

Mechanisms of Action of 17 β H-neriifolin on its Anticancer Effect in SKOV-3 Ovarian Cancer Cell Line

SITI-SYARIFAH MOHD MUTALIP¹, NURHANAN MURNI YUNOS¹, PUTERI SHAFINAZ AKMAR ABDUL-RAHMAN^{2,3}, MUHAMMAD HAFFIZ JAURI¹, ASIAH OSMAN¹ and MOHD ILHAM ADENAN¹

¹Natural Products Division, Forest Research Institute Malaysia, Kepong, Selangor, Malaysia;

²Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia;

³University of Malaya Centre for Proteomic Research, University of Malaya, Kuala Lumpur, Malaysia

Abstract. *Aim: Abnormalities in apoptotic signalling pathways often occur in cancer cells and limit the successful chemotherapy outcomes in cancers. Therefore, there is an urgent need to discover new anticancer agents with novel mechanisms of action to overcome the resistance effect in chemotherapy. Materials and Methods: In the present study, the anticancer effects and the mechanisms of action of 17 β H-neriifolin (cardiac glycoside) were evaluated by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay and a proteomic approach in treated and non-treated SKOV-3 ovarian cancer cells. Results: 17 β H-neriifolin was found to be active with IC₅₀ values of 0.01 \pm 0.001 in SKOV-3 ovarian cancer cell line, as evaluated by the sulforhodamine B (SRB) assay. Results from TUNEL assay indicated that 17 β H-neriifolin caused apoptosis in SKOV-3 cells in a dose-dependent manner. Based on differential analysis of treated and non-treated SKOV-3 two-dimensional electrophoresis (2-DE) profiles, four proteins, namely vimentin (VIM), pyruvate kinase, muscle (PKM), heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) and transgelin (TAGLN1) were identified to be involved in apoptosis. Other proteins including piggybac transposable element derived 5 (PGBD5), DENN/MADD domain containing 2D (DENND2D) and formin-like 1 (FMNL) have also been identified to be associated in SKOV-3 cell death induced by 17 β H-neriifolin. Conclusion: These findings may provide new insights on the potential of 17 β H-neriifolin's mechanism of action in killing ovarian cancer cells.*

Correspondence to: Siti-Syarifah Mohd Mutalip, Natural Products Division, Forest Research Institute Malaysia (FRIM), 52109 Kepong, Selangor, Malaysia. Tel: +60 362797657, e-mail: syarifah@frim.gov.my

Key Words: Cardiac glycosides, ovarian cancer, anticancer, apoptosis, proteomics.

Resistance to chemotherapy drugs in cancer treatments is a major hurdle that results from a variety of factors including altered drug metabolism or uptake, malfunctioned apoptotic cell death, increased repair of drug-induced damage, altered gene expression and drug target (1). For this reason, the search for better and more effective chemotherapeutic agents to improve disease outcome is in dire need. Apoptosis or programmed cell death was first described in 1972 by Ker *et al.* (2) to play a significant role in many biological processes including homeostatic balance in cell populations. The homeostatic imbalance between arisen new cells and apoptotic cell death may alter the apoptotic signaling pathways and contribute to the development of cancers. The alterations of these signaling pathways are due to modifications in some protein expression patterns, for example alterations in protein expression involved in apoptosis dealing with a over-expression of anti-apoptotic proteins (*e.g.* Bcl-2, IAPs and FLIP) and/or suppression or mutation of pro-apoptotic proteins (*e.g.* Bax, Apaf-1, caspase 8 and death receptors) (3).

Since the factors regulating apoptotic processes play a critical role in cancer sensitivity to chemotherapy, it may be rational to study the pattern of protein expression involved in the apoptotic pathway by discovering novel mechanisms of action(s) in inducing cancer cell death. Resistance of cancers occurs not only to a single chemotherapy drug, but also as a cross-resistance to a whole range of chemo-drugs that possess different structures and cellular targets. Thus, elucidation on the mechanisms of action of chemotherapy drugs with different unique targets and chemical structures has been an ultimate goal in cancer treatment. It may be noted that an extremely high number of proteins present in ovarian cancers remain to be studied; with some of them being possible targets that are unique to certain candidate, yet unidentified, drugs. Proteome studies that involve a combination of protein separation techniques like two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) have been widely used to identify

differentially-expressed proteins among treated and non-treated samples and post-translational modifications (4). Since the expressions of several proteins may not only involve a single pathway towards cell death, the application of proteomics technology will possibly provide major opportunities to elucidate new mechanisms of action and therapeutic targets.

The chemical diversity, structural complexity, affordability and inherent biological activity of natural products make them ideal candidates for new therapeutic approaches in cancer research. Cardiac glycosides (CGs) represent a group of naturally-derived compounds that share a unique set of two structures of glycone (sugar) and aglycone (steroid and lactone ring) moieties in which the latter is responsible for the pharmacokinetic activities of these compounds (5). Braunwald in 1985 (6) reported the use of CGs in the treatment of congestive heart failure while its anti-arrhythmic property was first described in 1785 by William Withering. However, in 1979 there was a new interest in studying the anticancer properties of these compounds when lower mortality rates of cancer patients receiving CG were observed (7). Numerous subsequent *in vitro* and *in vivo* studies verified that few CGs seem to be selective in killing cancer cells including skin, breast, oral, human small cell lung and pancreatic cancer cell lines but not normal cells (8-11). CG compounds, that were present in different plant families including digitoxin, digoxin, oleandrin, cerberin, ouabain, thevetin, proscillaridin and the one that had entered phase I clinical trial for cancer treatments, include digitoxin, digoxin and the semisynthetic drug coded as UNBS1450 (12). From our previous studies, 17 β H-neriifolin (Figure 1) is a compound of the CG group that had been isolated and characterised from the leaves of *Cerbera odollam* that was found to exhibit potent anticancer effects *in vitro* (13). The mechanisms of action of 17 β H-neriifolin, however, remain to be elucidated.

Further studies were performed to investigate the mechanisms of cell death induced by 17 β H-neriifolin in SKOV-3 ovarian cancer cells by using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay and proteomics approaches like 2-DE, mass spectrometry (MS) and proteomic database search (Mascot and IPA). Also, functional studies using Ingenuity Pathway Analysis (IPA) on proteins identified on 17 β H-neriifolin-treated and non-treated SKOV-3 cells may provide valuable information to in-depth understand the mechanisms of action of the compound of interest.

Materials and Methods

Materials and cells. The assay kits used in the present study include the Deadend™ Colorimetric Apoptosis Detection System Kit and DNase 1 (RNase – Free DNase) for TUNEL assay (Promega,

Madison, Wisconsin, USA), 2D-Quant kit (Amersham Biosciences, Uppsala, Sweden) for protein quantification and In-gel Tryptic Digestion Kit (Thermo Scientific, Rockford, Illinois, USA) for protein identification. The ovarian cancer cell line, namely SKOV-3, was purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA).

Test compound. 17 β H-neriifolin was isolated from the leaves of *C. odollam* based on the previously described method in Siti Syarifah *et al.* (13) with minor modifications. *C. odollam* plant species was authenticated by a botanist and deposited in the herbarium of Forest Research Institute Malaysia, Kepong, Selangor, Malaysia with voucher specimen number of AC5832-P.

