Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/jab

Original Research Article

Phenazine-1-carboxylic acid-induced programmed cell death in human prostate cancer cells is mediated by reactive oxygen species generation and mitochondrial-related apoptotic pathway

Valliappan Karuppiah[a,b,*], *Kumarappan Alagappan*[c], *Kannan Sivakumar*[b], *Lakshmanan Kannan*[b]

[a] *Research Centre for Plant Growth and Development, School of Life Sciences, University of KwaZulu-Natal Pietermaritzburg, Private Bag X 01, Scottsville 3209, South Africa*

[b] *CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai 608502, Tamil Nadu, India*

[c] *Accuvis Bio, Abu Dhabi University Campus, Khalifa City B, Abu Dhabi, United Arab Emirates*

ARTICLE INFO

Article history:
Received 10 October 2015
Received in revised form
11 January 2016
Accepted 11 January 2016
Available online 23 January 2016

Keywords:
Phenazine-1-carboxylic acid
Streptomyces
Prostate cancer
Programmed cell death
Reactive oxygen species
Mitochondria

ABSTRACT

Phenazine-1-carboxylic acid has extensive pharmacological activity, including antibiotic and immunomodulatory, but the anticancer activity remains unknown. Treatment of prostate cancer cell line (DU145) with phenazine-1-carboxylic acid stimulated inhibition of cell proliferation in concentration- and time-dependent manner. Dual staining confirmed phenazine-1-carboxylic acid stimulated prostate cancer programmed cell death in time-dependent manner. To investigate the exact mechanism, phenazine-1-carboxylic acid-stimulated oxidative stress and mitochondrial-related apoptotic pathway in human prostate cancer cells were examined in this study. Phenazine-1-carboxylic acid increased the generation of reactive oxygen species (ROS) in prostate cancer cell lines, which triggered the pro-apoptotic JNK signaling. Phosphorylated JNK stimulated the depolarization of mitochondrial membrane potential ($\Delta\Psi m$) and downregulation of anti-apoptotic protein Bcl-2 related with the upregulation of pro-apoptotic protein Bax. Downregulation of anti-apoptotic Bcl-2 family protein in corresponding with loss of $\Delta\Psi m$, stimulate the increased production of cytochrome c and programmed cell death inducing factor (AIF) from mitochondria, and ultimately induced the caspase-dependent and caspase-independent programmed cell death. Altogether, the present study suggests that phenazine-1-carboxylic acid showed an antitumor activity in prostate cancer cells by reactive oxygen species production and mitochondrial-related apoptotic pathway. The results of the present study offered an insight into the prospective of phenazine-1-carboxylic acid for prostate cancer therapy.

* *Corresponding author at*: University of KwaZulu-Natal, Research Centre for Plant Growth and Development, School of Life Sciences, Private Bag X 01, Scottsville 3209, South Africa. Tel.: +27 0817601823.
E-mail address: vkactinobacteria@gmail.com (V. Karuppiah).

1214-021X/$ – see front matter
http://dx.doi.org/10.1016/j.jab.2016.01.003

Introduction

Prostate cancer (PCA) is a largely detected malignancy in the matured males and the second top cancer-associated deaths in the US (Jemal et al., 2005). It has also developed as a prevailing disease in several Asian countries (Pu et al., 2004). Researchers are now effectively trying to identify a novel natural product that can significantly inhibit the proliferation of tumor cell growth without disturbing normal cells (Krishnan et al., 2003). With regard to PCA chemoprevention, natural products had more interest with chemoprevention (Gupta, 2007; Singh et al., 2002).

Phenazines, a group of heterocyclic nitrogenous compounds isolated from the bacteria including actinobacteria and *Pseudomonas* (Giddens et al., 2002; Mavrodi et al., 2006). It has several biological activities such as antibiotic (Abken et al., 1998; Kerr et al., 1999), antimalarial (Hussain et al., 2011; Neves-Pinto et al., 2002) and anticancer (Gao et al., 2012; Rewcastle et al., 1987). Phenazines with different structural features have been isolated from actinobacteria due to their potential bioactivity and low toxicity to normal healthy cells (Cimmino et al., 2012, 2013). Since phenazines are small molecules, they can easily invade into the tissues and/or organs with multiple targets (Gao et al., 2012, 2013). Previous studies were proved that phenazines generate high level of reactive oxygen species (ROS) and selectively kill the cancer cells (Cimmino et al., 2013; Pierson and Pierson, 2010). For instance, Sorensen et al. (1983) showed that ROS produced by pyocyanin inhibited the growth of human lymphocytes. In general, each cell contains balance level of ROS and antioxidants (Gao et al., 2012, 2013). Once this crucial balance is disturbed by excessive ROS generation and/or antioxidant reduction, oxidative stress can come to exist (Pierson and Pierson, 2010). The excessive generation of ROS could damage the cells by modifying intracellular or extracellular macromolecules and signaling pathways (Pierson and Pierson, 2010). Simultaneously, ROS could not affect the normal cells due to their better tolerance to oxidative stress and regular metabolism (Abdelfattah et al., 2011). Thus, ROS have a significant role in anticancer drug discovery.

Phenazine-1-carboxylic acid is considered as a metabolic precursor for other phenazine derivatives (Pierson and Pierson, 2010). It has been reported that phenazine-1-carboxylic acid exhibit antibiotic activity, but the anticancer activity of this compound has not been studied thus far (Pierson and Pierson, 2010). In the present study, we first detected that phenazine-1-carboxylic acid inhibited the proliferation of SKOV3, Hela, Hep2, Ht1080 and DU145 cell lines. To further characterize the anticancer mechanism of that phenazine-1-carboxylic acid in DU145 cell, we studied the intensity of intracellular ROS and mitochondrial membrane potential, which are strongly related with the programmed cell death signal transduction pathway and effect the chemosensitivity of tumor cells to anticancer agents. Our results revealed that phenazine-1-carboxylic acid is a potential candidate for prostate cancer treatment.

Materials and methods

Isolation and identification of the actinobacterial strain MNMon3

The actinobacterial strain MNMon3 was isolated from the coral reef sediment of the Manoli Island, Gulf of Mannar Biosphere Reserve, India using dilution plate technique in glucose yeast extract and malt extract agar and maintained in −80 °C. The strain MNMon3 was identified based on chemotaxonomy, micromorphology and 16S rRNA gene sequencing. Genomic DNA was extracted from the culture of MNMon3 grown on ISP2 broth as described by Sun et al. (2010). 16S rRNA gene of MNMon3 was amplified using the method of Karuppiah et al. (2011) and sequenced in Macrogen, Korea. The sequence was compared with the public databases using NCBI-BLAST program. Phylogenetic tree was constructed with the MEGA version 5 with the method of maximum likelihood and bootstrap analysis with 1000 resamplings. The sequence was deposited under the GenBank accession number KM079126.

Extraction and characterization of phenazines

Actinobacterial strain MNMon3 was inoculated in 1-L Erlenmeyer flasks containing 400 mL of the ISP2 medium and incubated at 28 °C on rotary shakers for 5 days. After fermentation the culture was filtered and extracted with equal volumes of ethyl acetate for three times. The crude extract was partitioned in silica gel (60–120 mesh) column chromatography using a step wise gradient from 100% dichloromethane to 100% methanol. Based on TLC analyses, fraction 4 was applied repeatedly to semipreparative RP-C18 (X Aqua-C18 5 μm, 10 × 250 mm) HPLC (Agilent Technologies, USA) and separated with a linear gradient from 20% to 100% methanol at a flow rate of 3 mL/min for 40 min to get phenazine-1-carboxylic acid from MNMon3 (tR = 30.7 min).

