Proteomic analysis reveals that pardaxin triggers apoptotic signaling pathways in human cervical carcinoma HeLa cells: cross talk among the UPR, c-Jun and ROS

Tsui-Chin Huang1,2,3,4 and Jyh-Yih Chen1,*

1Marine Research Station, Institute of Cellular and Organismic Biology, Academia Sinica, 23-10 Dahuen Road, Jiaushi, Ilan 262, Taiwan, 
2PhD Program for Cancer Biology and Drug Discovery, College of Medical Science and Technology, Taipei Medical University and Academia Sinica, Taipei 110, Taiwan and 
3Graduate Institute of Cancer Biology and Drug Discovery and 
4Center of Excellence for Cancer Research, Taipei Medical University, Taipei 110, Taiwan

*To whom correspondence should be addressed. Tel: +886 920802111; Fax: +886 3987035; Email: zoocyj@gate.sinica.edu.tw

Pardaxin, an antimicrobial peptide secreted by the Red Sea flatfish Pardachirus marmoratus, inhibits proliferation and induces apoptosis of human cancer cell lines. However, the underlying molecular mechanisms are only partially understood at present. In this study, we used proteomic approaches and network reconstruction to clarify the mechanism of pardaxin-induced apoptosis in human cervical carcinoma HeLa cells. We identified that pardaxin-regulated proteins predominantly function in the unfolded protein response, oxidative stress and cytoskeletal distribution. Molecular examination of signal transduction and cellular localization demonstrated that the activator protein-1 (AP-1) transcription factor was activated, which eventually caused apoptosis via both caspase- and apoptosis-inducing factor-dependent pathways. Scavenging of reactive oxygen species (ROS) alleviated c-Jun activation, and small interfering RNA knockdown of c-Jun abrogated pardaxin-induced caspase activation and cell death, thereby implicating ROS and c-Jun in pardaxin-induced apoptosis signaling. In summary, this study provides the first protein-interacting network maps and novel insights into the biological responses and potential toxicity of pardaxin.

Introduction

Antimicrobial peptides (AMPs) have evolutionarily conserved functions and can be produced by almost all organisms (1). As immunomodulators that target pathogens, AMPs enhance the innate immune response of hosts by acting as the first line of defense (2,3). Based on differences in the physical properties of cell membranes of normal cells and pathogens, AMPs are usually toxic to bacteria, fungi, viruses, mycoplasma and, in some cases, cancer cells (3). In general, AMPs bind to anionic and hydrophobic membranes of pathogens through electrostatic or hydrophobic interactions (4). After attaching to a membrane, AMPs insert themselves into membrane bilayers to form pores, or penetrate lipid bilayers to disrupt intracellular reactions, attacking cells in a lytic way or by disrupting homeostasis (4,5). Pardaxin is an AMP secreted by the Red Sea flatfish Pardachirus marmoratus (6). In addition to its utilization as a shark repellent, pardaxin has been shown to possess potent antibacterial, antiviral and antifungal activities (7,8). Recently, we revealed that pardaxin displays high-efficacy anticancer activity by inducing apoptosis in human cervical carcinoma HeLa cells (9). We also demonstrated that pardaxin activated caspase-3 and caspase-7 activities, disrupted mitochondrial membrane potential (MMP) and caused the accumulation of reactive oxygen species (ROS) produced by fibrosarcoma HT-1080 cells (10). Scavenging of ROS production and inhibition of caspase activities reduced pardaxin-induced effects (10).

Apoptosis is a cell death process that involves specific morphological changes, including plasma membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation and chromosomal DNA fragmentation (11). Mitochondria play a central role in initiating apoptosis by releasing small mitochondrion-derived activator of caspasess (SMAC) and cytochrome (Cyt) c into the cytosol. Cytosolic small mitochondrion-derived activator of caspases and Cyt c bind to the inhibitor of apoptosis proteins and apoptotic protease activating factor (Apaf)-1, respectively, to cooperatively activate caspase cascades and execute apoptosis. Another distinct apoptotic pathway is caspase independent, which is caused by the release of apoptosis-inducing factor (AIF) from intermembrane spaces of mitochondria; translocation of AIF to nuclei triggers chromatin condensation and DNA fragmentation (12).

The endoplasmic reticulum (ER) is a continuous membrane network in which secreted and membrane proteins mature with the assistance of ER luminal chaperone proteins, such as glucose-regulated protein, 78 kDa (GRP78/BiP), GRP94 and calreticulin. Accumulation of misfolded proteins in the ER activates the unfolded protein response (UPR), which causes an expansion of the ER membrane, upregulation of components involved in protein folding and quality control and attenuation of protein synthesis, to restore ER capacity. When ER homeostasis cannot be restored, a prolonged UPR can lead to apoptosis through activation of CHOP and caspase-3. Elevated CHOP transcription induces Bim, a proapoptotic BH3-only B-cell lymphoma 2 family member, which activates apoptosis (13).

