Chemico-Biological Interactions 183 (2010) 293–303

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Apoptosis induction in human leukemic cells by a novel protein Bengalin, isolated from Indian black scorpion venom: Through mitochondrial pathway and inhibition of heat shock proteins

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ARTICLE INFO

Article history:
Received 2 September 2009
Received in revised form 30 October 2009
Accepted 5 November 2009
Available online 12 November 2009

Keywords:
Bengalin
Apoptosis
Mitochondria
Heat shock protein
Caspases

ABSTRACT

Scorpion venom possesses protein toxins having numerous biological activities, some of which are potentially anticancerous. Previously we had reported antiproliferative activity of the venom of Indian black scorpion, *Heterometrus bengalensis* Koch. Here we have isolated and purified a novel protein named Bengalin (72 kDa) from the venom, responsible for antiproliferative and apoptogenic activities against human leukemic cells U937 (histiocytic lymphoma) and K562 (chronic myelogenous leukemia). N-terminal sequence of first 20 amino acids of Bengalin was G-P-L-T-I-L-H-I-N-D-V-H-A-A/R-F-E-Q/G-F/G-N-T. Bengalin induced cell growth inhibition at IC_{50} values of 3.7 and 4.1 μg/ml for U937 and K562 cells respectively did not significantly affect normal human lymphocytes. Inhibition of U937 and K562 cell proliferation occurred by apoptosis as evidenced from damaged nuclei, cell cycle arrest at sub G1 phase, increase of early apoptotic cells, augmentation of DNA fragmentation and also a reduction of telomerase activity. Further insights revealed that Bax:Bcl2 ratio was elevated after Bengalin treatment. Moreover Bengalin elicited loss of mitochondrial membrane potential (MMP) which commenced cytochrome *c* release in cytosol, decreased heat shock protein (HSP) 70 and 90 expression, activated caspase-9, caspase-3 and induced poly(ADP-ribose) polymerase (PARP) cleavage. We have also determined that HSP70 and 90 inhibitions correlated with Bengalin induced antiproliferation, caspase-3 upregulation, apoptogenesis and increased DNA fragmentation. These results hypothesize that Bengalin might provide a putative molecular mechanism for their anticancer effect on human leukemic cells which might be mediated by mitochondrial death cascade. Inhibition of HSPs might also play a crucial role in induction of apoptosis.

1. Introduction

Scorpion venom has the property of inhibiting growth of various types of cancers. The venom possesses different peptides and proteins that are chiefly responsible for the anticancer activities. Several antitumor peptides have been reported from scorpion venom showing cytotoxic activities [1,2]. Chlorotoxin a peptide isolated from the venom of *Leirus quinquestriatus* [3] specifically binds to glioma cells and prevent their migration [4]. Existence of chlorotoxin and its anticancer potential has also been reported from the venom of *Buthus martensii* Karsch [5]. It also binds to cancer cells from other origins as well with immense precision [6]. Recent report suggests that nanoparticles are being attached to chlorotoxin for targeted delivery of nanoprobes to tumor cells for inhibiting their invasive potentials [7].

Initiation and advancement of cancer occurs mostly due to uncontrolled cell division and malfunctioned apoptotic mechanisms and the resistance of cancer to existing chemotherapeutic measures is caused due to their property of apoptosis evasion. Apoptosis is a type of programmed cell death, initiated by several factors involving a cascade of intracellular events, leading to the termination of cell survival [8]. Telomerase activity has also been reported in almost 85% of tumors of human origin and in 100% of different cancer cell lines [9,10]. This indicates that telomerase can serve as a marker for cellular survivability and might be required for the continued division of cancer cells. Cell cycle arrest at various checkpoints results in activation of intracellular pathways which culminates in apoptosis [11]. There are two major pathways of apoptosis viz. extrinsic and intrinsic [12]. The extrinsic pathway involves the activation of death cascade, while the intrinsic mechanism involves mitochondrial mediation featured by the loss of mitochondrial membrane potential (MMP), release of cytochrome *c* and the subsequent activation of caspases [12]. Activated caspase-9 cleaves downstream procaspase-3 to active caspase-3, which subsequently cleaves poly(ADP-ribose) polymerase (PARP) [13,14]. It

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doi:10.1016/j.cbi.2009.11.006

is well known that the Bcl2 family of proteins is one of the most important mediators of mitochondrial death cascade [8,15]. During apoptosis, proapoptotic Bax is upregulated along with a down regulation of Bcl2.