The methanol solution of phenazine-1-carboxylic acid was detected on an ultra-performance liquid and quadrupole time of flight mass spectroscopy (UPLC-QTOF-MS Premier, Waters Corporation, USA). The phenazine-1-carboxylic acid were separated on a C18 RP-column (ACQUITY BEH-C18 1.7 μm, 2.1 × 100 mm, Waters Co.) with the linear gradient elution from 30% H_2O to 100% acetonitrile. Total ion chromatography (TIC) and mass spectrum of selected ion were acquired in positive electrospray ionization mass spectrum (ESI-MS) mode. Dereplication of substance was realized by comparison of MS data obtained by UPLC–MS analyses with the Antibase (Laatsch, 2007) and the Chapman & Hall/CRC Dictionary of Natural Products databases (Buckingham, 2008).

Chemical reagents and antibodies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RIPA lysis buffer, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and rhodamine 123 (Rh-123) were purchased from Thermo fisher Scientific (Massachusetts, USA) and Sigma-Aldrich Inc. (St. Louis, MO, USA). Caspase-3 Colorimetric Assay

Kit was purchased from Abcam, Cambridge, UK. High Pure Mitochondria Isolation Kit was purchased from Abcam, Cambridge, UK. Antibodies against Bax, Bcl-2, phosphorylated JNK, JNK, cytochrome c, programmed cell death inducing factor (AIF), CoxIV, and β-actin were obtained from Abcam, Cambridge, UK. The secondary antibodies, namely, horseradish peroxidase (HRP)-labeled anti-mouse IgG antibody and HRP-labeled anti-rabbit IgG antibody were purchased from Abcam, Cambridge, UK.

Cell cultures

Cancer cell lines of SKOV3, Hela, Hep2, Ht1080 and DU145 were purchased from ATCC (Manassas, VA, USA). DU145, Hela, Hep2 and Ht1080 cells were grown in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin/amphotericin B, SKOV3 cells were grown in RPMI 1640 and the other cell lines were grown in DMEM-F12, all supplemented with 10% fetal bovine serum and penicillin/streptomycin/amphotericin B.

Cell viability assay

The cells were cultured at a density of 4×10^4 cells per well in 96-well plates and treated with 10, 25, 50, 75, 100 μM of phenazine-1-carboxylic acid for 24 and 48 h. For evaluating cell viability, MTT assay was carried out as described by Karuppiah et al. (2013).

Measurement of intracellular ROS

ROS generation was examined with DCFH-DA, a cell-permeable dye. DCFH-DA enters into the cells and oxidized by intracellular H_2O_2 or low-molecular-weight peroxides to fluorescent DCF (Kuo et al., 2007). Subsequent treatment with phenazine-1-carboxylic acid, cells were incubated with 50 μM DCFH-DA dye in medium for 30 min in dark and washed with PBS. Fluorescent signals were measured with excitation and emission filters at 485 ± 10 and 530 ± 12.5 nm, respectively. Fluorescence microscopic images were taken using a blue filter (450–490 nm) on an epi-fluorescence microscope.

Measurement of mitochondrial membrane potential

After incubation of DU145 cells with phenazine-1-carboxylic acid for 24 and 48 h, fluorescent dye Rh-123 (10 μM/mL), a lipophilic cationic dye, highly specific for mitochondria was added to the cells and kept in an incubator for 30 min. Then the cells were rinsed with PBS and observed under a fluorescence microscope with a blue filter. Polarized mitochondria were distinct by orange–red fluorescence, and depolarized mitochondria, by green fluorescence (Nakano et al., 2006).

Caspase-3 activity assay

After treated with phenazine-1-carboxylic acid for 24 or 48 h, the caspase-3 activity in DU145 cell lysates were described using Colorimetric Assay Kit (Abcam, Cambridge, UK) according to the manufacturer's instruction.

Isolation of mitochondria

After treated with phenazine-1-carboxylic acid for 24 or 48 h, the mitochondria of DU145 cells were isolated by the High Pure Mitochondria Isolation Kit (Abcam, Cambridge, UK) according to the manufacture instructions.

Immunoblotting analysis

The immunoblotting studies were carried out as described by Chang et al. (2006). In concise, the cells were rinsed with PBS and lysed with the lysis buffer. The protein lystes (40 μM) were subjected to 10% SDS PAGE and transferred to nitrocellulose membrane. After blocking with 10% BSA, membranes were incubated with different primary antibodies, followed by incubated with secondary antibodies. Signals were observed by enhanced chemiluminescence kit (Bio-Rad, California, USA).

Statistics

Data are given as means ± SED and comparisons were made using Student's *t* test. A probability of 0.05 or less was taken statistically significant.

Results

Isolation and identification of marine actinobacteria

A marine actinobacterium MNMon8 strain was isolated from the coral reef sediments of Manoli island, Gulf of Mannar Biosphere Reserve, India. The strain MNMon3 showed the presence of LL-diaminopimelic acid and glycine with no distinguished sugar pattern, which reveals that this strain belonged to the cell wall chemotype-I. In general aerial mycelium and substrate mycelium was white and brown, respectively. Spore chains and surface were rectiflexibiles and smooth, respectively. Evaluation of the 16S rRNA gene sequence of the strain MNMon3 with previously deposited sequences in the GenBank (NCBI) indicated that this organism is belongs to the genus *Streptomyces*. The rooted phylogenetic tree showed that the strain MNMon3 was interlinked with *Streptomyces cebimarensis* DSM 41798T (AJ560629) (Fig. 1). Based on the morphology, chemotaxonomy and 16S rRNA gene sequencing, the strain MNMon3 was identified as the *Streptomyces* sp. The sequence was submitted to Gene Bank in NCBI with the accession number KM079126.

Extraction, purification and characterization of phenazine derivative

Based on purification of extract through silica gel column and preparative HPLC, the fermentation culture (10 L) of *Streptomyces* sp. MNMon3 yielded 7 mg of yellow powdered compound. The purity of the compound was confirmed in analytical HPLC with retention time of 30.7 min. The ESI-MS spectrum showed the molecular ion peak at m z-1 225.21 (M+H) (Fig. 2A). On the basis of spectral data, the comparative results of Antibase (Laatsch, 2007) and the Chapman & Hall/CRC Dictionary of

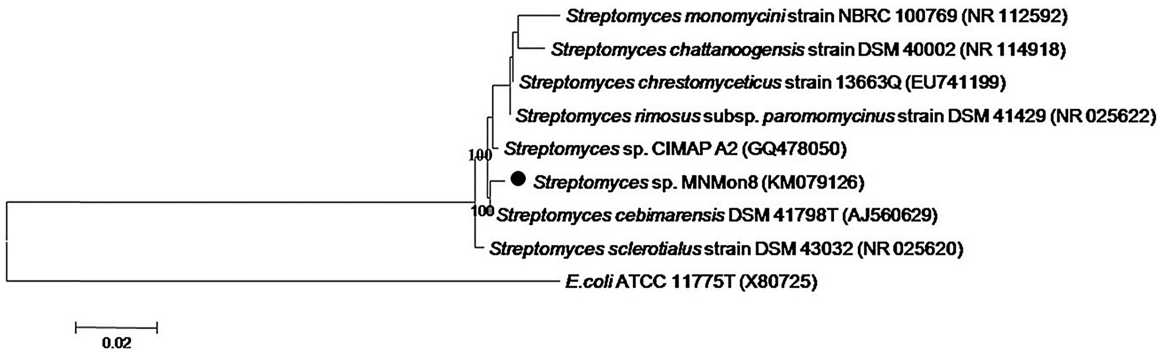

Fig. 1 – Phylogenetic tree based on 16S rRNA gene sequences showing the positions of MNMon3 and related strains. Bar 0.02 substitutions per nucleotide position.