The ER is a redox environment in which reduced and oxidized disulphide bonds are formed, which helps ensure that proteins assume the correct conformation during protein folding. Disruptions to the balance of the redox system through accumulation of ROS or inhibition of antioxidant-producing systems triggers the UPR, thereby helping cells adapt to oxidative stress (14). Activation of c-Jun contributes to several cellular responses, including apoptosis, proliferation and differentiation. The outcome of c-Jun N-terminal kinase (JNK)/c-Jun signaling strongly depends on the cell type and cellular context. Accumulation of ROS predominantly activates the mitogen-activated protein kinases (MAPKs), JNK and p38 MAPK, which result in c-Jun phosphorylation and apoptosis (15). ROS production activates c-Jun on one hand and induces the UPR on the other. Both c-Jun phosphorylation and UPR signaling contribute to apoptosis, when the cell cannot overcome oxidative stress.

In this study, we demonstrate the efficacy of pardaxin against several cervical cancer cell lines and determine the predominant effects of inducing apoptotic cell death in HeLa cells. By inducing oxidative stress, the UPR and phosphorylation of c-Jun, pardaxin triggered both caspase- and AIF-dependent apoptotic pathways. As oxidative stress, the UPR and c-Jun activation are double-edged swords, here we emphasize that pardaxin exhibits high cytotoxicity efficacy and may therefore be a promising anticancer drug that induces apoptosis in cervical cancer cells by regulating these three cellular modulators.

Materials and methods

A schematic representation of the study design is shown in Supplementary Figure 1, available at Carcinogenesis Online, and the methods are described in detail below.

Peptide synthesis

Pardaxin (H-GFFALPIPKISSPLFKTLSSAVGASLLSSGGQEOH) was synthesized and purified to >90% grade by GL Biochemistry (Shanghai, China).

Abbreviations: AIF, apoptosis-inducing factor; AMP, antimicrobial peptide; DAPI, 4′,6-diamidino-2-phenylindole; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; IEF, isoelectric focusing; JNK, c-Jun N-terminal kinase; MALDI, matrix-assisted laser desorption/ionization; MMP, mitochondrial membrane potential; MS, mass spectrometer; NAC, N-acetyl-L-cysteine; PBS, phosphate-buffered saline; PL, propidium iodide; ROS, reactive oxygen species; siRNA, small interfering RNA; TOF, time-of-flight; UPR, unfolded protein response.
Before use, the peptide was dissolved in deionized water at a concentration of 1 mg/ml and sterilized by filtration through a 0.2 μm filter.

**Cell lines and cell culture**

Human cervical carcinoma (HeLa, HeLa229, CaSkii and C33A) and human fibroblast (MRC-5) cell lines were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air in modified Eagle’s medium supplemented with 2 mM l-glutamine, 0.1 mM non-essential amino acids and 10% heat-inactivated fetal bovine serum.

**Cell viability assay**

The cytotoxicity of pardaxin was determined by a two-color analysis of annexin V (AV)-fluorescein isothiocyanate (FITC) binding and propidium iodide (PI) uptake by flow cytometry, according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Cells were harvested and washed twice in cold phosphate-buffered saline (PBS), stained with V-FITC and PI for 30 min in the dark and analyzed with a Cytomics FC 500 flow cytometer (Beckman Coulter). Cells equipped with an air-cooled argon laser with emission at 488 nm. Data were analyzed using FlowJo software (TreeStar, Ashland).

**Assessment of apoptosis by flow cytometry**

Apoptotic morphological changes in cells were detected by staining with Hoechst 33342 (1 μg/ml in PBS) for 15 min at 37°C. Fixed cells were stained with Hoechst 33342 (1 μg/ml in PBS) for 15 min at 37°C in the dark, then examined by fluorescence microscopy.

**Evaluation of the MMP (ΔΨm)**

The MMP was measured with JC-1 dye (Invitrogen). Briefly, cells were harvested and washed twice in cold PBS and resuspended in PBS containing 2 μM JC-1. After 30 min of incubation at 37°C, the cells were washed with PBS and resuspended in 500 μl PBS. Stained cells were analyzed on a Coulter, CA) equipped with an air-cooled argon laser with emission at 488 nm. Data were analyzed using FlowJo software (TreeStar, Ashland).

**Measurement of intracellular ROS**

Intracellular ROS levels were measured using a cell-permeable fluorescent dye, H₂DCFDA (Invitrogen). Cells were harvested and washed twice in cold PBS and incubated with 10 μM H₂DCFDA for 30 min at 37°C in the dark. Stained cells were analyzed with a Cytomics FC 500 flow cytometer (Beckman Coulter). The mean fluorescence intensity was obtained by histogram statistics using WinMDI 2.9.

**Western blot analysis**

For two-dimensional electrophoresis, isoelectric focusing (IEF) was performed using the Bio-Rad Protein IEF Cell (Bio-Rad, Hercules, CA). Total proteins at 350 μg were mixed with 300 μl rehydration buffer containing 7 M urea, 2% thiourea, 4% [3-cholamidopropyl(dimethylammonio)]-propane sulfonate, 65 mM diethyrlthritol, 1% carrier ampholytes (IPG buffer) and 0.002% bromophenol blue. Mixtures were loaded onto a 17 cm IPG ReadyStrip pH 4–7 (Bio-Rad). IEF parameters for separation were set to 50 μA per strip at 20°C, with a rehydration step for 14h. IEF was carried out as follows: 2h at 100 V, 30 min at 250V, 30min at 500 V, 30min at 1000 V and 30min at 4000 and 8000 V for 65 000 Vh. The focused strips were reduced with 65 mM dithioerythritol and alkylated with 55 mM iodoacetamide. Second-dimension separation was performed on a linear-gradient 10–15% polyacrylamide gel. The protein gel was fixed in 10% methanol/7% acetic acid and stained using the SYPRO® Ruby method (Invitrogen). Gels were then scanned using a Molecular Imager FX (Bio-Rad) and analyzed using the Prodigy SameSpot 2D software package (Nonlinear Dynamics, Newcastle, UK).