Cell viability assay. The cell line was cultured in T-75 cm² flask containing Dulbecco's Modified Eagle's (DMEM; Sigma-Aldrich, St. Louis, Missouri, USA) medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin, 0.25% amphotericin B and 1% gentamycin. Briefly, 4 to 6 \times 10³ cells/well were plated into the 96-well plates and incubated overnight in a humidified incubator at 37°C with 5% CO₂ to allow cells attachment. The SKOV-3 ovarian cancer cell line was treated at different concentrations of 17 β H-neriifolin and paclitaxel at 0.001, 0.002, 0.004, 0.008, 0.016 μ M and 0.01, 0.02, 0.04, 0.08, 0.16 μ M, respectively. The plates were then incubated for 72 hours in a humidified incubator at 37°C with 5% CO₂. The percentage of cells viability was determined using the sulforhodamine B (SRB) assay as previously described by Skehan *et al.* (14). The results of the treated and non-treated cells were read at an optical density (OD) of 492 nm using a Magellan V.4 microtiter plate reader (Tecan, Seestrasse, Männedorf, Switzerland). The percentage of living cells was calculated by the formula:

$$\left[\frac{\text{OD}_{492\text{nm}} \text{ of treated cells}}{\text{OD}_{492\text{nm}} \text{ of non-treated cells}} \right] \times 100$$

The IC₅₀ values were determined from the dose-response curve as resulted from living cells *versus* concentrations of compound or drug (μ M). Cell viability assay for each treatment were performed in triplicate in at least three independent experiments (n=9) and the IC₅₀ values and the percentage of apoptotic cells are given as mean \pm standard deviation (SD).

TUNEL assay. The apoptotic morphological changes in SKOV-3 cells treated with 17 β H-neriifolin and paclitaxel were observed under a fluorescence microscope after performing TUNEL assay. Briefly, 1 \times 10⁵ cells were plated into each chamber of four-Labtek® Chamber Slides (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and incubated overnight in a humidified incubator to allow cell attachment. The cells were treated with 0.01 μ M 17 β H-neriifolin and 0.032 μ M paclitaxel, respectively, for 4, 6, 12 and 24 hours prior to TUNEL assay. Another set of experiment was also conducted to study the effect of different concentrations of 17 β H-neriifolin (0.001, 0.01, 0.1 μ M) and paclitaxel (0.0032, 0.032, 0.32 μ M) both incubated for 24 hours. Following the treatment, the cells were subjected to the TUNEL assay protocol as recommended by the manufacturer (Promega). Generally, the addition of terminal deoxynucleotidyl transferase (TdT) enzyme to the treated cells catalyses the binding of fluorescence labeled deoxyuridine

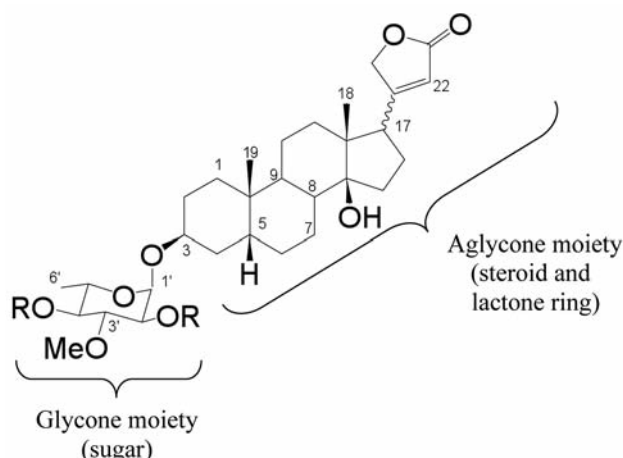


Figure 1. 17 β H-neriifolin, a cardiac glycoside isolated from *C. odollam* leaves.

triphosphate (dUTP) to the 3'OH ends of the fragmented DNA and therefore the cells undergoing DNA fragmentation can be detected as a yellow fluorescence signal in the cell nuclei when observed under the fluorescence microscope (BX53, Olympus, Japan) at 400 times magnification. Percentage of apoptotic cells is calculated using the formula:

$$\text{Apoptotic cells, \%} = \left[\frac{\text{apoptotic cell number}}{\text{total cell number}} \right] \times 100$$

Statistical significance for the apoptosis study was evaluated by paired sample *t*-test using the SPSS software version 16.0 (en.softonic.com/s/spss-16.0-free-download-full-version). The results were accepted as significant if the *p* value was <0.05.

Preparation of proteins from 17 β H-neriifolin-treated and non-treated SKOV-3 ovarian cancer cells. Briefly, SKOV-3 cells were grown in 75 cm² flasks up to at least ten million cells *per* flask. 0.01 μ M 17 β H-neriifolin-treated and non-treated cells were incubated for 24 hours prior to cell pellet collection. Cell pellets were re-suspended with 500 μ l of lysis buffer consisting of 6 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 65 mM dithiothreitol (DTT) and protease inhibitor cocktail. The pellets were lysed by using a 25-gauge needle and the lysed cells were then spun at 14,000 rpm for 30 minutes at 4°C. The protein concentrations were then determined using the 2D-Quant kit.

Two-dimensional electrophoresis (2D-E). Isoelectric focusing (IEF) was performed on the treated and non-treated SKOV-3 cells using immobilised drystrip (pH 3-10, 13 cm, non-linear, GE Healthcare, Uppsala, Sweden) using the Ettan IPGphor IEF System (GE Healthcare, Wauwatosa, Wisconsin, USA). Prior to that, 100 μ g of protein sample was reconstituted in a rehydration buffer containing 6 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer, 0.002% bromophenol blue, DTT and rehydrated for 17 h (passive rehydration) at 21°C. Isoelectric focusing was performed under the following

conditions: (i) 500 V, 1 h 20 min, step and hold; (ii) 1000 V, 1 h 30 min, gradient; (iii) 8000 V, 2 h 45 min, gradient and (iv) 8000 V, 1 h, step and hold. The temperature was maintained at 20°C and the current was kept at 50 μ A *per* strip. Following IEF, the gel strip was first equilibrated for 15 minutes in the equilibration buffer containing 6 M urea, 75 mM Tris-HCl, pH 8.8, 29.3% glycerol, 2% SDS 0.002% bromophenol blue and 2% DTT, followed by a second equilibration for 15 min in the same equilibration buffer containing 2.5% iodoacetamide instead of DTT. The second dimensional electrophoresis was performed in 12.5% acrylamide gels using the SE 600 Ruby electrophoresis system (GE Healthcare, Uppsala, Sweden) at a constant voltage of 50 V and 40 mA/gel for 30 min and then switched to 600 V and 40 mA/gel until the bromophenol blue marker was 1 mm away from the bottom of the gel. Silver stained gels were visualised and scanned using the ImageScanner™ III (GE Healthcare, Uppsala, Sweden).

Image and data analysis. The gel images of 17 β H-neriifolin-treated and non-treated SKOV-3 cells were analysed using Progenesis SameSpot software (Nonlinear Dynamics Ltd., Newcastle, UK). The software was used to subtract background, normalize and match gels including assigning an identification and intensity number of each protein spot. Each gel was assigned to a match set and matched to a selected reference gel. Spots shared between all gels were used as landmarks to improve match rate between gels. Statistical significance between treatments for proteomics study (*p*≤0.05, ANOVA) and presence in all 6 gels were the two criteria for acceptance of the differentially-expressed protein spots. Selected spots were filtered based on an average expression level change of at least 1.5-fold.

In-gel tryptic digestion. The in-gel digestion procedures of the silver stained gel plugs were conducted according to the manufacturer's protocol in the In-gel Tryptic Digestion Kit (Thermo Scientific). Protein spots of interest were manually excised and destained with 25 mM ammonium bicarbonate and 50% acetonitrile (ACN). The plugs were then reduced with 10 mM DTT at 60°C for 30 minutes and alkylated with 55 mM iodoacetamide in the dark at room temperature for 15 min. They were then washed twice with 25 mM ammonium bicarbonate and 50% ACN by incubating the plugs at 37°C for 15 min with shaking and dehydrated with 100% ACN for 15 min prior air-dried the gel pieces. Finally, the plugs were digested with 10 ng/ μ l trypsin in 25 mM ammonium bicarbonate at 37°C for at least 18 hours. The extraction solution was finally removed from the peptide mixtures by using Speedvac. The dried peptides were reconstituted with 10 μ l of 0.1% TFA prior to desalting using Zip Tip C18 tips following the manufacturer's protocol (Milipore, Billerica, MA, USA).