Natural Products databases (Buckingham, 2008), the compound was characterized as phenazine-1-carboxylic acid (Fig. 2B).

Effects of phenazine-1-carboxylic acid on cell viability

To explore the potential inhibition of cancer cells by phenazine-1-carboxylic acid, MTT assay was performed against five different malignant cell lines including SKOV3, Hela, Hep2, Ht1080 and DU145. After treating with phenazine-1-carboxylic acid (ranging from 10 to 100 μM) for 24 and 48 h, DU145, a prostate cancer line, showed the most prominent anti-proliferation activity in concentration- and time-dependent manner with an IC50 value of 19.5 and 12.5 μM/mL for 24 and 48 h respectively (Fig. 3A and B).

Phenazine-1-carboxylic acid induced DU145 programmed cell death

To investigate the underling apoptotic mechanism of the phenazine-1-carboxylic acid on DU145 cells, 15 μM of phenazine-1-carboxylic acid was selected for further studies. As shown in Fig. 4A and B, DU145 cells showed an alteration in the morphology after treatment with phenazine-1-carboxylic acid at 15 μM. Fluorescent DNA binding dyes including acridine orange and ethidium bromide were used to differentiate programmed cell death and necrosis of cells. Normal cells utilize acridine orange and fluoresces green, while nonviable apoptotic cells utilize both dyes and fluoresce orange. As observed in florescent microscope, DU145 cells in control reflect green, while cells treated with phenazine-1-carboxylic acid showed orange, which indicate programmed cell death. The apoptotic rate was calculated by analysing more than 400 cells in 12 different fields of each well. As illustrated in Fig. 4B, in DU145cells, programmed cell death was increased from 2% in control cells to 48 and 56% in phenazine-1-carboxylic acid treated cells after 24 and 48 h respectively.

Phenazine-1-carboxylic acid influenced the intensity of ROS and mitochondrial membrane potential (ΔΨm) in cells

To examine the ROS generation by phenazine-1-carboxylic acid, DU145 treated cells were incubated with DCFH-DA. As illustrated in Fig. 5A and B, DU145 cells treated with phenazine-1-carboxylic acid showed comparatively higher ROS generation than control cells.

The usage of Rh 123 is a valuable method for determining mitochondrial function. Rh 123 is a lipophilic cationic dye and highly specific for mitochondrial membrane. The Rh 123 transform fluorescence emission from red to green as ΔΨm reduces. Cells with intact membrane encourage the development of red fluorescence, and cells with low membrane potential show green fluorescence. As illustrated in Fig. 6, Rh 123 was accumulated in control cells, representing an adequate membrane potential, whereas Rh 123 was defectively accumulated in phenazine-1-carboxylic acid-treated cells, signifying the distraction of ΔΨm.

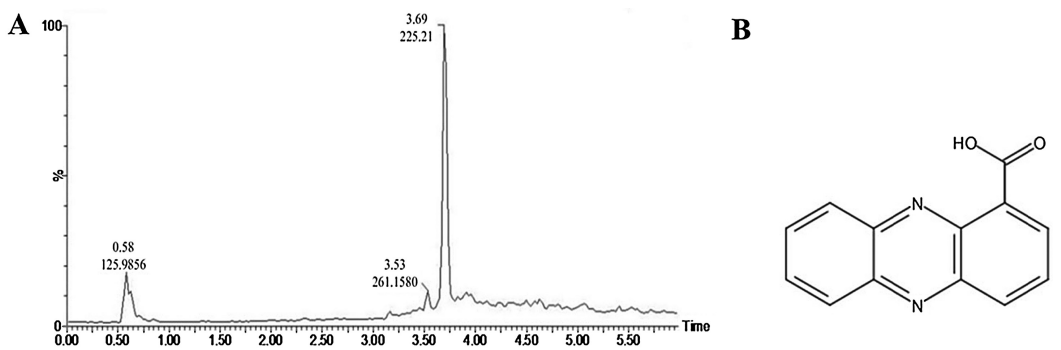

Fig. 2 – (A) MS-chromatogram of purified phenazine-1-carboxylic acid from *Streptomyces* sp. MNmon3, (B) chemical structure of phenazine-1-carboxylic acid.

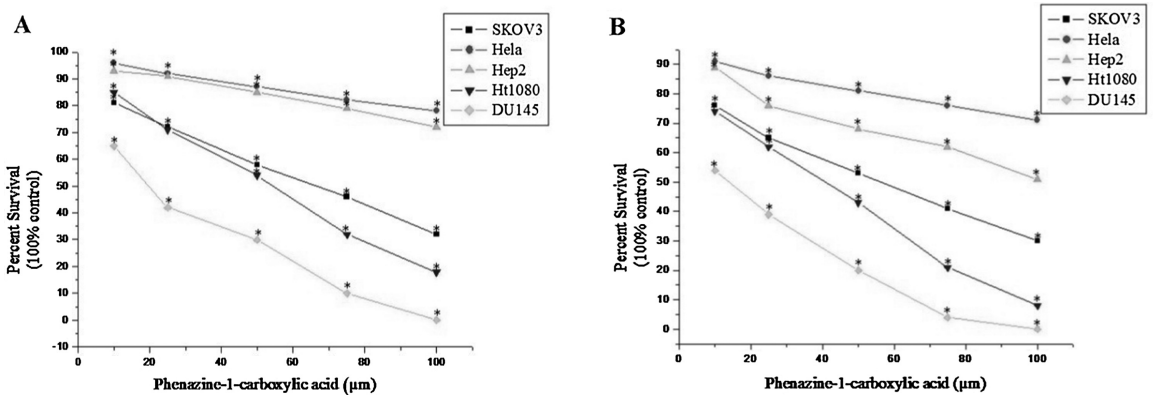

Fig. 3 – Phenazine-1-carboxylic acid inhibits cell viability of SKOV3, Hela, Hep2, Ht1080 and DU145 cells. The effect of phenazine-1-carboxylic acid on cell viability was estimated by MTT assay. (A) SKOV3, Hela, Hep2, Ht1080 and DU145 cells were treated with phenazine-1-carboxylic acid for 24 h. (B) SKOV3, Hela, Hep2, Ht1080 and DU145 cells were treated with phenazine-1-carboxylic acid for 48 h. Phenazine-1-carboxylic acid efficiently inhibited cell viability of all cell lines in a dose- and time-dependent manner. The experiments were carried out in triplicate and data showed as the mean ± SEM. $^{*}p \leq 0.05$, phenazine-1-carboxylic acid-treated group compared with control group.