**MALDI-TOF**

MALDI-TOF MS and MS/MS were performed on a dedicated Q-TOF Ultima MALDI instrument (Micromass, Manchester, UK), with fully automated data-directed acquisition using a predefined probe motion pattern, and a peak intensity threshold for switching over from the MS survey scan to MS/ MS, and from one MS/MS to the other. All individual MS/MS data points thus generated from a particular sample were then output as a single Mascot compatible peak list file. Within each sample well, parent ions that met the predefined criteria (any peak within the m/z 80–300 range with an intensity >10 counts ± inclusion/exclusion list) were selected for collision-induced dissociation MS/MS using argon as the collision gas, and a mass-dependent rolling collision energy of ±5 V until the end of the probe pattern was reached (all details are available at http://proteome.sinica.edu.tw). At a laser firing rate of 10 Hz, individual spectra from a 5 s integration period acquired for each MS survey and MS/MS performed were combined, smoothed, deisotoped (fast option) and centroided using Micromass ProteinLynx™ Global Server (PGS) 2.0 data processing software. Proteins were subsequently identified using the MASCOT search engine against the Swiss-Prot release 2011_06 database, as described previously. Database search parameters included the following: only trypptic peptides with up to one missing cleavage site were allowed, modifications were carbamidomethylation (C) and oxidation of methionine, the peptide mass tolerance was ±50 p.p.m. and the fragment mass tolerance was ±0.25 Da. For positive identification, the score of the result of [-10 log(P)] had to exceed the significance threshold level (P < 0.05).

**Transmission electron microscopy**

Cells were collected, washed with PBS and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate–HCl buffer (Sigma, St Louis, MO) for 2 h at 4°C. Next, cells were washed with 0.1 M sodium cacodylate–HCl buffer three times, postfixed for 2 h with 1% osmium tetroxide in 0.1 M sodium cacodylate–HCl buffer, and washed again with 0.1 M sodium cacodylate–HCl buffer. Cells were dehydrated through graded ethanol concentrations with final dehydration using propylene oxide. After dehydration, cells were embedded in epoxy resin. Samples were cut into ultrathin sections, double stained with uranium and lead citrate and observed under transmission electron microscopy operating at 75 kV (Hitachi H-7000, Tokyo, Japan).

**Immunofluorescence staining**

Cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, washed twice with PBS and permeabilized with 0.25% Triton X-100 in PBS for 10 min. Fixed cells were incubated with the blocking reagent (10% bovine serum albumin in PBS) for 30 min at room temperature. Cells were then washed and overlaid at 4°C with primary and Alexa-488-conjugated immunoglobulin G (IgG) for 30 min at room temperature, and cells were finally washed and mounted using the ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI). The primary antibodies were as follows: anti-c-Jun (Abcam, Cambridge, MA), anti-c-Fos (Cell Signaling Technology, Beverly, MA), anti-AIF (Abcam, anti-CHOP (Santa Cruz), anti-Bcl-2 (Santa Cruz), and anti-Bax (Santa Cruz). Fluorescent signals and bright-field images were captured using an inverted fluorescence microscope.

**Western blot analysis**

Cells were harvested, washed twice with ice-cold PBS and lysed in lysis buffer containing leupeptin and aprotinin inhibitor cocktail. The concentration of extracted proteins was determined using the Bio-Rad protein assay reagent. Protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After being transferred onto polyvinylidene difluoride membranes and blocked with...
Pardaxin, an antimicrobial peptide, targets cervical cancer cells

Dry skimmed milk, immunoblots were incubated with the primary antibody, followed by incubation with horseradish peroxidase-conjugated secondary antibodies and visualization by enhanced chemiluminescence detection. The primary antibodies were as follows: anti-RhoGDI (Abcam), anti-DJ-1 (Cell Signaling Technology), anti-Grp94 (Abcam), anti-calmodulin (Abcam), anti-β-actin (Millipore, Bedford, MA), anti-α-tubulin (Millipore), anti-PERK (Cell Signaling Technology), anti-calnexin (Cell Signaling Technology), anti-PDI (Cell Signaling Technology), anti-phospho-eIF2α (Cell Signaling Technology), anti-eIF2α (Cell Signaling Technology), anti-Nrf-2 (Santa Cruz Biotechnology), anti-phospho-JNK (Cell Signaling Technology), anti-JNK (Cell Signaling Technology), anti-phospho-c-Jun (Abcam), anti-c-Jun (Abcam), anti-phospho-c-Fos (Cell Signaling Technology), anti-AIF (Santa Cruz) and anti-cytochrome c (Santa Cruz).

Small interfering RNA transfection

ON-TARGETplus SMARTpool small interfering RNAs (siRNAs) targeting c-Jun were purchased from Dharmacon Research (Lafayette, CO). HeLa cells were seeded onto 12 well plates at 50–70% confluency and transfected with 50 pmol c-Jun siRNA or control siRNA using Lipofectamine 2000 (Invitrogen). After transfection for 36 h, cells were incubated in the presence or absence of pardaxin for 12 h, and cell viability or caspase-3/-7 activity was measured.