Mass spectrometry and database search. An equal volume of sample (0.5 μ l) and alpha cyano-4-hydroxycinnamic were mixed and spotted onto a Matrix-assisted laser desorption/ionization (MALDI) target plate (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada). Once the sample and matrix was air dried completely, peptide masses were analysed. The mass spectra were obtained using 4800 Plus MALDI TOF/TOF™ Analyser (Applied Biosystems, Concord, Ontario, Canada) and the peptide mass analysis was carried out using the GPS Explorer software (Applied Biosystems, Foster City, CA, USA). Protein identification using peptide mass fingerprinting was compared with the theoretical

masses derived from the sequences in MASCOT protein identification software (Matrix science) and the NCBI database. Few parameters were set for database searching including: Homo sapiens, mass ranges within 900-4000 Da, peptide tolerance at 75 ppm; carbamidometylation of cystein was set as a fixed modification and methionine oxidation was set as a variable modification.

Ingenuity pathway analysis (IPA). Functional analyses to predict networks that are affected by the differentially expressed proteins were carried-out using Interactive Pathway Analysis software (Ingenuity Systems, <http://www.ingenuity.com/>). This software uses computational algorithms to identify relationships between millions of different proteins, genes, cells, tissues, drugs and diseases. The accession numbers and fold changes of the differentially expressed proteins were tabulated in Microsoft Excel and imported into the IPA software. Each protein identifier was mapped to its corresponding protein object and was overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. The hypothetical networks were then build from the expressed proteins and generated as graphical representations. In the IPA analysis, the right-tailed Fisher Exact test is used to calculate *p*-value and score ($p\text{-score} = -\log_{10} p\text{-value}$) (Ingenuity Systems, <http://www.ingenuity.com/>). A score of 2 was established by the IPA to increase confidence that a particular protein was not randomly assembled into a network.

Results

Anticancer effects of 17 β H-neriifolin treated against ovarian cancer cells. The anticancer effect of 17 β H-neriifolin was evaluated against the SKOV-3 ovarian cancer cell line. Paclitaxel was incorporated for comparison studies. As illustrated in the dose-response curve in Figure 2, 17 β H-neriifolin exhibited a very significant anticancer effect with an IC_{50} value of $0.01 \pm 0.001 \mu\text{M}$ against SKOV-3 cells, while the IC_{50} value of paclitaxel in SKOV-3 was $0.032 \pm 0.002 \mu\text{M}$. It may be noted that 17 β H-neriifolin exhibited almost three-times higher anticancer effect compared to paclitaxel in SKOV-3 cells. In the subsequent assays, the concentration of 17 β H-neriifolin and paclitaxel used were based on the IC_{50} values.

Induction of apoptosis by 17 β H-neriifolin in SKOV-3 ovarian cancer cells. The results described above suggest that SKOV-3 ovarian cancer cell line show potent anticancer effects when treated with 17 β H-neriifolin. To determine the mechanisms of SKOV-3 cell death induced by 17 β H-neriifolin, the cells were treated with 17 β H-neriifolin and paclitaxel at 0.01 and 0.032 μM , respectively, and further treated at different time frames of 4, 6, 12 and 24 h. The 17 β H-neriifolin-treated and non-treated SKOV-3 cells were subjected to TUNEL assay and the fluorescence stained-cells were observed under fluorescence microscopy. Positive control cells were treated with DNase 1 used as potential marker to indicate and validate DNA fragmentation that had taken place which was ultimately demonstrated by the detection of a yellow fluorescence signal, whereas non-

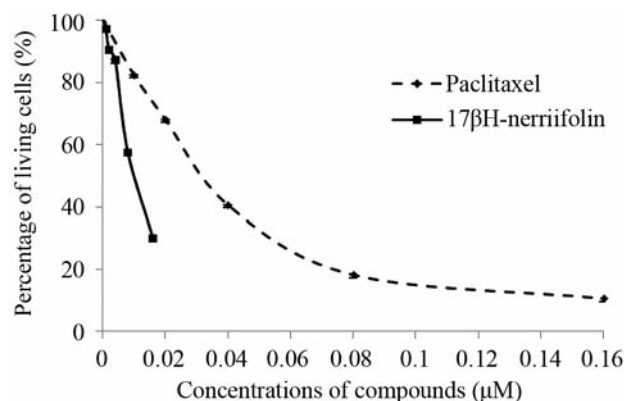


Figure 2. Anticancer effects of 17 β H-neriifolin and paclitaxel on SKOV-3 ovarian cancer cell determined by the SRB assay. Data are expressed as the mean IC_{50} values \pm standard deviation (S.D) ($n \geq 3$).

treated SKOV-3 cells displayed an ordinary epithelial shape with red background colour due to propidium iodide (PI) background staining. The occurrence of DNA fragmentation and formation of apoptotic bodies under fluorescence microscopy indicating apoptosis was clearly observed. Emergence of fluorescence-stained nuclei was detected as early as 4 h when SKOV-3 cells treated with 17 β H-neriifolin. From our results, it was found that, the number of yellow fluorescence signal indicating apoptotic cells increases as the incubation time with 17 β H-neriifolin increases. A similar observation was also recorded in the cells treated with paclitaxel used for comparison reasons.

Following the visualisation of some apoptotic events induced by 17 β H-neriifolin and paclitaxel on SKOV-3 ovarian cancer cells, the percentage of apoptotic cells were calculated. SKOV-3 cells treated with 17 β H-neriifolin at different time frames showed that a 24 h incubation produced the highest percentage of apoptotic cell death (Figure 3 D). The apoptotic effects of SKOV-3 treated with 17 β H-neriifolin and paclitaxel were evaluated at different concentrations (0.001, 0.01, 0.1 μM for 17 β H-neriifolin and 0.0032, 0.032, 0.32 μM for paclitaxel) at the same 24 h time point. It was found that 17 β H-neriifolin and paclitaxel induced apoptosis in a dose-dependent manner (Figure 4) in which, 17 β H-neriifolin and paclitaxel caused an increase of apoptotic cell death by 20.59, 41.94, 60.63% and 22.11, 45.61, 75.99%, respectively at 24 h of treatment. As shown in Figure 4, the percentage of apoptotic cells increased approximately 1.5-fold as the concentration of 17 β H-neriifolin increased. A similar trend was also observed in the SKOV-3 cells treated with paclitaxel. It was also found that more than 50% of apoptotic cells were detected at the highest concentrations of both compounds.