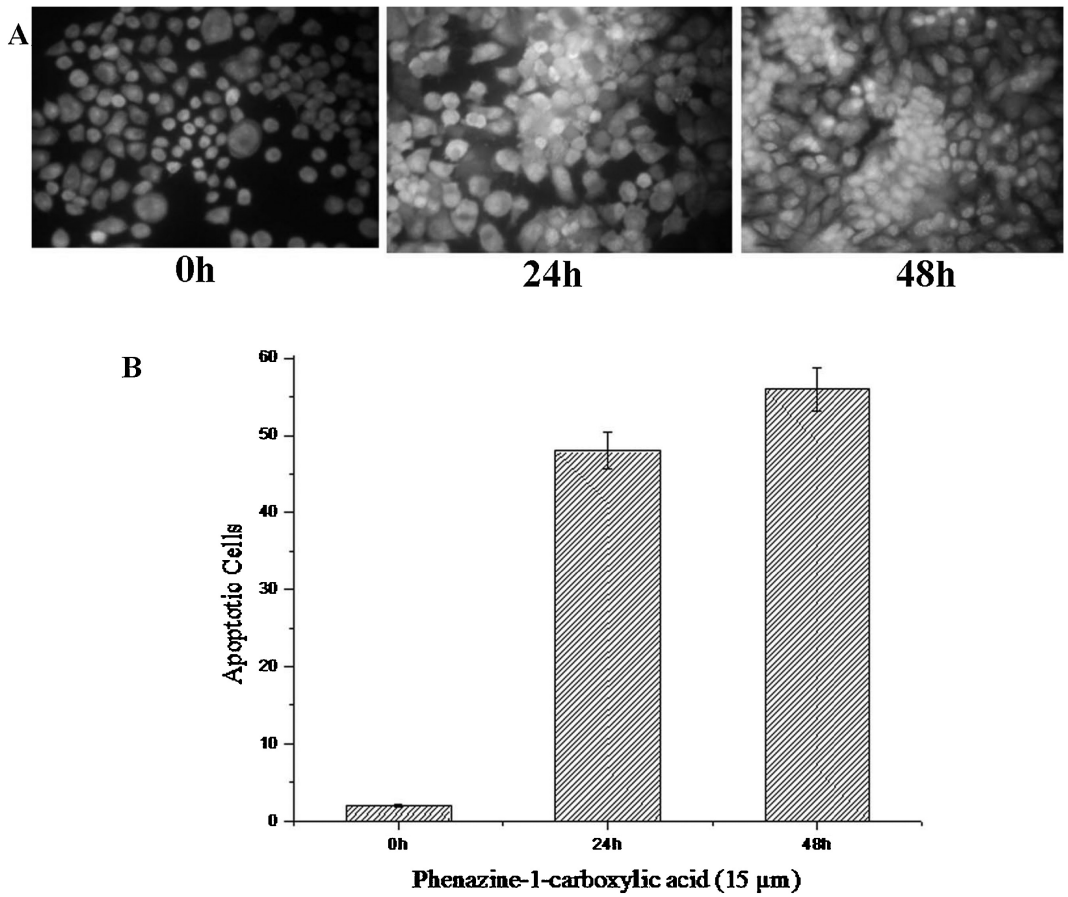

Fig. 4 – Phenazine-1-carboxylic acid induced DU145 programmed cell death. (A) Fluorescence microscopy images of DU145-stained cells showing the emergence of apoptotic morphology in phenazine-1-carboxylic acid-treated prostate cancer cells. In contrast to the control group, the group treated with phenazine-1-carboxylic acid showed characteristic apoptotic phenotypes. (B) The apoptotic rate was calculated by analysing at least 400 cells from 12 different fields per well. The data presented as mean ± SEM of four individual experiments. $^{*}p \leq 0.05$, Phenazine-1-carboxylic acid-treated group compared with control group.

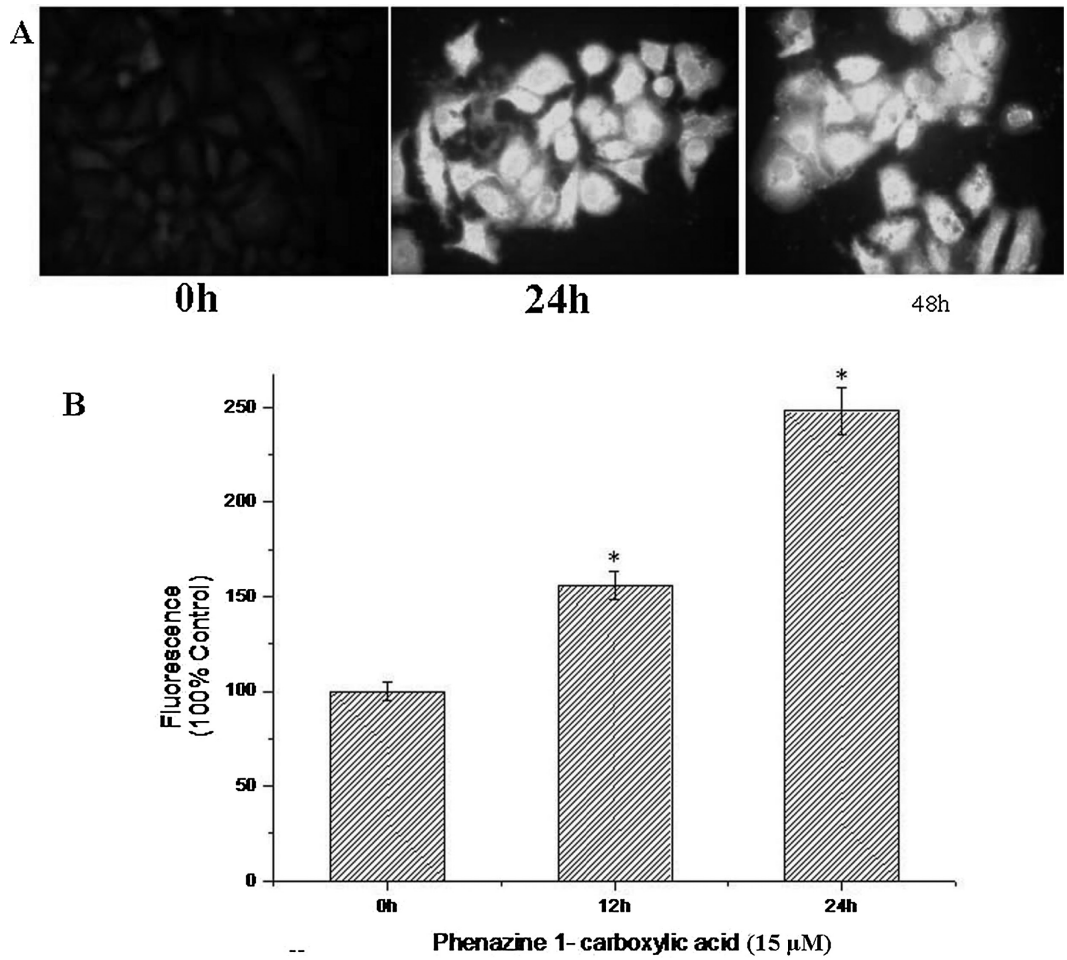

Fig. 5 – Phenazine-1-carboxylic acid induced generation of ROS in DU145 cells. Compared with control group, treatment with phenazine-1-carboxylic acid 15 μM for 12 and 24 h markedly increased ROS generation. The data showed as mean ± SEM of four individual experiments. $^{*}p \leq 0.05$, phenazine-1-carboxylic acid-treated group compared with control group.

Phenazine-1-carboxylic acid induced activation of JNK in DU145 cells

The elevation of ROS within cells could activate the JNK signaling (Nakano et al., 2006). Hence, phenazine-1-carboxylic acid mediated activation of JNK, and phosphorylation status of JNK in DU145 cells were determined by westernblotting. As shown in Fig. 7A and B, phenazine-1-carboxylic acid treated DU145 cells considerably raised the phosphorylation of JNK in DU145cells.