Statistical analysis

Data are shown as the mean ± standard deviation (SD). Statistical comparisons were performed using Student’s t-test, and differences between groups were considered significant at a P < 0.01. For cell viability, variance between dosages was analyzed by analysis of variance followed by Scheffe’s test, and variance between treated cells and the untreated control was analyzed by Student’s t-test.

Results

Pardaxin caused the death of cervical cancer cells

We observed that treatment with pardaxin caused dose- (0–20 µg/ml) and time-dependent (3–24h) death of HeLa, HeLa299, CaSk and C33A human cervical cancer cells. Pardaxin at 15 µg/ml significantly reduced viability of all four cell lines compared with the control within as little as 3 h (black bars) of exposure (Figure 1). Fifty percent inhibitory concentration (IC50) values were calculated by sigmoid-curve fitting (Supplementary Table 1, available at Carcinogenesis Online). The average IC50 values at 3–24 h posttreatment with pardaxin for HeLa, HeLa299, CaSk and C33A cells were 13.85 ± 1.24, 14.63 ± 1.37, 14.87 ± 0.42 and 14.14 ± 0.94 µg/ml, respectively. At 12 h, pardaxin exhibited the highest efficacy in HeLa cells (IC50 of 12.96 µg/ml). To confirm that the cytotoxic effect of pardaxin is limited to cervical cancer cells, we measured the cell viability of MRC-5 fibroblasts treated with 13 µg/ml pardaxin for 12 h. We report that pardaxin caused a decrease in the viability of HeLa, but not MRC-5 cells, suggesting selectivity of pardaxin for neoplastic cells (Supplementary Figure 2, available at Carcinogenesis Online).

Pardaxin-induced apoptosis in HeLa cells

To determine the type of cell death induced in HeLa cells by pardaxin, we measured the percentage of apoptotic HeLa cells using FITC-conjugated AV/PI and flow cytometry. Simultaneous staining of cells with FITC-AV (green fluorescence) and the non-vital dye PI (red

Fig. 1. Cytotoxic effects of pardaxin on human cervical carcinoma cell lines. Cells were treated with various concentrations of pardaxin for 3, 6, 12 and 24h. Results are presented as the percent cell viability as determined by an MTS assay. Cell viability is presented as the mean ± SD. Letters indicate differences in viability between pardaxin concentrations (analysis of variance, Scheffe’s test, P < 0.05). Asterisks indicate differences between treated and untreated cells (Student’s t-test, P < 0.01).
fluorescence) enabled the discrimination of intact (AV−/PI−), necrotic (AV−/PI+), early apoptotic (AV+/PI−) and late apoptotic cells (AV+/PI+). We found that pardaxin treatment significantly decreased intact cells (Supplementary Figure 3, available at Carcinogenesis Online, white bars), but did not affect the necrotic population (Supplementary Figure 3, available at Carcinogenesis Online, black bars). The decrease in intact cells reflected an increase in apoptotic cells. The percentage of apoptotic (both early and late) cells was markedly increased by pardaxin treatment, in a time-dependent manner. Total apoptotic cells significantly increased 3-fold from 3 to 24 h (Figure 2A). Pardaxin also increased the number of condensed nuclei from 0.71 to 33.42% (Figure 2B and C). These results indicate that pardaxin induces apoptotic cell death in HeLa cells.

Loss of MMP acts as a key regulator of the intrinsic apoptosis pathway (18), and as such, we examined whether pardaxin causes mitochondrial dysfunction in HeLa cells by measuring MMP using a JC-1 probe. MMP in HeLa cells significantly decreased after 3 h of treatment with 13 μg/ml pardaxin and continued to decrease over time (Figure 2D). When cells are induced to undergo apoptosis, AIF translocates from the mitochondrial intermembrane space to the cytosol and nuclei in a caspase-independent manner (19). In addition, loss of outer membrane integrity results in a massive release of Cyt

Fig. 2. Apoptotic effect of pardaxin on HeLa cells. (A) Pardaxin-treated and untreated HeLa cells were harvested after 3, 6, 12 or 24 h, and double stained with AV/PI, counted by flow cytometry and analyzed by FlowJo software. Percentages of early apoptotic (AV+, PI−) and late apoptotic cells (AV+, PI+) are shown. Data are presented as the mean ± SD (n = 3), *P < 0.01. (B) Morphology of HeLa cells treated with 13 μg/ml pardaxin for 12 h; nuclei were stained with Hoechst 33342. Arrows indicate apoptotic cells showing nuclear chromatin condensation. Scale bars indicate 50 μm. (C) The number of apoptotic cells was normalized to that of total cells. For each bar, the mean ± SD of three independent experiments is presented. *P < 0.01. (D) MMPs in untreated HeLa cells and HeLa cells treated with pardaxin for 3, 6, 12 or 24 h, as measured by JC-1 monomer/aggregate staining. Data are presented as the mean ± SD (*P < 0.01) of the ratio from three independent determinations. (E) Immunostaining with an antibody against AIF in HeLa cells treated with pardaxin. Cell nuclei were detected with the DNA-binding dye, DAPI. (F) Proteins were extracted from the cytosolic (C) and nuclear (Nu) fractions of cells treated or untreated with pardaxin for 12 h, and expression of AIF and cytochrome (Cyt) c were examined by western blot. Proteins were normalized to the level of β-actin in the same fraction, and the treated sample was subsequently normalized to the untreated control. (G) Pardaxin activates caspase-3/7 in HeLa cells. HeLa cells were treated with 13 μg/ml pardaxin for 12 h, stained with 5 μM CellEvent™ Caspase-3/7 Green detection reagent for 30 min at 37°C and immediately imaged. Caspase-3/7 activation was observed after pardaxin treatment. (H) Statistical analyses indicated a significant (*P < 0.01) increase in caspase-3/7 activity in pardaxin-treated cells. Data are presented as the mean ± SD (*P < 0.01) of three independent determinations.
Pardaxin, an antimicrobial peptide, targets cervical cancer cells