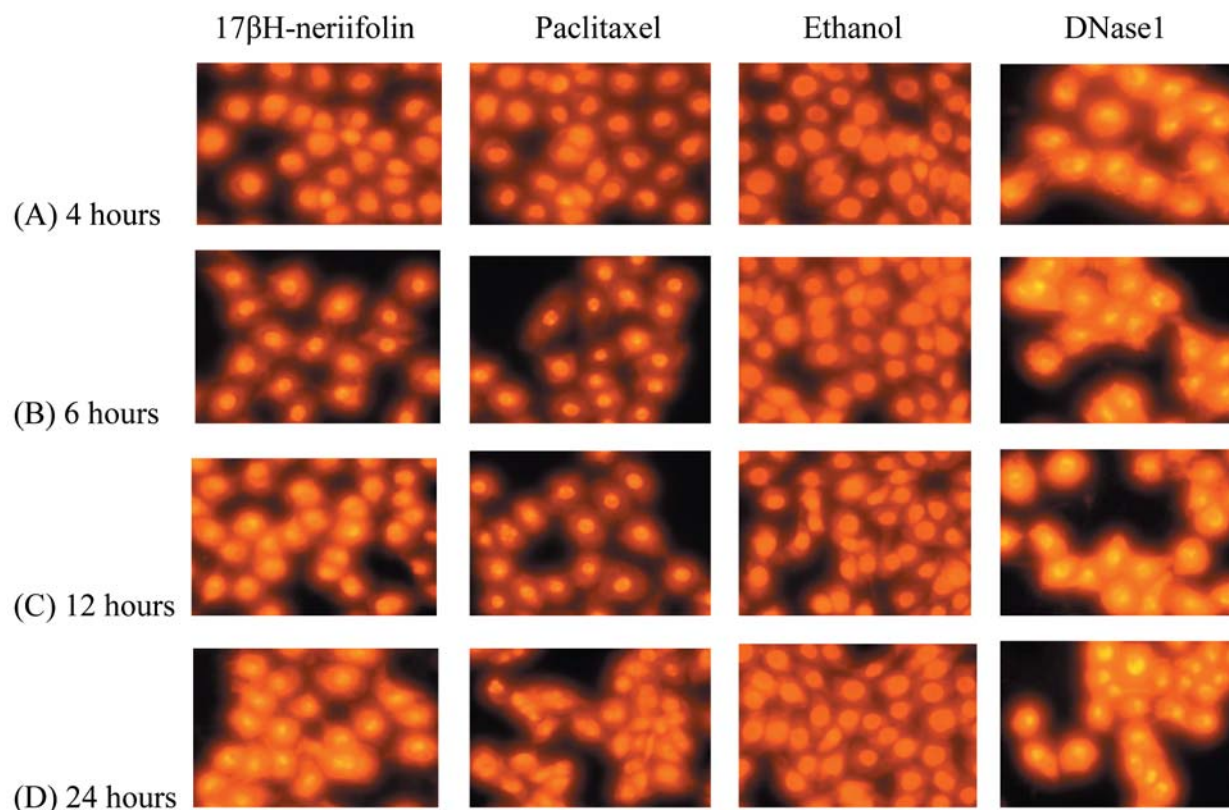


Figure 3. Representative images from morphological observations of 17 β H-neriifolin- and paclitaxel-treated SKOV-3 ovarian cancer cell line stained with fluorescence labeling (TUNEL) examined under fluorescence microscopy. SKOV-3 cells were treated with 0.01 μ M 17 β H-neriifolin, 0.032 μ M paclitaxel, ethanol (as negative control) and DNase1 (as positive control) for 4, 6, 12 and 24 h. Occurrence of DNA fragmentation indicated by the yellow fluorescence stained nuclei observed in both 17 β H-neriifolin- and paclitaxel-treated SKOV-3 cells (A-D) and formation of apoptotic bodies observed only in paclitaxel-treated SKOV-3 cells (C-D). Magnification: \times 400.

2-DE analysis of 17 β H-neriifolin-treated and non-treated SKOV-3 ovarian cancer cells. Proteome analysis was further performed to identify proteins involved in mechanisms of cell death *i.e.* apoptosis, when SKOV-3 cancer cells were treated with 17 β H-neriifolin. The differential protein expressions in 2-DE gel profiles of protein samples obtained from 17 β H-neriifolin-treated and non-treated SKOV-3 cancer cells were analysed by using the Progenesis SameSpot software. Overall, both the 2-DE gel profiles showed spot similarities however, there were significant differences in the expression of some individual proteins (Figure 5). More than 1,300 resolved protein spots were detected in both the 17 β H-neriifolin-treated and non-treated gel profiles. The protein spots from the 2-DE gel profiles of 17 β H-neriifolin-treated and non-treated SKOV-3 cells were matched prior to differential analysis and a great similarity was observed in which the matching rate was up to 90.3%. Statistical analysis of protein expression levels between treated and non-treated gel images were determined for each spot based on mean spot volume and differences in protein expression were

assessed by ANOVA. Protein spots that were present in all gels (n=6) with significant difference in expression ($p \leq 0.05$) that showed average expression level change of at least 1.5-fold in both treated and non-treated cells were selected. Based on these criteria, nine protein spots were found to be differentially expressed (Table I).

Identification of the differentially expressed proteins in SKOV-3 ovarian cancer cells. Based on the Progenesis SameSpot analysis, among the nine protein spots that were differentially expressed, four protein spots (spot number 576, 689, 950, 947) were up-regulated and five protein spots (spot number 417, 636, 672, 1039, 1037) down-regulated in SKOV-3 cells treated with 17 β H-neriifolin. A total of seven proteins were successfully identified by mass spectrometry and database search (Table II). Spot number 10 and 11 were not identified by MS/MS as their scores were lower than the cut-off value for positive inclusion criteria. The up-regulated proteins, namely vimentin (VIM - spot 576) and transgelin (TAGLN1 - spot 689), were up-regulated by 2.9- and 1.5-folds, respectively. The

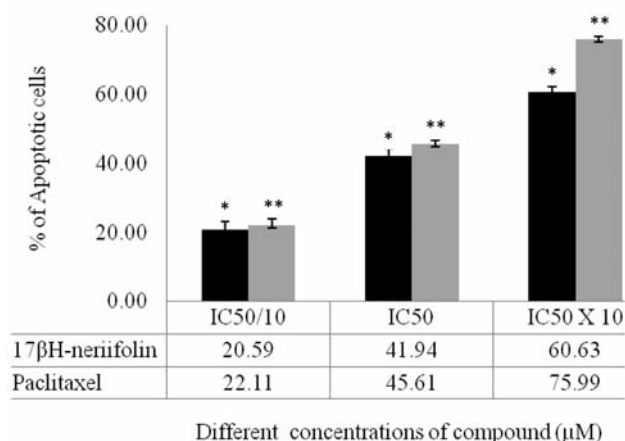


Figure 4. Induction of apoptosis in SKOV-3 ovarian cancer cells by 17βH-neriifolin and paclitaxel at different concentrations treated for 24 h as detected by the TUNEL assay. The asterisk indicates a significant difference as analysed by ANOVA paired sample t-test (* and ***p*<0.05). Each value represents means±SD from four independent experiments. Concentration of 17βH-neriifolin (IC₅₀/10: 0.001, IC₅₀: 0.01, IC₅₀ × 10: 0.1 μM) and paclitaxel (IC₅₀/10: 0.0032, IC₅₀: 0.032, IC₅₀ × 10: 0.32 μM). 17βH-neriifolin and paclitaxel-induced SKOV-3 ovarian cancer cell death via apoptosis is dose-dependent.

six down-regulated proteins, namely DENN/MADD domain (DENND2D - spot 417) and pyruvate kinase, muscle (PKM - spot 636) were down-regulated by 1.7-fold, piggybac transposable element-derived five (PGBD5 - spot 672) by 1.6-fold, heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1 - spot 1039) by 2.7-fold and formin-like 1 (FMNL - spot 1037) by 1.9-fold, as shown in Table II.

Ingenuity pathway analysis (IPA). The significantly up-regulated and down-regulated proteins were then analyzed using IPA to assess their functions, biological mechanisms and relevant interactions with other proteins in the form of global protein interaction network. According to IPA software, a score of 2 and above shows at least a 99% confidence of not being generated by random chance and a high score indicates higher confidence level. Based on IPA analysis, seven proteins, namely VIM, TAGLN1, DENND2D, PKM, PPID, PGBD5, HNRNPA1 and FMNL, are primarily associated in top networks of cell cycle, cellular movement and cancer development with a score of 20 (Figure 6). Interestingly, four proteins (VIM, TAGLN1, PKM and HNRNPA1) were found to be associated with other apoptotic-related proteins including FBJ murine osteosarcoma viral oncogene homolog (FOS), amyloid beta (A4) precursor protein (APP), ERK1/2 and CD44 through multiple signaling pathways including ovarian cancer signaling, apoptosis signaling, telomere extension by telomerase and glycolysis in

Table I. Relative expression of nine differentially expressed proteins by Progenesis SameSpot analysis.