It has been reported that, heat shock proteins (HSP) can be induced by various stresses [16]. Antiapoptotic HSP70 protects cells from various apoptotic stimuli and inhibits caspase-3 and SAPK/JNK activation [17,18]. Another antiapoptotic member HSP90 functions by accumulating Bcl2 along with the inhibition of apoptotic peptidase activating factor (Apaf) thereby inhibiting apoptosis [19].

Efforts are being carried out throughout the world to discover newer anticancer molecules from natural resources. In our earlier study we had reported the anticancer property of crude venom from *Heterometrus bengalensis*. In our present investigation we have isolated the fraction that is responsible for the antileukemic activity and elucidated its probable mode of molecular action on human leukemic cell lines.

2. Materials and methods

2.1. Scorpion venom purification, molecular weight determination and amino acid sequencing

Indian black scorpions (*H. bengalensis* Koch) were collected locally and were kept in wooden cages. Food and water were provided *ad libitum*. Venom from live animals was collected and stored as described earlier [20]. Venom was dissolved in 0.02 M phosphate buffer, pH 7.2 and centrifuged at 10,000 × *g* for 15 min to remove mucous and undissolved particles. Venom (200 mg) was loaded on a DEAE (diethyl aminoethyl) cellulose (Sigma–Aldrich, St. Louis, MO, USA) ion-exchange chromatography column (6.5 cm × 2 cm). Proteins were eluted with 0.02 M phosphate buffer, pH 7.2 containing stepwise increasing molarities of NaCl (0.02–1 M). Protein fraction having cytotoxic activity was further purified by high performance liquid chromatography (HPLC) using Waters HPLC Protein Pak 300SW column (7.5 mm × 300 mm) and 10 mM sodium phosphate buffer containing 0.1 M NaCl (pH 7) at a flow rate of 0.8 ml/min. Protein content was estimated by Bradfords's method [21]. Homogeneity of the protein fractions was determined by 12% SDS PAGE using a current of 100 V and 40 mA for 1.5 h at 4 °C. The gel was stained by Coomassie brilliant blue to detect protein bands.

Molecular weight of the fraction was compared with protein markers of known molecular weights run in a 12% SDS-PAGE as described above. Relative mobility value of the protein fraction was calculated to determine the molecular weight [22].

50 pg of purified protein was subjected to protein sequencing using Applied Biosystems Procise Sequencer by the process of Edman's degradation and data were analyzed using Smooth Degree 9, Interpolated Baseline [23].

2.2. Cell culture

Human leukemic cells, U937 (histiocytic lymphoma) and K562 (chronic myelogenous leukemia) were purchased from National Facility for Animal Tissue and Cell Culture, Pune, India. Cells were cultured in RPMI-1640 (Invitrogen, Grand Island, NY, USA), with 10% heat inactivated fetal bovine serum (Invitrogen, Grand Island), penicillin-streptomycin and gentamycin (Invitrogen, Grand Island). Normal human lymphocytes were isolated with histopaque (Sigma–Aldrich, USA) and cultured in RPMI-1640. Cells were incubated in a humidified CO_2 incubator having a temperature of 37 °C and 5% CO_2.

2.3. Cell growth inhibition assay

10^6 cells/ml of U937, K562 and normal human lymphocytes were seeded in a 96 well tissue culture plate. They were treated with increasing concentrations of Bengalin from 1 to 20 μg for 48 h and 3[4-dimethylthiazol-2-yl]-2-5-diphenyl tetrazolium bromide (MTT) (Sigma–Aldrich, St. Louis) assay was performed. Briefly both control and treated cells were incubated for 4 h with MTT. Produced purple colored formazan was dissolved in dimethyl sulfoxide (DMSO) (Sigma–Aldrich, St. Louis) and isopropanol (Sigma–Aldrich, St. Louis). Absorbance was recorded at 570 nm in a BioRad microplate reader 680XR (Japan) with a reference serving as blank [24]. Results obtained from the MTT assay was used to calculate the IC_{50} value of Bengalin [25].

2.4. Morphological studies

Fluorescence microscopic observation of the treated cells was carried out to assess the morphological changes. After incubation with Bengalin for 24 h, U937 and K562 cells were washed with phosphate buffer saline (PBS) and treated with ethidium bromide (EtBr) (Sigma–Aldrich, St. Louis) and acridine orange (AO) (Sigma–Aldrich, St. Louis) solution (both 100 μg/ml of PBS) and finally observed under a Zeiss fluorescence microscope (Gottingen, Germany).