Pardaxin, an antimicrobial peptide, targets cervical cancer cells from mitochondria in response to caspase activation (18). To assess the release of Cyt c and AIF, we measured the amounts of these proteins in cytosolic and nuclear fractions. Furthermore, we examined AIF translocation by immunofluorescence staining. We found that pardaxin treatment resulted in AIF translocation to nuclei (Figure 2E and F) and increased Cyt c in the cytosol (Figure 2F), indicating that pardaxin triggers caspase-dependent and caspase-independent apoptosis.

A decrease in MMP suggested that caspase-dependent apoptosis may occur in response to pardaxin. Caspase-3/-7 activation was detected by cleavage of a DNA probe containing the sequence DEVD. Of HeLa cells treated with 13 μg/ml pardaxin, 52.74% exhibited positive fluorescent signals in nuclei, indicating caspase-3/-7 activation (Figure 2G and H).

Identification of differentially expressed proteins in pardaxin-treated HeLa cells

To characterize the effects of pardaxin on cervical cancer cells, we performed two-dimensional electrophoresis and MALDI-Q-TOF to identify differentially expressed proteins in pardaxin-treated HeLa cells (Figure 3A). Protein expression profiles were analyzed, and spots with >1.4-fold change in expression were identified (Supplementary Table 2, available at Carcinogenesis Online). Among the differentially expressed proteins, 14 proteins were decreased (Figure 3B) and 13 proteins were increased (Figure 3C). Two of the downregulated proteins (RhoGDI and protein DJ-1) and two of the upregulated ones (Grp94 and calmodulin) were randomly selected to validate the proteomic results by western blotting (Figure 3D).

Pardaxin-induced protein folding, free-radical scavenging and cytoskeleton-associated protein alterations in HeLa cells

To associate biological functions with pardaxin-modulated proteins, we applied the software tool, Ingenuity Pathway Analysis. The list of identified proteins and corresponding multiples of change were uploaded to the Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA) for core analysis. According to the biofunction analysis, the top networks with associated molecular and cellular functions were protein folding, free-radical scavenging and cytoskeletal rearrangement (Supplementary Figure 4A, available at Carcinogenesis Online). Protein-folding processes mainly take place in the ER. Disruption of protein folding in the ER triggers the UPR to recover protein-folding capacity. Failure to restore ER homeostasis causes prolonged UPR, which leads to apoptosis. Induction of endogenous free-radical scavengers or antioxidants is beneficial under oxidative stress, which may be caused by ROS accumulation and imbalances between the prooxidative and antioxidant systems. MAPKs, such as JNK (20) and p38MAPK, are preferentially activated in response to ROS production, linking oxidative stress to apoptosis signaling (21). Cytoskeletal modification, arrangement and distribution are highly modulated by activated caspases during apoptosis. The initial process of apoptosis in cells involves partial detachment from the extracellular matrix, and the formation of a rounded morphology

Fig. 3. Differentially expressed proteins in pardaxin-treated HeLa cells. (A) Two-dimensional electrophoretic map of pardaxin-treated and untreated HeLa cells. Arrows indicate spots of (B) downregulated or (C) upregulated proteins in pardaxin-treated (T) and pardaxin-untreated (C) HeLa cells. (D) Confirmation of changes in protein expression by western blot.
as a consequence of a loss of stress fibers and reorganization of actin into a peripheral ring (22). In summary, enrichment of all three of these distinct molecular and cellular functions suggests that pardaxin triggers apoptosis.

In addition, we found that upstream regulators of upregulated proteins, including AP-1, c-Fos and Nrf-2, were highly connected (Supplementary Figure 4B, available at Carcinogenesis Online), suggesting that cooperative transcriptional regulation may have taken place. The transcription factor AP-1 consists of c-Jun and c-Fos. Activation of AP-1 implies increased JNK activity, whereas activation of Nrf-2 implies the initiation of antioxidation production (20). Both JNK and antioxidants undergo molecular regulation in response to oxidative stress; this suggests that pardaxin may cause an accumulation of ROS that contributes to oxidative stress.

To dissect the function of pardaxin in HeLa cells, we analyzed upregulated and downregulated proteins by gene ontology annotation, and discovered that pardaxin resulted in increased levels of UPR proteins (Supplementary Figure 5A, available at Carcinogenesis Online) and decreased levels of proteins involved in oxidative stress (Supplementary Figure 5B, available at Carcinogenesis Online). Overall, our results indicate that pardaxin caused oxidative stress and activated the UPR, which resulted in cytoskeletal disassembly and apoptosis.