Protein No	Average normalised volumes		Fold-change	ANOVA (p)
	Treated	Non-treated		
576	3.38×10 ⁶	1.17×10 ⁶	+2.9	1.609×10 ⁻¹²
689	14.73×10 ⁶	9.51×10 ⁶	+1.5	7.927×10 ⁻⁸
950	3.52×10 ⁶	2.34×10 ⁶	+1.5	2.376×10 ⁻⁴
947	8.75×10 ⁶	4.48×10 ⁶	+2.0	8.149×10 ⁻¹⁴
417	2.37×10 ⁶	4.11×10 ⁶	-1.7	2.722×10 ⁻⁶
636	2.79×10 ⁶	4.83×10 ⁶	-1.7	4.585×10 ⁻¹⁴
672	1.17×10 ⁶	1.82×10 ⁶	-1.6	2.846×10 ⁻⁷
1039	4.33×10 ⁶	11.69×10 ⁶	-2.7	8.478×10 ⁻⁹
1037	5.79×10 ⁶	10.92×10 ⁶	-1.9	5.914×10 ⁻⁸

(+) positive values show up-regulation and (-) negative values show down-regulation in the fold change in 17βH-neriifolin-treated SKOV-3 cells.

causing apoptotic cell death in SKOV-3 cells treated with 17βH-neriifolin. Although three proteins, namely FMNL, DENND2D and PGBD5, were not directly involved in apoptotic mechanisms, they were found to be involved as key regulator proteins assisting the regulation of other essential proteins that participate in cancer cell death.

Discussion

Many plant-based, clinically used, anticancer chemo-drugs belong to different groups of compounds including vinca alkaloids (vinblastine, vincristine), taxanes (paclitaxel), camptothecin derivatives (camptothecin, irinotecan) and podophyllotoxins (15). In recent years, more interest has been geared to study the anticancer effects of cardiac glycoside (CG) compounds due to their unique mechanisms of action (12). It was found in the present study that a CG compound, namely 17βH-neriifolin, isolated from the leaves of *C. odollam* demonstrated anticancer effects on SKOV-3 ovarian cancer cells *in vitro* at potent IC₅₀ values of 0.01 μM. These results were also supported by other studies on the anticancer effects of various CG compounds, namely Proscillaridin A, digitoxin, digoxin, ouabain and oleandrin, with their IC₅₀ values ranging from 0.03 to 10 μM in a panel of cancer cell lines including breast, prostate, renal, cervical, ovarian, skin, oral and lung cancer cells, leukaemia and melanoma cells (8, 10-11, 16).

Apoptosis (programmed cell death) is a key process in cancer development and progression that plays an important role in both human embryonic development and adult tissue homeostasis. Impairment in apoptosis due to de-activation of pro-apoptotic proteins, expression of certain onco-proteins and other associated proteins involved in cell proliferation,

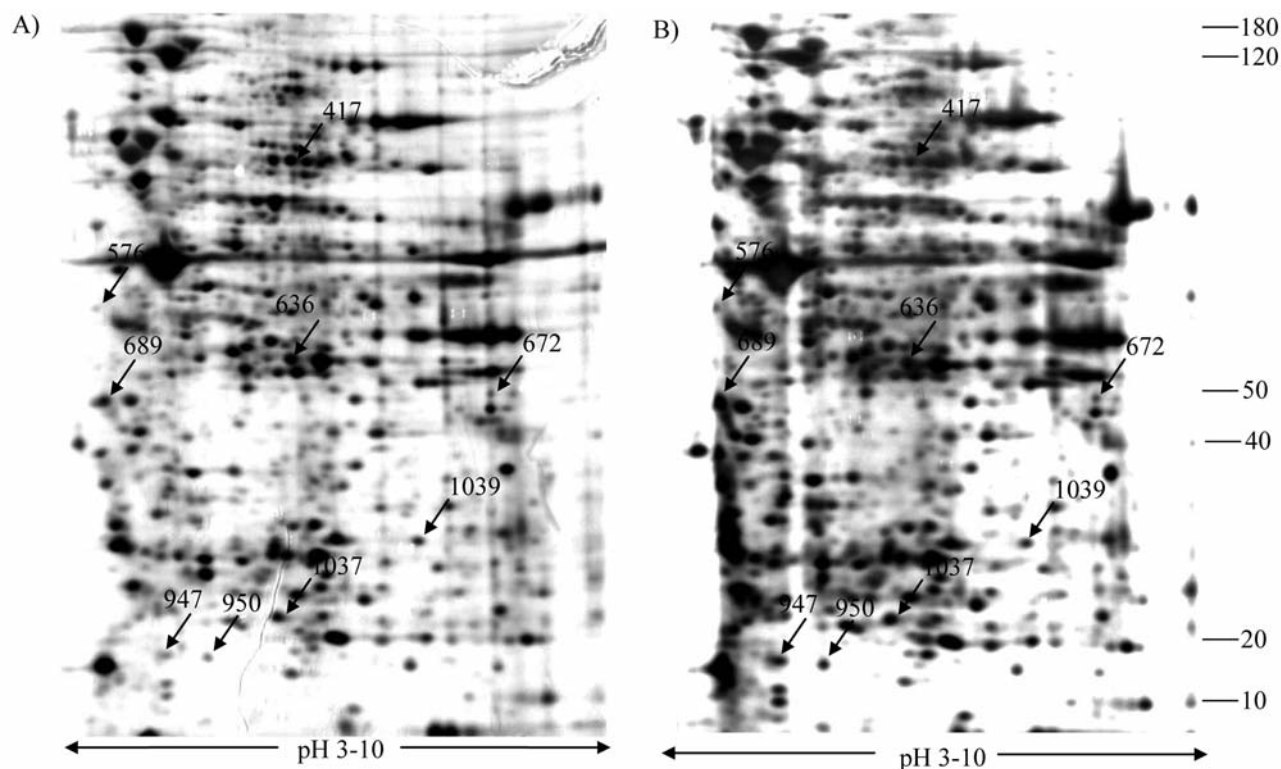


Figure 5. Representative 2-DE gel profiles of (A) non-treated and (B) 17 β H-neriifolin-treated SKOV-3 ovarian cancer cells. Approximately 1,300 spots per gel within the pH 3-10 were detected. Nine protein spots that were differentially expressed ($p \leq 0.05$) and showed an average expression level change of at least 1.5-fold in both gel profiles are indicated by arrows and labeled with the same spot number in Table I.

growth, division, migration and cell cycle (17). The search for new drug candidates for treating ovarian cancer is still on-going due to chemo-resistance effects; thus, the bioactive compounds re-inducing apoptosis in cancer cells appear to have a potential to become anticancer agents. In order to address the question on whether the anticancer effects of 17 β H-neriifolin may cause apoptotic cell death, SKOV-3 cells treated with 17 β H-neriifolin were subjected to TUNEL assay. SKOV-3 cells treated with 17 β H-neriifolin exhibited a few cellular morphological changes indicating apoptosis including the formation of apoptotic bodies and DNA fragmentation, as indicated by the yellow fluorescence-stained nuclei. In addition, 17 β H-neriifolin induced apoptosis in SKOV-3 cells in a dose-dependent manner. A few CG compounds, namely Tanghinigenin, β -D-Glucosyl-(1-4)- α -thevetosides of 17 β digitoxigenin and 2'-epi-2'-O-Acetylthevetin B, were reported to induce apoptosis at different stages in human promyelocytic leukemia HL-60 cells and in human hepatocellular carcinoma HepG2 cells (18-20). Digitoxin and oleandrin have also been reported to cause apoptotic cell death *in vitro* based on the emergence of apoptotic cell features examined by fluorescent-dye assay

(7, 11, 21). Additionally, in an *in vivo* animal model, application of oleandrin to mouse with tumor skin resulted in significant expression of few proteins that regulated the expression of specific genes that participated in inducing apoptosis (22).