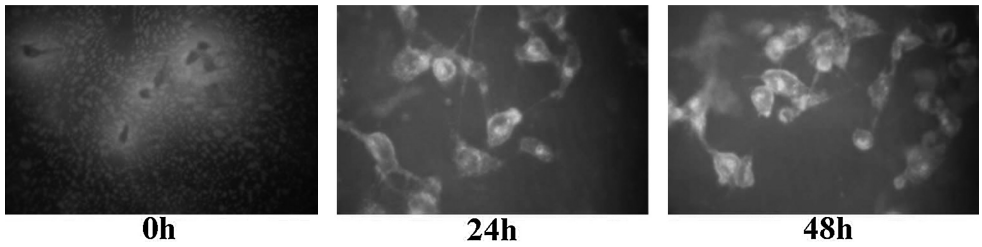

Fig. 6 – Phenazine-1-carboxylic acid induced loss of mitochondrial membrane potential in DU145 cells. MCF-7 andMDA-MB-231 cells were stained with a mitochondria-specific dye, rhodamine 123, and its florescence was monitored using fluorescence microscopy. rhodamine 123 was accumulated in control cells, where it displayed a bright-red fluorescence indicating a high potential. In contrast, rhodamine 123 was poorly accumulated in phenazine-1-carboxylic acid-treated cells, which displayed green fluorescence, indicating the loss of the mitochondrial membrane potential.

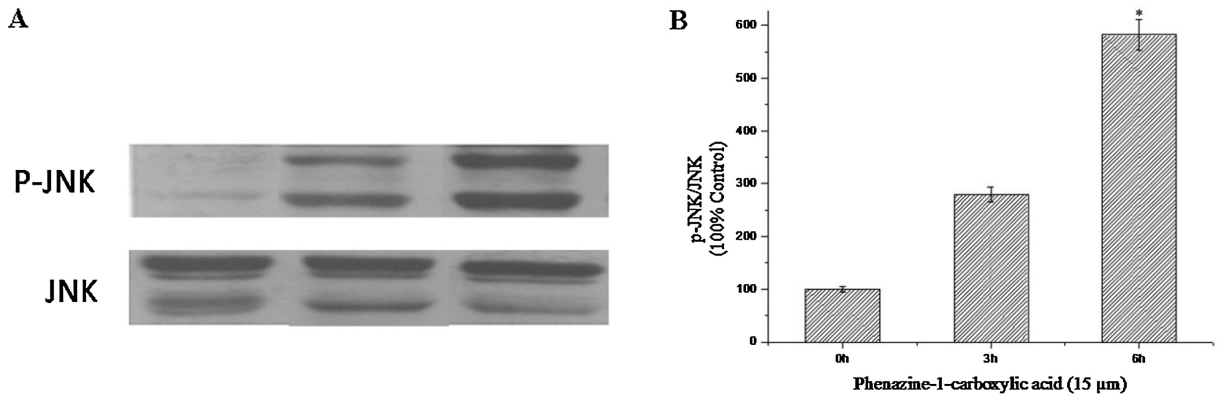

Fig. 7 – Phenazine-1-carboxylic acid induced activation of JNK in DU145 cells. Cells were incubated with phenazine-1-carboxylic acid for 3 and 6 h, and then, cell proteins were acquired and detected with antiphospho-JNK anti-JNK antibodies by Western blot. Phenazine-1-carboxylic acid extensively increased the phosphorylation of JNK. $^{*}p \leq 0.05$, phenazine-1-carboxylic acid-treated group compared with control group.

Modification of anti-apoptotic and pro-apoptotic protein expression level in DU145 cells by phenazine-1-carboxylic acid

The level of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax were determined to characterize the mitochondrial mediated programmed cell death induced phenazine-1-carboxylic acid. The results of western blot analysis (Fig. 8) showed the decreased and incresed level of Bcl-2 and Bax, respectively. It implied that programmed cell death of DU145 cells by phenazine-1-carboxylic acid was mediated by alteration in the level of pro- and anti-apoptotic proteins of the Bcl-2 family.

Phenazine-1-carboxylic acid induced releasing of pro-apoptotic factors from mitochondria of DU145 cells

ROS could induce programmed cell death through the mitochondrial pathway by increasing loss of $\Delta\Psi m$ and liberate mitochondrial pro-apoptotic proteins including cytochrome c and programmed cell death inducing factor (AIF) into cytosol (Chandra et al., 2000). To verify the role of mitochondrial pathway involved in the programmed cell death of DU145 cells induced by phenazine-1-carboxylic acid, we analyzed the level of mitochondrial AIF and cytochrome c in DU145 cells. As illustrated in Fig. 9, level of cytochrome c and AIF in

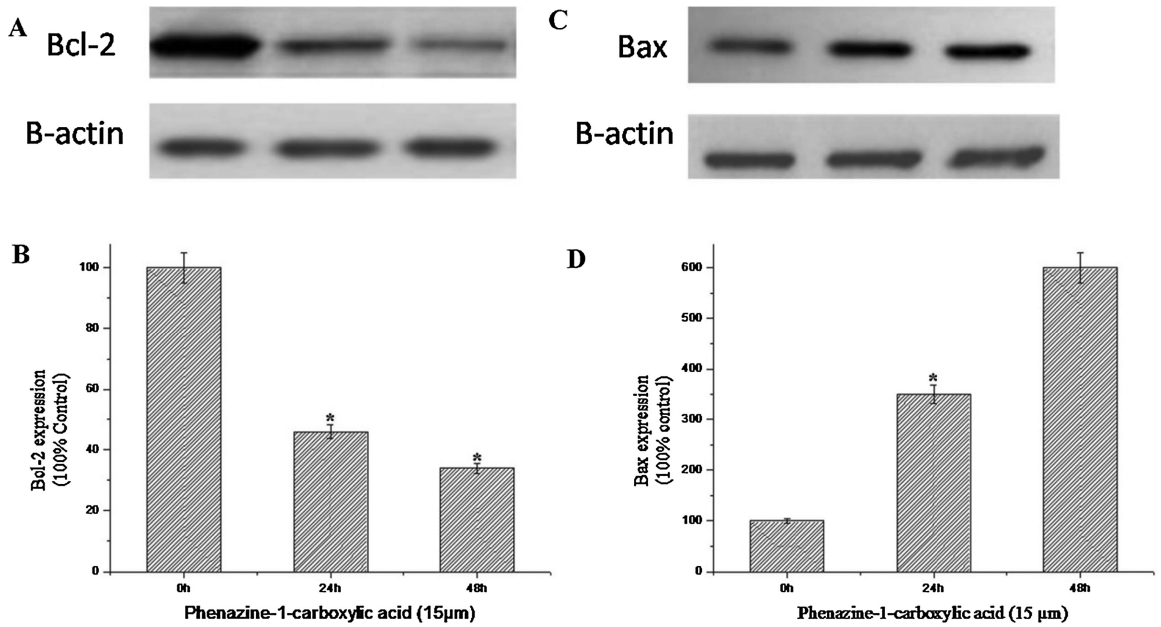

Fig. 8 – Phenazine-1-carboxylic acid induced alteration of anti-apoptotic and pro-apoptotic protein expression level in DU145 cells. Cells were treated with phenazine-1-carboxylic acid for 24 and 48 h, and then, cell proteins were acquired and detected with anti-Bcl-2 and anti-Bax antibodies by Western blot. Treatment of DU145 cells with phenazine-1-carboxylic acid resulted in a decreased expression of Bcl-2 (A and B) and an increased expression of Bax (C and D). $^{*}p \leq 0.05$, phenazine-1-carboxylic acid-treated group compared with control group.