**Pardaxin caused cytoskeletal rearrangement**

We proceeded to confirm the effect of pardaxin on cytoskeletal rearrangement, a process which is related to the initiation of apoptosis. Actin filaments and microtubules are the two major components of the cytoskeleton and they control cell shape and movement. To monitor changes in the cytoskeleton, we examined the immunofluorescence of actin filaments and microtubules, using phalloidin and an α-tubule antibody, respectively. Interestingly, we observed that strong stress fiber bundles predominantly formed along cell edges in the presence of pardaxin, whereas untreated cells harbored thinner actin fibers both in central areas and at the edges of cells (Figure 4A). In addition, microtubules exhibited strong perinuclear staining and fragmentation in the cytoplasm after 12 h of pardaxin treatment (Figure 4A), implying that failure of cell division may have occurred.

**Pardaxin elevated UPR-regulated proteins**

To examine whether pardaxin triggered the UPR, we measured levels of UPR proteins and monitored the morphology of the ER, where proteins are folded. Transmission electron microscopy analysis demonstrated that the ER was dilated in response to 6 h of pardaxin treatment (Figure 4B), prior to the emergence of typical signs of apoptosis, such as nuclear condensation. Western blot analysis indicated that the UPR was activated within 3 h of pardaxin treatment, as demonstrated by increases in PERK, IRE1, and ATF6 proteins, and by eIF2α phosphorylation (Figure 4C). Furthermore, we found that pardaxin treatment induces translocation of two transcription factors, ATF6 (Figure 4D) and CHOP (Figure 4E), to nuclei; these transcription factors mediate apoptotic signaling from the UPR. These data indicate that pardaxin causes ER stress, which may account for the increase in apoptotic cells.

**Pardaxin repressed ROS-associated enzymes and stimulated ROS generation by HeLa cells**

Among the identified downregulated proteins, the gene ontology term ‘cellular response to hydrogen peroxide’ was enriched, indicating that this process may be closely associated with ROS signaling (Supplementary Figure 5B, available at Carcinogenesis Online). Hence, we reasoned that pardaxin treatment may cause aberrant ROS signaling in cervical cancer cells. We used DCFDA staining to measure intracellular ROS production by HeLa cells at the indicated time points in response to 13 μg/ml pardaxin treatment. As expected, pardaxin treatment enhanced ROS generation within 1 h compared with the untreated control (P < 0.01; Figure 4F). We next examined the response of an oxidative response transcription factor, Nrf-2, to pardaxin treatment. Western blot revealed that levels of Nrf-2 were increased by pardaxin (Figure 4G). These data imply that pardaxin may induce ROS accumulation through suppressing the expression of ROS-associated enzymes, thus causing oxidative stress.

**Pardaxin promoted AP-1 phosphorylation and translocation to nuclei**

AP-1 consists of two transcription factors, c-Jun and c-Fos, and regulates cell growth, proliferation, transformation and apoptosis. The Ingenuity Pathway Analysis transcription factor analysis predicted that c-Jun and c-Fos were costimulated (Supplementary Figure 4B, available at Carcinogenesis Online). To validate activation of AP-1, we examined the phosphorylation and nuclear localization of c-Jun and Nrf-2. In response to pardaxin treatment, both c-Jun and c-Fos were phosphorylated and translocated to nuclei (Figure 5A and Supplementary Figure 6, available at Carcinogenesis Online). Because c-Jun is phosphorylated by JNK, we next confirmed whether JNK was activated by phosphorylation under the same conditions. Indeed, JNK was phosphorylated after at least 3 h of pardaxin treatment (Figure 5B). Phosphorylation of JNK and c-Jun was dramatically enhanced, suggesting that pardaxin may induce apoptosis via stimulation of the JNK pathway.

**Pardaxin-triggered c-Jun activation was mediated via ROS production**

It was previously reported that increased ROS levels contribute to AP-1 activation (23); thus, we next examined whether pardaxin-induced c-Jun phosphorylation was mediated by ROS. To address this question, we pretreated cells with N-acetyl-l-cysteine (NAC), an ROS scavenger which helps protect against oxidative stress, before pardaxin treatment. Pardaxin-induced phosphorylation and translocation of c-Jun were significantly attenuated by treatment with 10 mM NAC for 1 h (Figure 5C), indicating that pardaxin activates AP-1 via ROS production.

**Pardaxin-induced cytotoxicity and caspase-3/-7 activation are mediated by c-Jun**

To determine whether c-Jun is involved in pardaxin-mediated cytotoxicity, we examined the viability of c-Jun knockdown cells in response to pardaxin. About 31% of control siRNA-treated HeLa cells survived pardaxin treatment (Figure 5D, white bars), whereas >65% of c-Jun-knockdown cells survived (Figure 5D, black bars), indicating that pardaxin-triggered apoptosis may be mediated by c-Jun. We also investigated the involvement of c-Jun in the pardaxin-mediated increase in caspase cascade signaling. Knockdown of c-Jun caused a decrease in caspase-3/-7 activation by pardaxin. Only 31% of pardaxin-treated cells exhibited caspase-3/-7-activation under c-Jun knockdown conditions compared with 55% of control siRNA cells (Figure 5E). These results indicate that c-Jun participates in pardaxin-mediated apoptosis.