The ability of CGs in inducing *in vitro* and *in vivo* cancer cell death have provided evidence on the potential of these compounds to be new candidates for cancer drug development. The mechanisms of action of CGs in killing cancer cells can be further elucidated *via* proteomics approaches since this method facilitates large-scale analysis of complete proteomes and enables comparisons between distinct conditions from different biological sources (30). Since 17 β H-neriifolin was shown to induce apoptosis, further in-depth mechanisms of action studies were performed to identify potential proteins involved in cell death. The new era of 'omics', revealed that most diseases especially cancer, results from dysregulation of many proteins by different mechanisms of action (23). Thus, this study is the first to employ proteomic techniques as tools to globally search for the proteins being affected (*i.e.*, up-regulated or down-regulated) by 17 β H-neriifolin in causing ovarian cancer cell

Table II. Identification of differentially expressed proteins induced by 17βH-neriifolin in 17βH-neriifolin-treated SKOV-3 cells (compared to non-treated cells) by MALDI-MS/MS.

Spot number	Protein name	Accession number	Expression change (fold) ^a	Theoretical pI ^b /MW (kDa) ^c	Matched peptide sequences	Sequence Coverage (%) ^d	Mascot score ^e	Molecular and cellular function ^f
576	Vimentin (VIM)	ACA06103.1	+2.9	5.19/49.7	14-28; 37-50; 146-155; 224-236; 295-304; 391-401	20	111	Cellular proliferation, migration, cell spreading, apoptosis
689	Transgelin (TAGLN1)	ACA06038.1	+1.5	10.29/3.8	21-43; 68-88	74	37	Cellular differentiation, migration, invasion
417	DENN/MADD domain containing 2D (DENND2D)	EAW56475.1	-1.7	8.16/21.9	88-95; 161-181	15	26	Others
636	Pyruvate kinase, muscle (PKM)	BAG57589.1	-1.7	7.96/66.5	29-49; 107-130; 148-163; 216-225; 305-320; 353-368; 497-517	20	67	Cellular invasion, apoptosis
672	Piggybac transposable element derived 5 (PGBD5)	EAW69912.1	-1.6	9.33/63.1	91-110; 400-420; 427-439	9	51	Others
1039	Heterogeneous Nuclear Ribonucleoprotein A1 (HNRNPA1)	AAI03708.1	-2.7	9.19/29.5	13-29; 91-103; 105-138; 145-159	29	115	Cellular proliferation, invasion, tumor development, apoptosis
1037	Formin-like 1 (FMNL)	AAI11565.1	-1.9	5.28/69.7	1-23; 489-508	6	49	Others

^aPositive values show up-regulation against non-treated cells and negative values show down-regulation in the fold change; ^bpI: isoelectric point; ^cMW (kDa): molecular mass in kilodalton; ^dSequence coverage %: percentage of sequence coverage obtained with identified peptides with Mascot software; ^eMascot Score: identification score obtained with Mascot software; ^fbased on the IPA database search. Spot numbers 947 and 950 were not listed since they have not been identified as explained earlier in the text. Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS/MS).

death. In order to examine the mechanisms of action of 17βH-neriifolin in inducing apoptosis in SKOV-3 cells, proteomics analyses were performed by conducting differential analysis from 2-DE profiles of 17βH-neriifolin-treated and non-treated SKOV-3 protein samples, to identify proteins affected by the treatment. Among thousands of proteins that were detected in the 2-DE profiles of 17βH-neriifolin-treated and non-treated SKOV-3 ovarian cancer cells, nine proteins were found to be differentially expressed. Four proteins were found to be up-regulated, namely VIM and TAGLN1, whilst two spots were not successfully identified due to limitations of the Mascot database. In addition, five proteins were found to be down-regulated due to 17βH-neriifolin treatment in SKOV-3 cells, namely DENND2D, PKM, PGBD5, HNRNPA1 and FMNL.

Based on the IPA, four out of the seven identified proteins, namely HNRNPA1, PKM, TAGLN1 and VIM, were shown to interact with up-stream and down-stream proteins through multiple signaling pathways in inducing cell death in SKOV-3 cells. The pyruvate kinase muscle (PKM) isoenzyme was found to be down-regulated in the 17βH-neriifolin-treated SKOV-3 cells as compared to the non-treated SKOV-3 cells.

PKM exists mainly as a dimeric form in tumor cells and is also known as Tumor M2-PK. It has been reported that over-expression of PKM plays a key role in tumor cell growth and cancer metabolism. Earlier studies in cancer cells, including HepG2 and SKOV-3, have shown that the knockdown of PKM resulted in decreased cancer cells viability which eventually induces apoptosis (24). Since, PKM is an isoenzyme of the glycolytic enzyme pyruvate kinase which plays as a key regulator for glycolysis, Tamada *et al.* (25) had reported on the capability of PKM in causing apoptotic cell death in a glucose supply-dependent manner. Glycolytic defect causes oxidative stress and hypoxia-like signal activation which eventually elevates intracellular reactive oxygen species (ROS) and causes DNA damage and lipid oxidation. These cellular responses could be accountable for the apoptosis induces by glycolytic defect (34). Thus, in this study, the down-regulation of PKM in SKOV-3 ovarian cancer cells treated with 17βH-neriifolin may possibly suggest that the compound killed the SKOV-3 cells by triggering the glucose-starvation pathway leading to disruption of glucose uptake, eventually causing apoptotic cell death.

