro-apoptotic mechanisms.

HspB1, also known as heat shock protein 27 (Hsp27), is another essential heat shock protein which should be emphasized. This protein has been indicated as a critical marker in several cancer cells. HspB1 has also been implicated to have tumorigenic, metastatic, chemoresistance, and anti-apoptotic properties [77, 78]. Higher expression of HspB1 has been shown in different cancer cells or patients such as cervical cancer/neoplasia, laryngeal cancer cells, head and neck squamous cell carcinoma, and leukemia patients [68,78–80]. Both Hsp60 and HspB1 have been demonstrated as predictors of recurrence for prostate cancer patients after radical prostatectomy [73] while acquirement of expression of HspB1 was showed to have novel diagnostic and prognostic information on the survival of individual prostate cancer patient at the time of primary diagnosis [81]. Regarding apoptosis, HspB1 is normally believed to increase proteasomal p53 degradation [82]. In adenocarcinoma cells, HspB1 protected UV-induced apoptosis by Akt and p21-dependent pathways of survival and proteins of p53 target genes including p21, Bcl-2, and Akt represented positive correlation with HspB1 level. HspB1 siRNA knockdown led to upregulation of Bax and downregulation of Bcl-2 [83]. In our study, proteomic data displayed 3-fold reduction of HspB1 in melanoma cells after sinularin treatment. Western blotting data further presented with downregulation of HspB1 and Bcl-2 and upregulation of p53, Bax, and p21. These data suggest that the decreased HspB1 should play an important role in apoptosis or reduced anti-apoptotic capacity of melanoma cells. Our data further implicate that p53, Bcl-2, Bax, and p21 are involved in the induction of apoptosis after sinularin treatment in melanoma cells. In addition, HspB1 seems to be a valuable marker for melanoma.

4.6 Conclusion

The anti-tumor effects of sinularin on A2058 melanoma cells have been extensively investigated in this study. The inhibitory effects on tumor cells were examined by MTT assay, cell migration assay, and wound healing assay. The induction of apoptosis and cell cycle delay has also been determined by annexin V-FITC/PI staining on a flow cytometer. Sinularin possesses evident inhibitory effects against A2058 melanoma cell proliferation and migration. Treatment with sinularin dose-dependently enhances apoptotic rate and causes G2/M phase delay in A2058 melanoma cells. Comparative proteomic analysis provides a broad and effective approach to detect changes of protein expression profiling in A2058 cells treated with sinularin. A total of 35 differential proteins (13 upregulated and 22 downregulated) in A2058 cells treated with sinularin have been identified by LC-MS/MS analysis. The differential proteins such as annexin A1, VDAC1, prohibitin, Prx2, Hsp60, and HspB1 are associated with anti-proliferation, induction of apoptosis as well as oxidative-stress protection while the changes of these proteins were verified by Western blot. Regarding these proteins, the changes of several pro-apoptotic factors were further examined. Enhanced expression of p53, cleaved-caspase-3, cleaved-caspase-8, cleaved-caspase-9, p21, and Bax as well as reduced expression of Bcl-2 were then verified by Western blot. Together with anti-tumor effects and the findings on p53, caspase-3, caspase-8, caspase-9, p21, Bax, and Bcl-2, characteristic modulation of several critical proteins including annexin A1, VDAC1, prohibitin, Prx2, Hsp60, and HspB1 suggest that sinularin is capable of inducing apoptosis in which p53, caspase-3, caspase-8, caspase-9, p21, Bax, and Bcl-2 are involved in A2058 melanoma cells. The results in our study demonstrate that proteomic analysis is a feasible approach to investigate and discover potential markers in melanoma cells. Moreover, these data may help realize the potent inhibitory and apoptosis-induced effects of sinularin on A2058 melanoma cells at the molecular level. Further studies on the modulation of annexin A1, VDAC1, prohibitin, Prx2, Hsp60, and HspB1 will be promising and helpful to unveil the anti-tumor mechanisms of sinularin. The findings in our study also benefit the development of other new target drugs against melanoma.