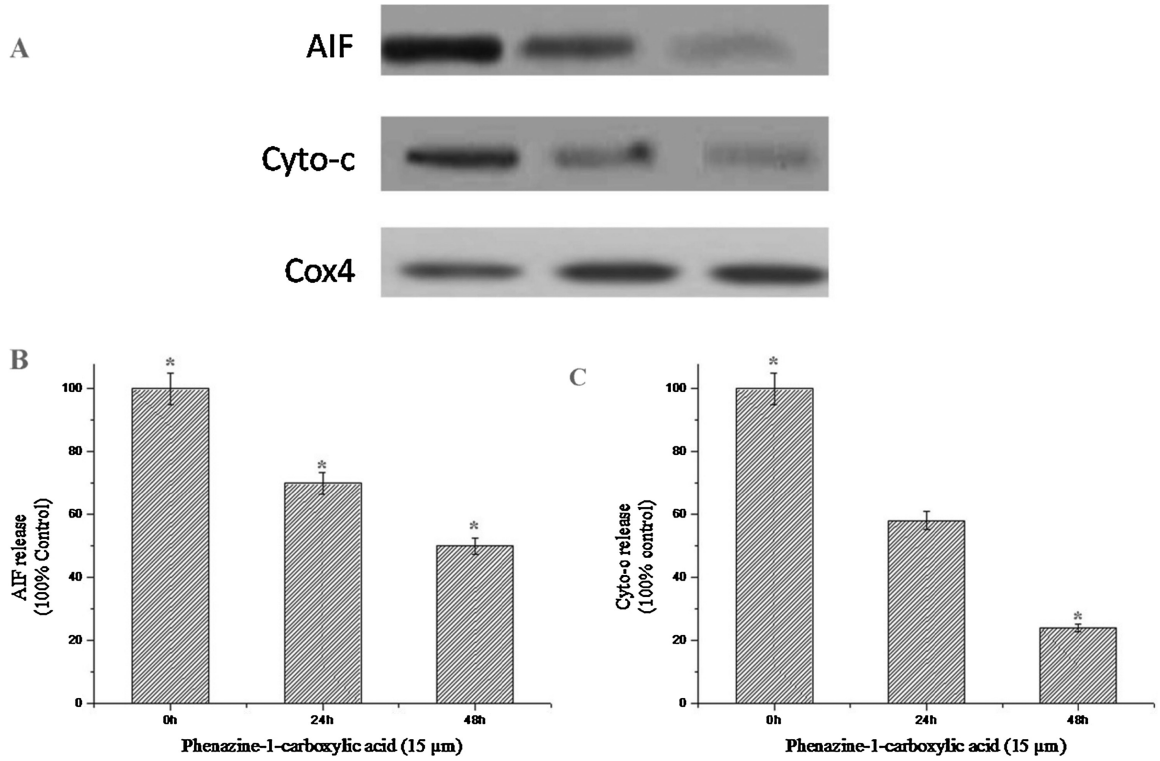

Fig. 9 – Phenazine-1-carboxylic acid induced releasing of cytochrome c and AIF from mitochondria in DU145 cells. Cells were treated with phenazine-1-carboxylic acid for 24 and 48 h, then, mitochondrial extracts were acquired and detected with anti-cytochrome c (A) or anti-AIF (B) antibody by Western blot. Treatment of DU145 cells with phenazine-1-carboxylic acid resulted in a marked liberation of cytochrome c and AIF from mitochondria. $^{*}p \leq 0.05$, Phenazine-1-carboxylic acid-treated group compared with control group.

mitochondria was declined in DU145 cells treated with 15 μM of phenazine-1-carboxylic acid for 24 and 48 h. It suggests that phenazine-1-carboxylic acid improved the loss of cytochrome c and AIF from mitochondria and consequently incresed the level of cytochrome c and AIF in cytosol, which ultimately activate the caspase-dependent and caspase-independent programmed cell death pathway, respectively.

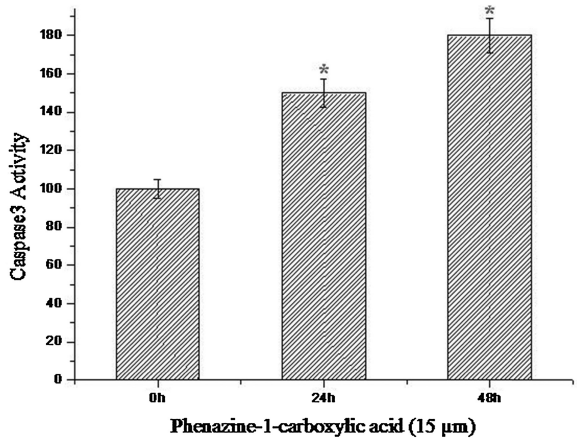

Fig. 10 – Phenazine-1-carboxylic acid induced activation of caspase-3 in both DU145 cells. Cells were treated with phenazine-1-carboxylic acid for 24 and 48 h, then, the cell lysate were analyzed by Caspase-3 Colorimetric Assay Kit. Treatment of DU145 cells with phenazine-1-carboxylic acid resulted in activation of caspase-3. $^{*}p \leq 0.05$, phenazine-1-carboxylic acid-treated group compared with control group.

Phenazine-1-carboxylic acid induced activation of caspase-3 in DU145 cells

Activation of caspase-3 indicates the unalterable or completing stage of programmed cell death. To further study the role of caspase-3 in DU145 cells programmed cell death by phenazine-1-carboxylic acid, the level of caspase-3 was identified as illustrated in Fig. 10. It showed that caspase-3 was notably stimulated after phenazine-1-carboxylic acid treatment for 24 and 48 h. DU145 cells treated with 15 μM of phenazine-1-carboxylic acid for 24 and 48 h significantly increased the caspase-3 activity by 150 and 180% above the control cells, respectively.

Discussion

Phenazine-1-carboxylic acid is one of the major antibiotic compounds isolated from *Pseudomonas* and *Streptomyces*. *Streptomyces* are ubiquitous in marine environment and well known for their valuable synthesis of secondary metabolites with pharmic activity such as antibiotics, anticancer, and immunomodulators (Karuppiah et al., 2015). In the present

study, phenazine-1-carboxylic acid was isolated from the *Streptomyces* sp. MNMon3, which was related to the type strain *Streptomyces cebimarensis* DSM 41798T (AJ560629). Previous studies have reported that phenazine-1-carboxylic acid have the antimicrobial activity and several biological effects in the human airway epithelial cells (Rane et al., 2007). However, the anticancer activity of phenazine-1-carboxylic acid has not been studied yet. To our knowledge, this is the first report on the anticancer activity of phenazine-1-carboxylic acid against prostate cancer.

In the present study, molecular mechanisms of phenazine-1-carboxylic acid induced DU145 cells programmed cell death have been studied. As shown in Fig. 3, phenazine-1-carboxylic acid inhibitrd the proliferation of DU145 cells in dose- and time-dependent manner. Additionally, phenazine-1-carboxylic acid induced the programmed cell death in time-dependent manners.

Since stimulation of programmed cell death shows to be the most significant property for potential anticancer drug, the morphological changes was characterised. The programmed cell death induced by phenazine-1-carboxylic acid was identified by the symptoms such as cell shrinkage, chromatin condensation, and fragmentation. These interpretations recommend that phenazine-1-carboxylic acid may be an efficient chemotherapeutic drug against prostate cancer. Additional studies were executed to explain the mechanism behind the inhibition of cell proliferation through programmed cell death.

ROS are believed to involve in a several cellular functions, such as cell proliferation, differentiation, and programmed cell death (Liou and Storz, 2010). Increase in ROS level generation leads to potentially cytotoxic ''oxidative stress''. ROS and oxidative stress are identified as programmed cell death inducers and modulators (Trachootham et al., 2008) and they have been revealed to play vital role in chemotherapy (Liou and Storz, 2010). Mitochondrial electron transport chain is a most important internal resource of ROS, of which NADH is the important factor. The extreme generation of ROS from mitochondria cause dysfunction of electron transport chain. Fig. 5 illustrated that phenazine-1-carboxylic acid triggered programmed cell death associated with excessive ROS generation in DU145cells, demonstrating that phenazine-1-carboxylic acid might exhibit anticancer activity by excessive generation of ROS.