Nuclear damage was observed under a confocal laser-scanning microscope (Leica Microsystem, Heidelberg, Germany) as described earlier [18]. After 24 h incubation with Bengalin both the cell lines were washed with chilled PBS and treated with propidium iodide (PI) (Sigma–Aldrich, St. Louis). Nuclear integrity was visualized by argon-krypton laser using a 590 nm filter.

2.5. Detection of cell cycle arrest, apoptosis and DNA fragmentation employing FACS

Flow cytometric analysis was done to determine the cell cycle stage in both U937 and K562 cells. Initially 10^6 cells were incubated with Bengalin at respective ½ IC_{50} (1.85 μg/ml for U937 and 2.05 μg/ml for K562 cells) and IC_{50} (3.7 μg/ml for U937 and 4.1 μg/ml for K562 cells) concentrations for 24 h. Then cells were washed in ice cold PBS and permeabilized. Next nuclear DNA was labeled with PI by CycleTEST PLUS DNA Reagent kit (BD Biosciences, San Diego, CA, USA). The amount of red fluorescence is indicative of the quantity of intracellular DNA and was measured in a Becton Dickinson fluorescence-activated cell sorting (FACS) (San Diego, CA, USA) at 488 nm argon laser light source with 623 nm band pass filter. Data were analyzed using CellQuest software (Becton Dickinson).

Analysis for apoptotic induction was done using a FACS. Initially 10^6 cells from both U937 and K562 cell lines were treated with their respective ½ IC_{50} and IC_{50} doses of Bengalin for 24 h. Normal human lymphocytes were also incubated with the IC_{50} dose of Bengalin for both U937 (3.7 μg/ml) and K562 (4.1 μg/ml) cells for 24 h. Apoptosis was measured by AnnexinV-PI Apoptosis Detection Kit (Sigma–Aldrich). Cells were acquired in a Becton Dickinson FACS at an excitation wavelength of 488 nm. For AnnexinV-FITC and PI detection, the band pass filters were 515 and 623 nm, respectively. Results were analyzed using Becton Dickinson Cell Quest software.

To substantiate the nature of tumor killing by Bengalin, U937 and K562 cells were fixed, permeabilized and incubated with TdT (terminal deoxynucleotidyl transferase) enzyme and FITC-dUTP after 48 h of treatment. Apo-direct TUNEL ASSAY KIT (Chemicon International Inc., Temecula, CA, USA) was used to label the fragmented DNA of apoptotic cells. The cells were then washed and analyzed on FACS (equipped with 488 nm Argon laser light source; 515 nm band pass filter, FL1-H, and 623 nm band pass filter, FL2-H) using CellQuest software (Becton Dickinson).

2.6. Assay for telomerase activity

Modulations in the telomerase activity due to Bengalin action were assayed using the TRAPEZE®XL telomerase Detection Kit (Chemicon) as per manufacturer's protocol. Briefly, 2×10^6 cells from both U937 and K562 were treated with Bengalin at concentrations of ½ IC_{50} and IC_{50} for 24 h and then cell lysates were obtained. The reaction mix was prepared according to protocol. Next elongation of telomeric DNA was performed in a PCR machine for 30 min at 30 °C, amplification was conducted for 10 min at 94 °C, then 36 cycles of 30 s at 94 °C, 59 °C for 30 s, 72 °C for 1 min followed by 3 min extension at 72 °C with a final incubation of 25 min at 55 °C. After completion, the reaction was maintained at 4 °C. Telomerase activity was measured in a luminescence spectrometer (Parkin Elmer-LS55, UK) at 495 nm/516 nm for fluorescein and 600 nm/620 nm sulforhodamine detection.

2.7. Assay for MMP

Changes in the MMP due to Bengalin treatment was measured by the 5,5′6,6′-tetracholoro-1,1′3,3′-tetraethylbenzimidazolylcarbocyanin iodide (JC-1) kit (BD Biosciences, San Diego). JC-1 is a fluorescent dye which gives signal after detection of the loss of MMP. U937 and K562 cells were treated with Bengalin at ½ IC_{50} and IC_{50} concentrations for 24 h. 10^6 cells from each treatment of both the cell lines were incubated with JC-1 as per manufacturer's protocol and fluorescent intensity was determined by a Becton Dickinson FACS with an excitation at 488 nm and emission at 525 nm. Data was presented as percentage of cells with altered MMP.

2.8. Isolation of cytosolic and mitochondrial fractions and measurement of cytochrome c level

U937 and K562 cells were treated with Bengalin at concentrations of ½ IC_{50} and IC_{50} for 24 h. Mitochondrial and cytosolic fractions of cells were isolated using Mitochondria/Cytosol fractionation kit (BioVision, Mountain View, CA, USA) according to the protocol provided. ELISA was performed to determine cytochrome *c* level from both isolated cytosolic and mitochondrial fractions using the Cytochrome C ELISA kit (Chemicon International Inc.) as per manufacturer's protocol.