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The authors have no conflict of interest related to this study.

5 References

[1] Sneyd, M., Cox, B., *N. Z. Med. J.* 2006, *119*, U2169.

[2] Parkin, D. M., Bray, F., Ferlay, J., Pisani, P., *CA Cancer J. Clin.* 2005, *55*, 74–108.

[3] Houghton, A. N., Polsky, D., *Cancer Cell.* 2002, *2*, 275–278.

[4] Fuglede, N. B., Brinck-Claussen, U. O., Deltour, I., Boesen, E. H., Dalton, S. O., Johansen, C., *Brit. J. Dermatol* 2011, *165*, 349–353.

[5] Tawbi, H. A., Buch, S. C., *Clin. Adv. Hematol. Oncol.* 2010, *8*, 259–266.

[6] Sasse, A. D., Sasse, E. C., Clark, L. G., Ulloa, L., Clark, O. A., *Cochrane Database Syst. Rev.* 2007, CD005413.

[7] Elias, G. E., Joanne, H. H., Sharma, B. S., *Cancers* 2010, *2*, 165–189.

[8] Essner, R., *Surg. Clin. North Am.* 2003, *83*, 109–156.

[9] Lavie, A., Desouches, C., Casanova, D., Bardot, J., Grob, J. J., Legre, R., Magalon, G., *Ann. Chir. Plast. Esthet.* 2007, *52*, 1–13.

[10] Chang, J. W., *Chang Gung Med. J.* 2010, *33*, 602–612.

[11] Treisman, J., Garlie, N., *Clin. Plast. Surg.* 2010, *37*, 127–146.

[12] Bleehen, N. M., Newlands, E. S., Lee, S. M., Thatcher, N., Selby, P., Calvert, A. H., Rustin, G. J., Brampton, M., Stevens, M. F., *J. Clin. Oncol.* 1995, *13*, 910–913.

[13] Acquavella, N., Kluger, H., Rhee, J., Farber, L., Tara, H., Ariyan, S., Narayan, D., Kelly, W., Sznol, M., *J. Immunother.* 2008, *31*, 569–576.

[14] Radhika, P., Rao, P. R., Archana, J., Rao, N. K., *Biol. Pharm. Bull.* 2005, *28*, 1311–1313.

[15] Ruggieri, G. D., *Science* 1976, *194*, 491–497.

[16] Faulkner, D. J., *Nat. Prod. Rep.* 2001, *18*, 1–49.

[17] Faulkner, D. J., *Nat. Prod. Rep.* 2002, *19*, 1–48.

[18] Poza, J. J., Fernandez, R., Reyes, F., Rodriguez, J., Jimenez, C., *J. Org. Chem.* 2008, *73*, 7978–7984.

[19] Kamel, H. N., Ferreira, D., Garcia-Fernandez, L. F., Slattery, M., *J. Nat. Prod.* 2007, *70*, 1223–1227.

[20] Hassan, H. M., Khanfar, M. A., Elnagar, A. Y., Mohammed, R., Shaala, L. A., Youssef, D. T., Hifnawy, M. S., El Sayed, K. A., *J. Nat. Prod.* 2010, *73*, 848–853.

[21] Chiang, P. C., Chien, C. L., Pan, S. L., Chen, W. P., Teng, C. M., Shen, Y. C., Guh, J. H., *J. Hepatol.* 2005, *43*, 679–686.

[22] Chiang, P. C., Kung, F. L., Huang, D. M., Li, T. K., Fan, J. R., Pan, S. L., Shen, Y. C., Guh, J. H. *Eur. J. Pharmacol.* 2006, *542*, 22–30.

[23] Lopez, L., Villavicencio, M. A., Albores, A., Martinez, M., de la Garza, J., Melendez-Zajgla, J., Maldonado, V., *J. Ethnopharmacol.* 2002, *80*, 115–120.