The ROS generated by mitochondria within cells play a vital role in the programmed cell death; recently it becomes important target for cancer drug discovery (Nogueira and Hay, 2013). In recent years, several studies have been reported that mitochondria are the major place of action for JNK in programmed cell death. Increase in ROS within cells inactivates MAPK phosphatases (MKPs) by oxidation of their catalytic cysteine, which directs to continuous activation of JNK (Son et al., 2011). JNK acts as an essential pro-apoptotic system in mitochondria mediated programmed cell death in cells. Initially, phosphorylated JNK shift from cytosol to mitochondria and stimulates mitochondrial membrane depolarization. Followed by, phosphorylated JNK performs its pro-apoptosis by regulating Bcl-2 family members to induce the cell death. In the present study, DU145 cells treated with Phenazine-1-carboxylic acid showed a significant activation of JNK, which leads to the loss of ΔΨm and regulation of Bcl-2 family members. After treated with phenazine-1-carboxylic acid in DU145 cells, down regulation of Bcl-2 expression was identified, and this downregulation was connected with the upregulation of Bax. This results revealed that phenazine-1-carboxylic acid might induce DU145 programmed cell death by ROS-mediated activation of JNK.

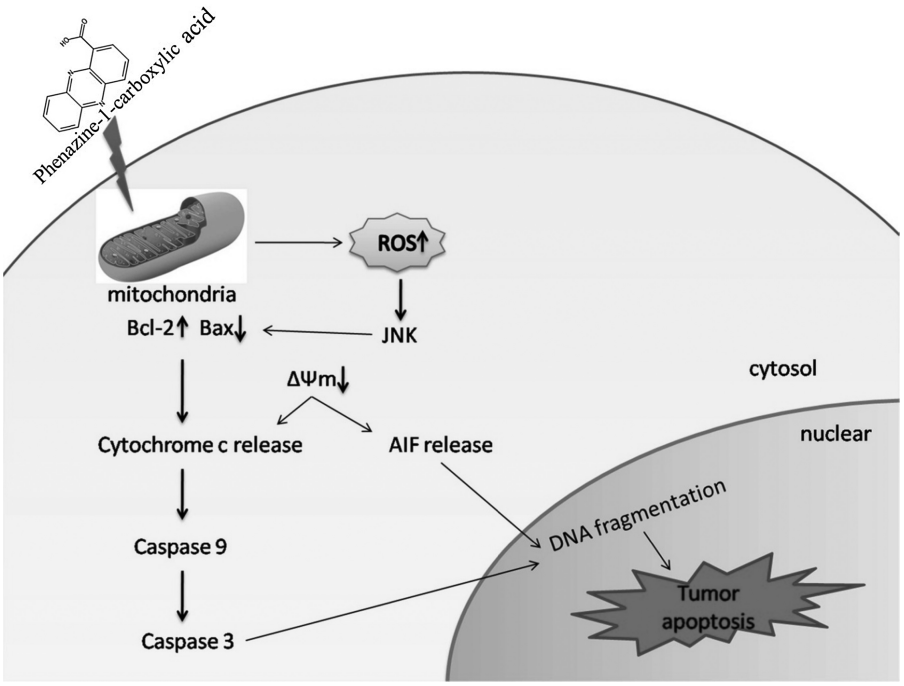

Fig. 11 – Schematic model for the action of phenazine-1-carboxylic acid-induced antitumor activity. Phenazine-1-carboxylic acid-triggered ROS production activated the pro-apoptotic stress kinase JNK, inhibited the anti-apoptotic Bcl-2 family protein, and resulted in loss of ΔΨm, which consequently improved the liberation of cytochrome c and AIF from mitochondria, and ultimately induced the caspase-dependent and caspase-independent programmed cell death pathway.

Several studies have showed that stimulation of downstream caspase activity was associated with cytochrome c release from mitochondria, which was regulated by ΔΨm and Bcl-2 family members (Arnoult et al., 2003). It is believed that distraction of ΔΨm is the initiation of mitochondrial membrane transition pores (MPTP) configuration. ΔΨm decrease and MPTP production support the localization of cytochrome c from mitochondria to cytoplasm to regulate the activity to access substrates or interacting partners (Ajenjo et al., 2004). In recent times, Bcl-2 proteins were considered to control the mitochondrial-mediated apoptotic pathway entirely (Elmore, 2007). Down regulation of Bcl-2 and up-regulation of Bax also induced the migration of cytochrome c from the mitochondria into the cytosol. Followed by, mitochondrial protein cytochrome c was observed in the cytosol. The level of cytochrome c in cytosol was drastically improved after phenazine-1-carboxylic acid treatment. Release of mitochondrial cytochrome c into the cytoplasm enhanced the formation of cytochrome c and Apaf-1 oligomeric complex called apoptosome. This complex triggers the effector caspase-3 (Fig. 8) and subsequently degrade substrates responsible for cellular death (McIlwain et al., 2013).

Surprisingly, AIF was also upregulated in the cytoplasm after phenazine-1-carboxylic acid treatment in DU145 cells. AIF encourages caspase-independent cell death principally (Cregan et al., 2004). Subsequent AIF relocation from the mitochondria to the nucleus, typical apoptotic characters, such as phosphatidyl serine exposure, partial chromatin condensation, and nuclear condensation, take place in the lack of caspase activation. Results of the present study reveled that both caspase-dependent and caspase-independent mechanism take part in the phenazine-1-carboxylic acid induced DU145 cell death.

In conclusion, the results of the present study revealed that phenazine-1-carboxylic acid inhibited proliferation and induced programmed cell death in DU145 cells. Based on our results, we proposed a model by which phenazine-1-carboxylic acid mediated release of ROS leading to DU145 programmed cell death (Fig. 11). The model demonstrated that phenazine-1-carboxylic acid acted on mitochondria, and induced ROS generation, which activated the pro-apoptotic stress kinase JNK, repressed the anti-apoptotic Bcl-2 family protein, and consequently resulted in loss of ΔΨm. The loss of ΔΨm improved the liberation of cytochrome c and AIF from mitochondria, and ultimately activated the caspase-dependent and caspase-independent programmed cell death pathway. This study might offer an insight into the prospective of phenazine-1-carboxylic acid for prostate cancer therapy.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We are thankful to Accvisbio, Abu Dhabi, UAE and the authorities of Annamalai University, India for providing necessary facility to carry out the research work.

REFERENCES

Abdelfattah, M.S., Kazufumi, T., Ishibashi, M., 2011. Isolation and structure elucidation of izuminosides AeC: a rare phenazine glycosides from *Streptomyces* sp. IFM 11260. J. Antibiot. 64, 271–275.

Abken, H.J., Tietze, M., Brodersen, J., Baumer, S., Beifuss, U., Deppenmeier, U., 1998. Isolation and characterization of methanophenazine and function of phenazines in membrane-bound electron transport of *Methanosarcina mazei* Go1. J. Bacteriol. 180, 2027–2032.

Ajenjo, N., Canon, E., Sanchez-Perez, I., Matallanas, D., Leon, J., Perona, R., Crespo, P., 2004. Subcellular localization determines the protective effects of activated ERK2 against distinct apoptogenic stimuli in myeloid leukemia cells. J. Biol. Chem. 279, 32813–32823.

Arnoult, D., Gaume, B., Karbowski, M., Sharpe, J.C., Cecconi, F., Youle, R.J., 2003. Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. EMBO J. 22, 4385–4399.

Buckingham, J., 2008. Dictionary of Natural Products on CD-ROM 16.2. Chapman and Hall, London.