**Discussion**

In this study, we have used a proteomic approach to uncover molecular explanations for pardaxin-induced apoptosis in cervical cancer HeLa cells (Supplementary Figure 1, available at Carcinogenesis Online). We identified that pardaxin-regulated proteins predominantly function in the UPR, oxidative stress and cytoskeletal distribution (Figure 4). Examination of signal transduction and cellular localization at the molecular level demonstrated that c-Jun activation contributes to pardaxin-activated caspase-3/-7 in apoptotic signaling (Figure 5).

In general, AMPs are peptides composed of cationic and amphipathic amino acids. AMPs can directly interact with anionic membranes, which most bacteria possess, by electrostatic binding, and subsequently disrupt the membrane structure or inhibit metabolism, thus killing the pathogen (2,24,25). Membrane lipid compositions greatly differ between bacteria and animal cells (26). The compositions of the inner and outer membrane...
Fig. 4. Validation that pardaxin affects the cellular processes identified by proteomic approaches. (A) HeLa cells were incubated in the presence or absence of 13 μg/ml pardaxin for 12h. Cells were counterstained with F-actin, α-tubulin and DAPI. Representative images were captured by fluorescence microscopy.
Fig. 5. Pardaxin-induced cell death and a caspase cascade were mediated by c-Jun activation in HeLa cells. (A) Immunostaining with a phospho-c-Jun antibody after pardaxin treatment of HeLa cells. Cell nuclei were detected with the DNA-binding dye, DAPI. (B) Expression of phospho-JNK and phospho-c-Jun proteins in HeLa cells treated or untreated with pardaxin was examined by western blot. Protein expression levels were quantified by densitometry and normalized to β-actin and total JNK or c-Jun. (C) Pretreatment with NAC abolished pardaxin-induced c-Jun phosphorylation in HeLa cells. HeLa cells were pretreated with 10 mM NAC for 1 h and treated with 13 μg/ml pardaxin for 12 h. Phosphorylation of c-Jun was analyzed by immunostaining. Cell nuclei were detected with the DNA-binding dye, DAPI. (D and E) siRNA knockdown of c-Jun alleviated pardaxin-induced cell death and caspase-3/7 activation. Cells were transfected with c-Jun siRNA or a scrambled control for 36 h and incubated in the presence and absence of pardaxin for a further 12 h. (D) Cell viability was assessed by an MTS assay. (E) Caspase-3/7 activity was assayed using the CellEvent™ Caspase-3/7 Green detection reagent, and image data were acquired by fluorescent microscopy from three random fields of view. Cell viability and caspase activity data were normalized to the untreated group with control siRNA. Asterisks indicate significant differences from control siRNA-harboring cells, at \( P < 0.001 \). Scale bars indicate 50 μm. (B) HeLa cells were incubated in the presence and absence of 13 μg/ml pardaxin for 3 h, then fixed, and processed for electron microscopy. Cells were imaged at ×20 000 magnification. Electron microscopic analysis of pardaxin-treated HeLa cells revealed a dilated ER. Black arrows indicate the ER. Scale bars indicate 500 nm. (C) Expression of ER stress-related proteins in pardaxin-treated and untreated HeLa cells was determined by western blot. Protein expression levels were quantified by densitometry and normalized to β-actin. The positions of phosphorylated (activated) PERK (PERK<sup>P</sup>) and hypophosphorylated (inactive) PERK (PERK<sup>β</sup>) and hypophosphorylated (inactive) PERK (PERK<sup>β</sup>) are indicated. (D) HeLa cells incubated in the presence and absence of 13 μg/ml pardaxin for 12 h were counterstained with ATF6 and DAPI. Representative images were captured by fluorescence microscopy. Scale bars indicate 50 μm. (E) HeLa cells incubated in the presence and absence of 13 μg/ml pardaxin for 12 h were counterstained with CHOP and DAPI. Representative images were captured by fluorescence microscopy. Scale bars indicate 50 μm. (F) HeLa cells were treated with 13 μg/ml pardaxin for 1 h. Intracellular ROS levels were analyzed by H<sub>2</sub>DCFDA staining and fluorescence-activated cell sorting analysis. All experiments were performed in triplicate. Histogram analysis of pardaxin-treated and control cells was carried out with WinMDI 2.9. Data are presented as the mean ± SD of the fluorescence intensity (MFI) from three independent experiments. An asterisk indicates a significant difference between control and pardaxin-treated cells, at \( P < 0.01 \). (G) Expression of the oxidative stress-response transcription factor Nrf-2 was normalized to β-actin and to an untreated sample, at 3, 6 and 12 h after pardaxin treatment.
Pardaxin, an antimicrobial peptide, targets cervical cancer cells

During transformation, the total amount of phospholipids increases (29). Tumor cells often reside in acidic environments, as their rapid duplication is supported by oxidative glycolysis, producing enormous levels of lactate (30). As a result, elevated phospholipids may alter the net charge of plasma membranes in acidic environments inhabited by tumor cells. Therefore, amphipathic AMPs may selectively bind to tumor cells, and subsequently penetrate or disrupt their plasma membranes (31). The different affinities of AMPs for tumor and normal cells result in target specificity for tumor tissues.