[24] Wang, G. H., Chou, T. H., Lin, R. J., Sheu, J. H., Wang, S. H., Liang, C. H., *J. Toxicol.* 2009, *2009*, 634868.

[25] Tseng, M. Y., Liu, S. Y., Chen, H. R., Wu, Y. J., Chiu, C. C., Chan, P. T., Chiang, W. F., Liu, Y. C., Lu, C. Y., Jou, Y. S., Chen, J. Y., *Oral Oncol.* 2009, *45*, 771–776.

[26] Chiu, C. C., Chen, J. Y., Lin, K. L., Huang, C. J., Lee, J. C., Chen, B. H., Chen, W. Y., Lo, Y. H., Chen, Y. L., Tseng, C. H., Lin, S. R., *Cancer Lett.* 2010, *295*, 92–99.

[27] Wu, Y. J., Chen, H. M., Wu, T. T., Wu, J. S., Chu, R. M., Juang, R. H., *Proteomics* 2006, *6*, 5898–5902.

[28] Huang, H. H., Brennan, T. C., Muir, M. M., Mason, R. S., *J. Cell. Physiol.* 2009, *220*, 267–275.

[29] Liu, C. I., Chen, C. C., Chen, J. C., Su, J. H., Huang, H. H., Chen, J. Y., Wu, Y. J., *Mar. Drugs* 2011, *9*, 1254–1272.

[30] Blackwell, G. J., Carnuccio, R., Di Rosa, M., Flower, R. J., Parente, L., Persico, P., *Nature* 1980, *287*, 147–149.

[31] Hirata, F., del Carmine, R., Nelson, C. A., Axelrod, J., Schiffmann, E., Warabi, A., De Blas, A. L., Nirenberg, M., Manganiello, V., Vaughan, M., Kumagai, S., Green, I., Decker, J. L., teinberg, A. D., *Proc. Natl. Acad. Sci. USA* 1981, *78*, 3190–3194.

[32] Perretti, M., Croxtall, J. D., Wheller, S. K., Goulding, N. J., Hannon, R., Flower, R. J., *Nat. Med.* 1996, *2*, 1259–1262.

[33] Lim, L. H., Solito, E., Russo-Marie, F., Flower, R. J., Perretti, M., *Proc. Natl. Acad. Sci. USA* 1998, *95*, 14535–14539.

[34] Lim, L. H., Pervaiz, S., *FASEB J.* 2007, *21*, 968–975.

[35] Bastian, B. C., Sellert, C., Seekamp, A., Romisch, J., Paques, E. P., Brocker, E. B., *J. Invest. Dermatol.* 1993, *101*, 359–363.

[36] Croxtall, J., Flower, R., Perretti, M., *J. Lipid Mediat.* 1993, *6*, 295–302

[37] Croxtall, J. D., Waheed, S., Choudhury, Q., Anand, R., Flower, R. J., *Int. J. Cancer* 1993, *54*, 153–158.

[38] Sakamoto, T., Repasky, W. T., Uchida, K., Hirata, A., Hirata, F., *Biochem Biophys Res. Commun.* 1996, *220*, 643–647.

[39] Arur, S., Uche, U. E., Rezaul, K., Fong, M., Scranton, V., Cowan, A. E., Mohler, W., Han, D. K., *Dev. Cell.* 2003, *4*, 587–598.

[40] Turko, I. V., Murad, F., *J. Biol. Chem.* 2003, *278*, 35844–35849.

[41] Mostyn, A., Pearce, S., Stephenson, T., Symonds, M. E., *Exp. Clin. Endocrinol Diabetes* 2004, *112*, 2–9.

[42] Yuqi, L., Lei, G., Yang, L., Zongbin, L., Hua, X., Lin, W., Rui, C., Mohan, L., Yi, W., Minxin, G., Shiwen, W., *BMC Med. Genet.* 2009, *10*, 114.

[43] Ozaki, T., Yamashita, T., Ishiguro, S., *Biochim. Biophys Acta.* 2009, *1793*, 1848–1859.