Chandra, J., Samali, A., Orrenius, S., 2000. Triggering and modulation of apoptosis by oxidative stress. Free Radic. Biol. Med. 29, 323–333.

Chang, H.L., Hsu, H.K., Su, J.H., Wang, P.H., Chun, Y.F., Chia, Y.C., Tsai, L.Y., Wu, Y.C., Yuan, S.S., 2006. The fractionated *Toona sinensis* leaf extract induces apoptosis of human ovarian cancer cells and inhibits tumor growth in a murine xenograft model. Gynecol. Oncol. 102, 309–314.

Cimmino, A., Andolfi, A., Evidente, A., 2013. Microbion phenazines. In: Chincholkar, T. (Ed.), Phenazine as an Anticancer Agent. Springer, Heidelberg, pp. 217–243.

Cimmino, A., Evidente, A., Mathieu, V., Andolfi, A., Lefranc, F., Kornienko, A., Kiss, R., 2012. Phenazines and cancer. Nat. Prod. Rep. 29, 487–501.

Cregan, S.P., Dawson, V.L., Slack, R.S., 2004. Role of AIF in caspase-dependent and caspase-independent cell death. Oncogene 23, 2785–2796.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495–516.

Gao, X., Lu, Y., Xing, Y., Ma, Y., Lu, J., Bao, W., Wang, Y., Xi, T., 2012. A novel anticancer and antifungus phenazine derivative from a marine actinomycete BM-17. Microbiol. Res. 167, 616–622.

Gao, X., Lu, Y., Fang, L., Fang, X., Xing, Y., Gou, S., Xi, T., 2013. Synthesis and anticancer activity of some novel 2-phenazinamine derivatives. Eur. J. Med. Chem. 69, 1–9.

Giddens, S.R., Feng, Y., Mahanty, H.K., 2002. Characterization of a novel phenazine antibiotic gene cluster in Erwinia herbicola Eh1087. Mol. Microbiol. 45, 769–783.

Gupta, S., 2007. Prostate cancer chemoprevention: current status and future prospects. Toxicol. Appl. Pharmacol. 224, 369–376.

Hussain, H., Specht, S., Sarite, S.R., Saeftel, M., Hoerauf, A., Schulz, B., Krohn, K., 2011. A new class of phenazines with activity against a chloroquine resistant Plasmodium falciparum strain and antimicrobial activity. J. Med. Chem. 54, 4913–4917.

Jemal, A., Murray, T., Ward, E., Samuels, A., Tiwari, R.C., Ghafoor, A., Feuer, E.J., Thun, M.J., 2005. Cancer statistics 2005 Canada. Cancer J. Clin. 55, 10–30.

Karuppiah, V., Aarthi, C., Sivakumar, K., 2011. Enhancement of PCR amplification of actinobacterial 16S rRNA gene using an adjuvant, dimethyl sulphoxide. Curr. Sci. 101, 22–23.

Karuppiah, V., Aarthi, C., Sivakumar, K., Kannan, L., 2013. Statistical optimization and anticancer activity of a red

pigment isolated from *Streptomyces* sp. PM4. Asian Pac. J. Trop. Biomed. 3, 650–656.
Karuppiah, V., Li, Y., Sun, W., Feng, G., Li, Z., 2015. Functional gene-based discovery of phenazines from the actinobacteria associated with marine sponges in the South China Sea. Appl. Microbiol. Biotechnol. 99, 5939–5950.
Kerr, J.R., Taylor, G.W., Rutman, A., Hoiby, N., Cole, P.J., Wilson, R., 1999. *Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit fungal growth. J. Clin. Pathol. 52, 385–387.
Krishnan, K., Campbell, S., Abdel-Rahman, F., Whaley, S., Stone, W.L., 2003. Cancer chemoprevention drug targets. Curr. Drug Targets 4, 1389–4501.
Kuo, P.L., Chen, C.Y., Hsu, Y.L., 2007. Isoobtusilactone A induces cell cycle arrest and apoptosis through reactive oxygen species/apoptosis signal-regulating kinase 1 signaling pathway in human breast cancer cells. Cancer Res. 67, 7406–7420.
Laatsch, H., 2007. Antibase 2007 SciDex: The Natural Products Identifier, first ed. Wiley-VCH, Weinheim, Germany.
Liou, G.Y., Storz, P., 2010. Reactive oxygen species in cancer. Free Radic. Res. 44, 479–496.
Mavrodi, D.V., Blankenfeldt, W., Thomashow, L.S., 2006. Phenazine compounds in fluorescent *Pseudomonas* spp. biosynthesis and regulation. Annu. Rev. Phytopathol. 44, 417–445.
McIlwain, D.R., Berger, T., Mak, T.W., 2013. Caspase functions in cell death and disease. Cold Spring Harb. Perspect. Biol. 5, a008656.
Nakano, H., Nakajima, A., Sakon-Komazawa, S., Piao, J.H., Xue, X., Okumura, K., 2006. Reactive oxygen species mediate crosstalk between NF-kappaB and JNK. Cell Death Differ. 13, 730–737.
Neves-Pinto, C., Malta, V.R.S., Pinto, M.D.C.F.R., Santos, R.H.A., de Castro, S.L., Pinto, A.V., 2002. A trypanocidal phenazine derived from β-lapachone. J. Med. Chem. 45, 2112–2115.
Nogueira, V., Hay, N., 2013. Molecular pathways: reactive oxygen species homeostasis in cancer cells and implications for cancer therapy. Clin. Cancer Res. 19, 4309–4314.
Pierson, L.S., Pierson, E.A., 2010. Metabolism and function of phenazines in bacteria: impacts on the behaviour of bacteria in the environment and biotechnological processes. Appl. Microbiol. Biotechnol. 86, 1659–1670.
Pu, Y.S., Chiang, H.S., Lin, C.C., Huang, C.Y., Huang, K.H., Chen, J., 2004. Changing trends of prostate cancer in Asia. Aging Male 7, 120–132.
Rane, M.R., Sarode, P.D., Chaudhari, B.L., Chincholkar, S.B., 2007. Detection, isolation and identification of phenazine-1-carboxylic acid produced by biocontrol strains of *Pseudomonas aeruginosa*. J. Sci. Ind. Res. 66, 627–631.
Rewcastle, G.W., Denny, W.A., Baguley, B.C., 1987. Potential antitumor agents. 51. Synthesis and antitumor activity of substituted phenazine-1-carboxamides. J. Med. Chem. 30, 843–851.
Singh, R.P., Dhanalakshmi, S., Agarwal, R., 2002. Phytochemicals as cell cycle modulators: a less toxic approach in halting human cancers. Cell Cycle 1, 56–161.
Son, Y., Cheong, Y.K., Kim, N.H., Chung, H.T., Kang, D.G., Pae, H. O., 2011. Mitogen-activated protein kinases and reactive oxygen species: how can ros activate mapk pathways? J. Signal. Transduct. 2011, 792639.
Sorensen, R.U., Klinger, J.D., Cash, H.A., Chase, P.A., Dearborn, D. G., 1983. *In vitro* inhibition of lymphocyte proliferation by *Pseudomonas aeruginosa* phenazine pigments. Infect. Immun. 41, 321–330.
Sun, W., Dai, S.K., Jiang, S.M., Wang, G.H., Liu, G.H., Wu, H.B., Li, X., 2010. Culture-dependent and culture-independent diversity of Actinobacteria associated with the marine sponge *Hymeniacidon perleve* from the South China Sea. Antonie Van Leeuwenhoek 98, 65–75.
Trachootham, D., Lu, W., Ogasawara, M.A., Valle, N.R.D., Huang, P., 2008. Redox regulation of cell survival. Antioxid. Redox Signal. 10, 1343–1374.