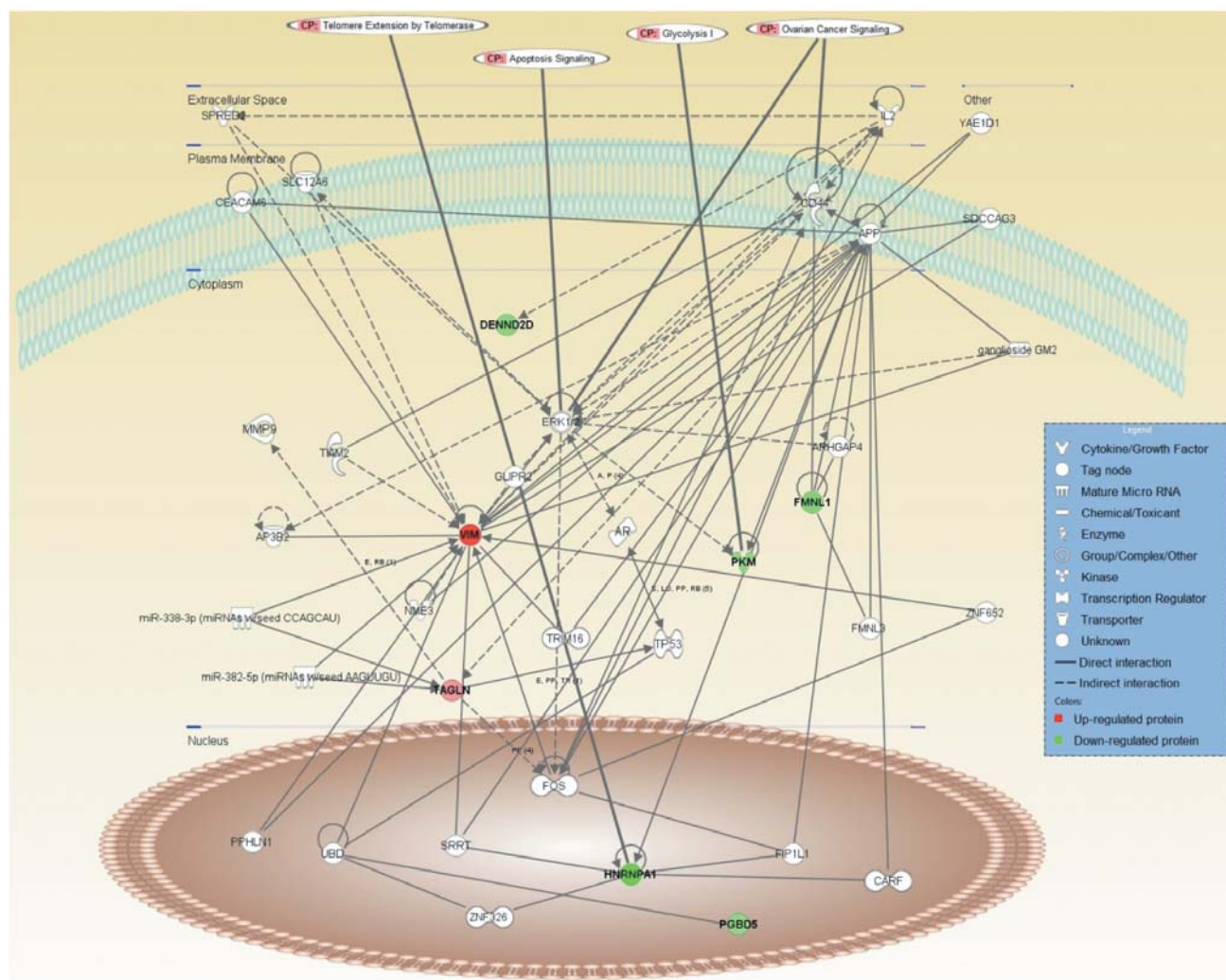


Figure 6. The protein-protein interaction network of the differentially expressed proteins in SKOV-3 ovarian cancer cells treated with 17 β H-neriifolin. The network of seven IPA mapped proteins is displayed graphically as nodes (proteins) and edges (the biological relationships between the nodes). Nodes in red indicate up-regulated proteins while those in green represent down-regulated proteins. Nodes without colors indicate unaltered expression. Various shapes of the nodes represent the functional class of the proteins. The different arrow shapes represent different types of interactions. Edges displayed with various labels describe the nature of the relationship between the nodes.

Heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) was found to be down-regulated in the 17 β H-neriifolin-treated SKOV-3 cells. HNRNPA1 was claimed to be an onco-protein involved in tumor development and progression including telomere biogenesis, inhibition of apoptosis, angiogenesis and cell invasion (26). Up-regulation of HNRNPA1 and A2/B1 in cancer cells helps in promoting cancer cell growth through the binding of HNRNPA1 and A2/B1 to telomere sequences. Consequently, down-regulation of HNRNPA1 and A2/B1 would hinder cancer cell growth by the failure of the telomere capping process due to low concentrations of HNRNPs (27, 28). As a result, the loss of telomeric capping leads to chromosome end-to-end fusion and triggers cell-cycle arrest

and apoptosis. Interestingly, in the present study, down-regulation of the onco-protein HNRNPA1 in SKOV-3 cells treated with 17 β H-neriifolin may involve a similar mechanism in an attempt to induce apoptosis.

In addition, transgelin (TAGLN1) was found to be up-regulated in the 17 β H-neriifolin-treated SKOV-3 cells by 1.5-fold compared to non-treated SKOV-3 ovarian cancer cells. This protein, which is also known as a tumor suppressor protein, is involved in cell differentiation and cell migration. Recent studies have revealed that TAGLN is down-regulated in cancer cells including human prostate cancer (29) and also in breast and colon cancer (30) tissues, whilst its up-regulation mediated TOV-81D and TOV-112D epithelial

ovarian cancer cell death (31). Interestingly, up-regulation of TAGLN has been reported to induce cell death in the human prostate cancer cell (LNCaP) cell line through a dual effect of androgen receptor (AR) inhibition and a p53 activation. Up-regulation of TAGLN was shown to help prevent the binding of AR to the co-regulator, ARA54, and thereby blocking the nuclear transformation that result in suppression of androgen stimulation on cancer cell growth. Zhang *et al.* (32) have reported that the up-regulation of TAGLN interacts with the activation of tumour suppressor gene, p53, that results in translocation of p53 from the nucleus to cytoplasm leading to the accumulation of cytoplasmic p53 which eventually induces apoptosis in the LNCaP cell line. Furthermore, accumulation of cytoplasmic p53 *in vivo* up-regulates the expression of Bax and down-regulates Bcl-2 which in turn triggers caspase-3 expression to induce apoptosis. Interestingly, from the IPA analysis, up-regulation of TAGLN showed a dual effect of AR and p53 gene in which could be one of the possibly related cell death mechanisms involved in the SKOV-3 cells treated with 17 β H-neriifolin.

Vimentin (VIM) is another up-regulated protein found in SKOV-3 17 β H-neriifolin-treated cells. The role of VIM as an anticancer molecular therapeutic target has been highlighted in certain studies (33-34). Even though, vimentin's overexpression in cancer correlates with increased tumor growth, invasion and poor prognosis, the present study showed contradictory results in which VIM was up-regulated in 17 β H-neriifolin-treated SKOV-3 cells. To the best of our knowledge, this finding has not been previously reported elsewhere. In the present study, VIM has been found to directly and indirectly interact with other apoptotic-related proteins such as ERK1/2, FOS, CD44 and ubiquitin D (UBD) in causing cancer cell death. Hence, up-regulation of VIM in this study may provide new insights in discovering the potential of VIM in inducing cell death. The discovery on the up-regulation of VIM, that explains the pro-apoptotic effects induced by 17 β H-neriifolin in SKOV-3 cells, is ground-breaking since to date it has been linked to increase the proliferation of cancer cells.

As mentioned previously, the piggybac transposable element derived 5 (PGBD5), DENN/MADD domain containing 2D (DENND2D) and Formin-like 1 (FMNL) were also affected in the cell death process induced by 17 β H-neriifolin although the IPA analysis did not link them directly to apoptotic cell death. However, changes in expressions of some of these proteins have triggered signals in some of pro-apoptotic proteins. From the IPA database search, the PGBD5 protein was reported to belong to the subfamily of PGBD proteins. PGBD5 that appears to be novel with no obvious relationship to other known protein families has been found to be down-regulated due to the treatment with 17 β H-neriifolin. The down-regulation of PGBD5 was found to trigger the

binding of UBD to tumor suppressor protein p53 (TP53), enolase-1 (ENO1), ubiquitin-like modifier activating enzyme 1 (UBA1) and other proteins. It may be noted that both TP53 and UBA1 is a pro-apoptotic protein while ENO1 is a glycolytic enzyme that may be associated in inducing apoptotic cell death. In addition, 17 β H-neriifolin also appeared to cause down regulation of DENND2D protein in SKOV-3 cells. This protein was regulated by interleukin 2 (IL2) which is a pro-inflammatory cytokine, important for the proliferation of T and B lymphocytes responsible for immune functions. FMNL is another protein which also appears to be down-regulated in SKOV-3 cells treated with 17 β H-neriifolin. Based on the IPA analysis, this protein was found to be directly bound to other proteins in the network including amyloid beta (A4) precursor protein (APP), CD44 and Rho GTPase activity protein 4 (ARHGAP4). The suppression of all these three proteins may be the key regulatory point of action that ultimately triggers the up-stream and down-stream protein activities in the SKOV-3 cells treated with 17 β H-neriifolin.

Conclusion

In conclusion, our study showed that 17 β H-neriifolin induces apoptotic cell death in a dose-dependent manner. Proteins, namely VIM, TAGLN1, DENND2D, PKM, PGBD5, HNRNPA1 and FMNL, were successfully identified in the present study. Among them, few proteins, namely VIM, TAGLN1, PKM and HNRNPA1, were found to be involved in apoptotic cell death induced by 17 β H-neriifolin *via* several biochemical pathways. These mechanisms of action will be validated in future studies using *in vitro* and *in vivo* approaches towards future drug development.

Acknowledgements

The authors gratefully acknowledge the Ministry of Science, Technology and Innovation for financial assistance and support through the e-Science Fund (31-31-10-07-001). Further acknowledgement goes to all the staff of the Drug Discovery Centre, FRIM and the members of Proteomics Lab in Faculty of Medicine, University Malaya for their invaluable assistance throughout the study.