[44] Scharstuhl, A., Mutsaers, H. A., Pennings, S. W., Russel, F. G., Wagener, F. A., *PLoS One* 2009, *4*, e6688.

[45] Yamagata, H., Shimizu, S., Nishida, Y., Watanabe, Y., Craigen, W. J., Tsujimoto, Y., *Oncogene* 2009, *28*, 3563–3572.

[46] Lan, C. H., Sheng, J. Q., Fang, D. C., Meng, Q. Z., Fan, L. L., Huang, Z. R., *J. Dig. Dis.* 2010, *11*, 43–49.

[47] Wolff, S., Erster, S., Palacios, G., Moll, U. M., *Cell. Res.* 2008, *18*, 733–744.

[48] Yu, J., Qian, H., Li, Y., Wang, Y., Zhang, X., Liang, X., Fu, M., Lin, C., *Cancer Biol. Ther.* 2007, *6*, 580–586.

[49] Winter, A., Kamarainen, O., Hofmann, A., *Proteins* 2007, *68*, 353–362.

[50] Wang, S., Fusaro, G., Padmanabhan, J., Chellappan, S. P., *Oncogene* 2002, *21*, 8388–8396.

[51] Wang, S., Zhang, B., Faller, D. V., *EMBO J.* 2002, *21*, 3019–3028.

[52] Wang, S., Zhang, B., Faller, D. V., *EMBO J.* 2004, *23*, 2293–2303.

[53] Zhang, B., Chambers, K. J., Faller, D. V., Wang, S., *Oncogene* 2007, *26*, 7153–7157.

[54] Fusaro, G., Wang, S., Chellappan, S., *Oncogene* 2002, *21*, 4539–4548.

[55] Fusaro, G., Dasgupta, P., Rastogi, S., Joshi, B., Chellappan, S., *J. Biol. Chem.* 2003, *278*, 47853–47861.

[56] Joshi, B., Rastogi, S., Morris, M., Carastro, L. M., DeCook, C., Seto, E., Chellappan, S. P., *Biochem. J.* 2007, *401*, 155–166.

[57] Nuell, M. J., Stewart, D. A., Walker, L., Friedman, V., Wood, C. M., Owens, G. A., Smith, J. R., Schneider, E. L., Dell' Orco, R., Lumpkin, C. K., Danner, D. B., McClung, J. K., *Mol. Cell. Biol.* 1991, *11*, 1372–1381.

[58] Rajalingam, K., Wunder, C., Brinkmann, V., Churin, Y., Hekman, M., Sievers, C., Rapp, U. R., Rudel, T., *Nat. Cell. Biol.* 2005, *7*, 837–843.

[59] Nijtmans, L. G., de Jong, L., Artal Sanz, M., Coates, P. J., Berden, J. A., Back, J. W., Muijsers, A. O., van der Spek, H., Grivell, L. A., *EMBO J.* 2000, *19*, 2444–2451.

[60] Lohrum, M. A., Woods, D. B., Ludwig, R. L., Balint, E., Vousden, K. H., *Mol. Cell. Biol.* 2001, *21*, 8521–8532.

[61] Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., Moll, U. M., *Mol. Cell.* 2003, *11*, 577–590.

[62] Chipuk, J. E., Green, D. R., *Cell. Cycle* 2004, *3*, 429–431.

[63] Zhang, B., Wang, Y., Su, Y., *Cancer Lett.* 2009, *286*, 154–160.

[64] Yo, Y. D., Chung, Y. M., Park, J. K., Ahn, C. M., Kim, S. K., Kim, H. J., *Exp. Mol. Med.* 2002, *34*, 273–277.

[65] Zhao, W., Fan, G. C., Zhang, Z. G., Bandyopadhyay, A., Zhou, X., Kranias, E. G., *Basic Res. Cardiol.* 2009, *104*, 377–389.

[66] Kim, J. H., Bogner, P. N., Baek, S. H., Ramnath, N., Liang, P., Kim, H. R., Andrews, C., Park, Y. M., *Clin. Cancer Res.* 2008, *14*, 2326–2333.