Pardaxin is a helical AMP with an overall positive charge, and 45% of its residues are hydrophobic. We demonstrated that pardaxin is cytotoxic to various cervical cancer cells (Figure 1), but has only small effects on normal cells (9,10), implying that pardaxin is an effective AMP for cancer targeting. The cytotoxicity of pardaxin was identified as arising from apoptotic cell death accompanied by mitochondrial dysfunction, including depletion of the MMP and release of AIF and Cyt c (Figure 2). Mitochondrial permeability is a key regulator of intrinsic apoptosis (32) and is under the control of antiaiaptotic proteins of the B-cell lymphoma 2 family. Once a death signal is transmitted, B-cell lymphoma 2 proteins are translocated to mitochondria and promote the release of Cyt c and other soluble proteins that reside in the mitochondrial intermembrane space (33). Our data indicate that pardaxin-induced release of Cyt c may account for activation of caspase-3/-7 (Figure 2E–H). Another intermembrane space protein, AIF, was reported to be sufficient to induce caspase-independent apoptosis (19). We found that AIF was released by mitochondria into the cytoplasm following pardaxin treatment (Figure 2E and F). In addition, pardaxin treatment resulted in chromatin condensation, as revealed by Hoechst nuclear staining (Figure 2B and C). RhoGDI is upregulated in multiple cancers. Silencing of RhoGDI sensitized cancer cells to chemotherapeutic agent-induced apoptosis (34), suggesting that RhoGDI plays a critical role in the development of drug resistance in cancer cells. Our proteomic analysis revealed that RhoGDI was decreased by pardaxin treatment (Figure 3D). Together, these results indicate that pardaxin-triggered apoptosis involves both caspase-dependent and caspase-independent pathways.

To investigate the upstream signals that activate apoptosis, we performed two-dimensional electrophoresis and network analysis, to identify differentially expressed proteins and reveal pardaxin-regulated biological functions (Supplementary Figure 4, available at Carcinogenesis Online). The top enriched function revealed by functional annotation analysis was protein folding, suggesting that the UPR may be induced by pardaxin (Supplementary Figure 5A, available at Carcinogenesis Online). Proteins involved in the UPR reside in the ER, where proteins are synthesized, folded, glycosylated and secreted (35). The UPR is activated in response to an accumulation of unfolded proteins in the ER, which exhibits a typical stressed morphology of an enlarged and dilated lumen (36,37). We showed that pardaxin treatment caused ER dilation, suggesting that pardaxin initiated the UPR (Figure 4B). Upregulation of UPR response proteins within 3 h of pardaxin treatment validated the proteomics data (Figure 4C). The UPR alleviates stress by upregulating protein folding and an ER-associated degradation pathway, and inhibiting protein synthesis (38). Western blot analysis revealed that pardaxin treatment increased proteins involved in protein folding, including Grp94, calnexin and PDI, and upregulated levels of PERK and CHOP and phosphorylation of eIF2α, indicating that protein translation was inhibited, consistent with the consequences of UPR activation.

The UPR is induced by various stimuli in cells (38). Oxidative stress is a product of various stimuli that disrupt protein folding and induce the UPR (39). A prolonged UPR eventually causes ER stress, accompanied by the release of calcium into the cytosol (38,40). A rise in the concentration of cytosolic calcium is modulated by a mitochondrial calcium unipporter into mitochondria, resulting in an increase in mitochondrial calcium (41,42). An overload of mitochondrial matrix calcium can lead to enhanced ROS generation, transition pore permeability and Cyt c release, resulting in apoptosis (42). ROS, on the other hand, activate the JNK pathway, which also leads to cell apoptosis (14,43). We found that NAC pretreatment restored c-Jun activation (Figure 5C), and c-Jun knockdown compromised pardaxin-caused cell death and caspase activation (Figure 5D and E), implying that...
JNK signaling is downstream of ROS generation and upstream of caspase activation and cell death.

In Figure 6, we present a model of pardaxin action. We hypothesize that pardaxin selectively targets cancer cells through electrostatic binding, which causes ROS production that results in oxidative stress and activation of the UPR; this in turn induces JNK/c-Jun and PERK/eIF2α/CHOP signal transduction (44,45), which leads to caspase- and AIF-dependent apoptotic events (20), such as loss of the MMP (36), a decrease in RhoGDI (postulated to generate the initial morphology of apoptosis by regulating actin polymerization (22,46)) and chromatin condensation (36). In summary, we have uncovered the molecular mechanisms underlying pardaxin-induced apoptosis. In addition, we have demonstrated the feasibility of combining proteomic approaches with network analysis to reveal the complex biological pathways and dynamic regulatory processes that modulate the effects of AMPs on cancer cells.

Supplementary material

Supplementary Tables 1 and 2 and Figures 1–6 can be found at http://carcin.oxfordjournals.org/

Funding

Development Program of Industrialization for Agricultural Biotechnology (to Dr C.-F.Hui and J.-Y.Chen); Taipei Medical University (TMU101-AE1-B38); Department of Health to Taipei Medical University—Center of Excellence for Cancer Research (TMU-CECR, DOH101-TD-C-111-008).

Acknowledgements

We thank Mr J.-F.Lee for help in performing experiments with AV/PI and MMP using the Cytomics FC 500 flow cytometer.

Conflict of Interest Statement: None declared.

References


Received August 14, 2012; revised April 4, 2013; accepted April 15, 2013.