References

- 1 Yan XD, Pan LY, Yuan Y, Lang JH and Mao N: Identification of platinum-resistance associated proteins through proteomic analysis of human ovarian cancer cells and their platinum-resistant sublines. *J Proteome Res* 6: 772-780, 2007.
- 2 Kerr JFR, Wyllie AH and Currie AR: Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26: 239-257, 1972.
- 3 Gossiau A and Chen KY: Nutraceuticals, apoptosis, and disease prevention. *Nutrition* 20: 95-102, 2004
- 4 Anderson NL and Anderson NG: The human plasma proteome: history, character and diagnostic prospects. *Mol Cell Proteomics* 1: 845-867, 2002.

- 5 Křen V and Martínková L: The role of glycosidic residue in biological activity. *Curr Med Chem* 8: 1303-1328, 2001.
- 6 Braunwald E: Effects of digitalis on the normal and the failing heart. *J Am Coll Cardiol* 5: 51A-59A, 1985.
- 7 Haux J, Lam J, Marthinsen ABL, Strickert T and Lundgren S: Digitoxin, in non toxic concentrations induces cell death in Jurkat T cells *in vitro*. *J Oncol* 31: 14-20, 1999.
- 8 Therien AG and Blostein R: Mechanisms of sodium pump regulation. *Am J Physiol* 279: 541-567, 2000.
- 9 Yeh JY, Huang WJ, Kan SF and Wang PS: Inhibitory effects of digitalis on the proliferation of androgen dependent and independent prostate cancer cells. *J Urol* 166: 1937-1942, 2001.
- 10 Laphookhieo S, Cheenpracha S, Karalai C, Chantrapromma S, Rat-a-pay Y, Ponglimanont C and Chantrapromma K: Cytotoxic cardenolide glycoside from the seeds of *Cerbera odollam*. *Phytochemistry* 65: 507-510, 2004.
- 11 López-Lázaro M, Pastor N, Azrak SS, Ayuso MJ, Austin CA and Cortés F: Digitoxin inhibits the growth of cancer cell lines at concentrations commonly found in cardiac patients. *J Nat Prod* 68: 1642-1645, 2005.
- 12 Newman RA, Yang P, Pawlus AD and Block KI: Cardiac glycosides as novel cancer therapeutic agents. *Mol Intervent* 8(1): 36-49, 2008.
- 13 Siti Syarifah MM, Nurhanan MY, Muhd Haffiz J, Mohd Ilham A, Getha K, Asiah O, Norhayati I, Lili Sahira H and Anee Suryani S: Potential anticancer compound from *Cerbera odollam*. *J Trop Forest Sci* 23(1): 89-96, 2011.
- 14 Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S and Boyd MR: New colorimetric assay for anticancer-drug screening. *J Natl Cancer Inst* 82: 1107-1112, 1990.
- 15 Prakash O, Kumar A, Kumar P and Ajeet: Anticancer potential of plants and natural products: A review. *Am J Pharm Sci* 1(6): 104-115, 2013.
- 16 Felth J, Rickardson L, Rosén J, Wickstrom M, Fryknäs M, Lindskog M, Bohlin L and Gullbo J: Cytotoxic effects of cardiac glycosides in colon cancer cells, alone and in combination with standard chemotherapeutic drugs. *J Nat Prod* 72: 1969-1974, 2009.
- 17 Ashkenazi A: Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* 2: 420-430, 2002.
- 18 Wang GF, Guo YW, Feng B, Li L, Huang CG and Jiao BH: Tanghinigenin from seed of *Cerbera manghas* L. induces apoptosis in human promyelocytic leukemia HL-60 cells. *Environ Toxicol Pharmacol* 30: 31-36, 2010.
- 19 Feng B, Guo Yue-Wei, Huang Cai-Guo, Li L, Chen Ruo-Hua and Jiao Bing-Hua: 2'-epi-2'-O-Acetylthevetin B extracted from seeds of *Cerbera manghas* L. induces cell cycle arrest and apoptosis in human hepatocellular carcinoma HepG2 cells. *Chem Biol Interact* 183: 142-153, 2010.
- 20 Feng B, Guo YW, Huang CG, Li L and Jiao BH: β -D-Glucosyl-(1-4)- α -L-thevetosides of 17 β -digitoxigenin from seeds of *Cerbera manghas* L. induces apoptosis in human hepatocellular carcinoma HepG2 cells. *Exp Toxicol Pathol* 64(5): 403-410, 2012.
- 21 Sreenivasan Y, Sarkar A and Manna SK: Oleandrin suppresses activation of nuclear transcription factor-kB and activator protein-1 and potentiates apoptosis induced by ceramide. *Biochem Pharmacol* 66: 2223-2239, 2003.
- 22 Afaq F, Saleem M, Aziz MH and Mukhtar H: Inhibition of 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion markers in CD-1 mouse skin by oleandrin. *Toxicol Appl Pharmacol* 195: 361-369, 2004.
- 23 Kunnumakkara AB, Anand P and Aggarwal BB: Curcumin inhibits proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell signaling proteins. *Cancer Lett* 269(2): 199-225, 2008.
- 24 Goldberg MS and Sharp PA: Pyruvate kinase M2-specific siRNA induces apoptosis and tumor regression. *J Exp Med* 209(2): 217-224, 2012.
- 25 Tamada M, Suematsu M and Sya H: Pyruvate Kinase M2: Multiple faces for conferring benefits on cancer cells. *Clin Cancer Res* 18: 5554-5561, 2012.
- 26 Carpenter B, MacKay C, Alnabulsi A, MacKay M, Telfer C, Melvin WT and Murray GI: The roles of heterogeneous nuclear ribonucleoproteins in tumour development and progression. *Biochim Biophys Acta* 1765: 85-100, 2006.
- 27 Patry C, Bouchard L, Labrecque P, Gendron D, Lemieux B, Toutant J, Lapointe E, Wellinger R and Chabot B: Small interfering RNA-mediated reduction in Heterogeneous Nuclear Ribonucleoparticulate A1/A2 proteins induces apoptosis in human cancer cells but not in normal mortal cell lines. *Cancer Res* 63: 7679-7688, 2003.
- 28 He Y, Brown MA, Rothnagel JA, Saundres NA and Smith R: Roles of Heterogeneous Nuclear Ribonucleoparticulate A and B in cell proliferation. *J Cell Sci* 118: 3173-3183, 2005.
- 29 Yang Z, Chang Yu-Jia, Miyamoto H, Ni J, Niu Y, Chen Z, Chen Yuh-Ling, Yao JL, di Sant' Agnese PA and Chang C: Transgelin functions as a suppressor *via* inhibition of ara54-enhanced androgen receptor transactivation and prostate cancer cell growth. *Mol Endocrinol* 21(2): 343-358, 2007.
- 30 Shields JM, Rogers-Graham K and Der CJ: Loss of transgelin in breast and colon tumors and in RIE-1 cells by Ras deregulation of gene expression through Raf-independent pathways. *J Biol Chem* 277: 9790-9799, 2002.
- 31 Gagné Jean-Philippe, Éthier C, Gagné P, Mercier G, Bonicalzi Marie-Eve, Mes-Masson Anne-Marie, Droit A, Winstall E, Isabelle M and Poirier GG: Comparative proteomic analysis of human epithelial ovarian cancer. *Proteomic Sci* 5(16): 1-15, 2007.
- 32 Zhang Zhe-Wei, Yang Zhi-Ming, Zheng Yi-Chun and Chen Zhao-Dian: Transgelin induces apoptosis in human prostate LNCaP cells through its interaction with p53. *Asian J Androl* 12: 186-195, 2010.
- 33 Lahat G, Zhu Quan-Sheng, Huang Kai-lieh, Wang S, Bolshakov S, Liu J, Torres K, Langley RR, Lazar AJ, Hung MC and Lev D: Vimentin is a novel anticancer therapeutic target: insights from *in vitro* and *in vivo* mice xenograft studies. *Plos One* 5(4): 1-19, 2010.
- 34 Satelli A and Li S: Vimentin in cancer and its potential as a molecular target for cancer therapy. *Cell Mol Life Sci* 68(18): 3033-3046, 2011.

Received May 25, 2014

Revised June 27, 2014

Accepted June 29, 2014