[67] Lee, T. H., Kim, S. U., Yu, S. L., Kim, S. H., Park, D. S., Moon, H. B., Dho, S. H., Kwon, K. S., Kwon, H. J., Han, Y. H., Jeong, S., Kang, S. W., Shin, H. S., Lee, K. K., Rhee, S. G., Yu, D. Y., *Blood* 2003, *101*, 5033–5038.

[68] Lomnytska, M. I., Becker, S., Bodin, I., Olsson, A., Hellman, K., Hellstrom, A. C., Mints, M., Hellman, U., Auer, G., Andersson, S., *Brit. J. Cancer* 2011, *104*, 110–119.

[69] Chen, Y. T., Chen, C. L., Chen, H. W., Chung, T., Wu, C. C., Chen, C. D., Hsu, C. W., Chen, M. C., Tsui, K. H., Chang, P. L., Chang, Y. S., Yu, J. S., *J. Proteome Res.* 2010, *9*, 5803–5815.

[70] Fujita, Y., Nakanishi, T., Hiramatsu, M., Mabuchi, H., Miyamoto, Y., Miyamoto, A., Shimizu, A., Tanigawa, N., *Clin. Cancer Res.* 2006, *12*, 6415–6420.

[71] Putz, S. M., Vogiatzi, F., Stiewe, T., Sickmann, A., *Mol. Cancer* 2010, *9*, 254.

[72] Creagh, E. M., Sheehan, D., Cotter, T. G., *Leukemia* 2000, *14*, 1161–1173.

[73] Glaessgen, A., Jonmarker, S., Lindberg, A., Nilsson, B., Lewensohn, R., Ekman, P., Valdman, A., Egevad, L., *AP-MIS* 2008, *116*, 888–895.

[74] Guimaraes, A. J., Frases, S., Gomez, F. J., Zancope-Oliveira, R. M., Nosanchuk, J. D., *Infect Immun.* 2009, *77*, 1357–1367.

[75] Castagna, A., Antonioli, P., Astner, H., Hamdan, M., Righetti, S. C., Perego, P., Zunino, F., Righetti, P. G., *Proteomics* 2004, *4*, 3246–3267.

[76] Ghosh, J. C., Dohi, T., Kang, B. H., Altieri, D. C., *J. Biol. Chem.* 2008, *283*, 5188–5194.

[77] Gibert, B., Hadchity, E., Czekalla, A., Aloy, M. T., Colas, P., Rodriguez-Lafrasse, C., Arrigo, A. P., Diaz-Latoud, C., *Oncogene* 2011, *30*, 3672–3681.

[78] Lee, J. H., Sun, D., Cho, K. J., Kim, M. S., Hong, M. H., Kim, I. K., Lee, J. S., *J. Cancer. Res. Clin. Oncol.* 2007, *133*, 37–46.

[79] Norton, J. A., Weinberger, P. M., Waller, J. L., Merkley, M. A., Jackson, L. L., Dynan, W. S. *Laryngoscope* 2010, *120* Suppl 4, S172.

[80] Sedlackova, L., Spacek, M., Holler, E., Imryskova, Z., Hromadnikova, I., *Tumour Biol.* 2011, *32*, 33–44.

[81] Cornford, P. A., Dodson, A. R., Parsons, K. F., Desmond, A. D., Woolfenden, A., Fordham, M., Neoptolemos, J. P., Ke, Y., Foster, C. S. *Cancer Res.* 2000, *60*, 7099–7105.

[82] Kanagasabai, R., Karthikeyan, K., Vedam, K., Qien, W., Zhu, Q., Ilangovan, G., *Mol. Cancer Res.* 2010, *8*, 1399–1412.

[83] Kanagasabai, R., Krishnamurthy, K., Druhan, L. J., Ilangovan, G., *J. Biol. Chem.* 2011, *286*, 33289–33300.