2.9. Assay for caspase activity

Caspase-8, caspase-9 and caspase-3 activity in the treated cells were estimated using caspase-8 and caspase-3 colorimetric assay kits (Chemicon International Inc.) and caspase-9 colorimetric assay kit (R&D Systems, Minneapolis, MN, USA). 2×10^6 cells from both U937 and K562 were treated with Bengalin at concentrations of ½ IC_{50} and IC_{50} for 24 h. Caspase generations were quantified as per the manufacturer's protocol. Caspase-8 generation was measured as the amount of *p*-nitroaniline produced due to cleavage of the substrate Acetyl-Ile-Glu-Thr-Asp-p-nitroaniline by caspase-8. Similarly amount of caspase-9 and caspase-3 generation was measured by the production of *p*-nitroaniline upon cleaving the substrates Leu-Glu-His-Asp p-nitroaniline and acetyl-Asp-Glu-Val-Asp p-nitroanilide, respectively. Inhibitor study using IETD-CHO (Calbiochem, La Jolla, CA, USA) for caspase-8, LEHD-CHO (Calbiochem) for caspase-9 and DEVD-CHO (Calbiochem) for caspase-3 were also performed.

2.10. Western blot analysis

Expression of different apoptosis associated proteins was measured by Western blotting. 10^6 cells from both the cell lines were incubated with Bengalin at ½ IC_{50} and IC_{50} concentrations for 24 h. Cells were lysed with RIPA buffer (Sigma–Aldrich, St. Louis), containing protease inhibitor cocktail (Amresco, Solon, OH, USA). Equal amount of protein from each sample was run in a 10% SDS-PAGE. The proteins were next electrophoretically transferred to a nitrocellulose membrane and blocking of nonspecific sites was done by 5% skimmed milk in 20 mM (Tris buffer saline) TBS (pH 7.4) containing 0.1% Tween-20 (Sigma–Aldrich, St. Louis). Thereafter, the membrane was incubated with primary antibodies Bcl2, Bax, HSP70, HSP90, Apaf-1, PARP and β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) followed by alkaline phosphatase conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz). Color was developed using NBT-BCIP mix (Sigma–Aldrich, St. Louis).

2.11. Statistical analysis

Statistical analyses were done by GraphPad InStat software (La Jolla, CA, USA). Data were expressed as mean ± S.D. of three independent experiments. The differences between the treated and control groups were analyzed by one-way ANOVA and posttests were done using Dunnett's multiple comparison test to determine the significant levels.

3. Results

3.1. Scorpion venom purification

Ion exchange chromatography of crude venom yielded four main peaks (Fig. 1A). The third peak (P3) eluting with 0.2 M NaCl showed significant cytotoxicity in comparison to other peaks as confirmed by MTT assay (data not shown) and was considered for further analysis and purification by HPLC. A single sharp peak having retention time of 8.6 min with absorbance maxima at 280 nm, confirmed the purity of the P3 (Fig. 1B). The fraction collected from the column was reinjected in the same column and a single sharp peak with retention time of 8.64 min was obtained. The protein was named Bengalin. Bengalin showed a single sharp band with SDS-PAGE analysis suggesting its homogenity (Fig. 1C). Bengalin accounted for 3.22 ± 0.06% of the total protein content of the whole venom. Molecular weight of Bengalin was calculated to be 72 kDa (Fig. 1D). The first 20 N-terminal amino acids obtained were G-P-L-T-I-L-H-I-N-D-V-H-A-A/R-F-E-Q/G-F/G-N-T.

3.2. Effect of Bengalin treatment on proliferation of U937, K562 and normal human lymphocytes

The incubation of cells from both the cell lines with Bengalin for 48 h resulted in a dose dependant reduction of cell viability without significantly affecting the normal human lymphocytes (Fig. 2A). From the observed results of the MTT assay, the IC_{50} value was calculated to be 3.7 and 4.1 μg/ml for U937 and K562 cells, respectively (Fig. 2A).

3.3. Morphological studies of U937 and K562 cells after treatment with Bengalin

Fluorescence microscopy revealed U937 and K562 cells showing apoptosis (Fig. 2B) and nuclear damage (Fig. 2C) after treatment with Bengalin for 24 h. The control cells had undamaged and distinct nuclei.

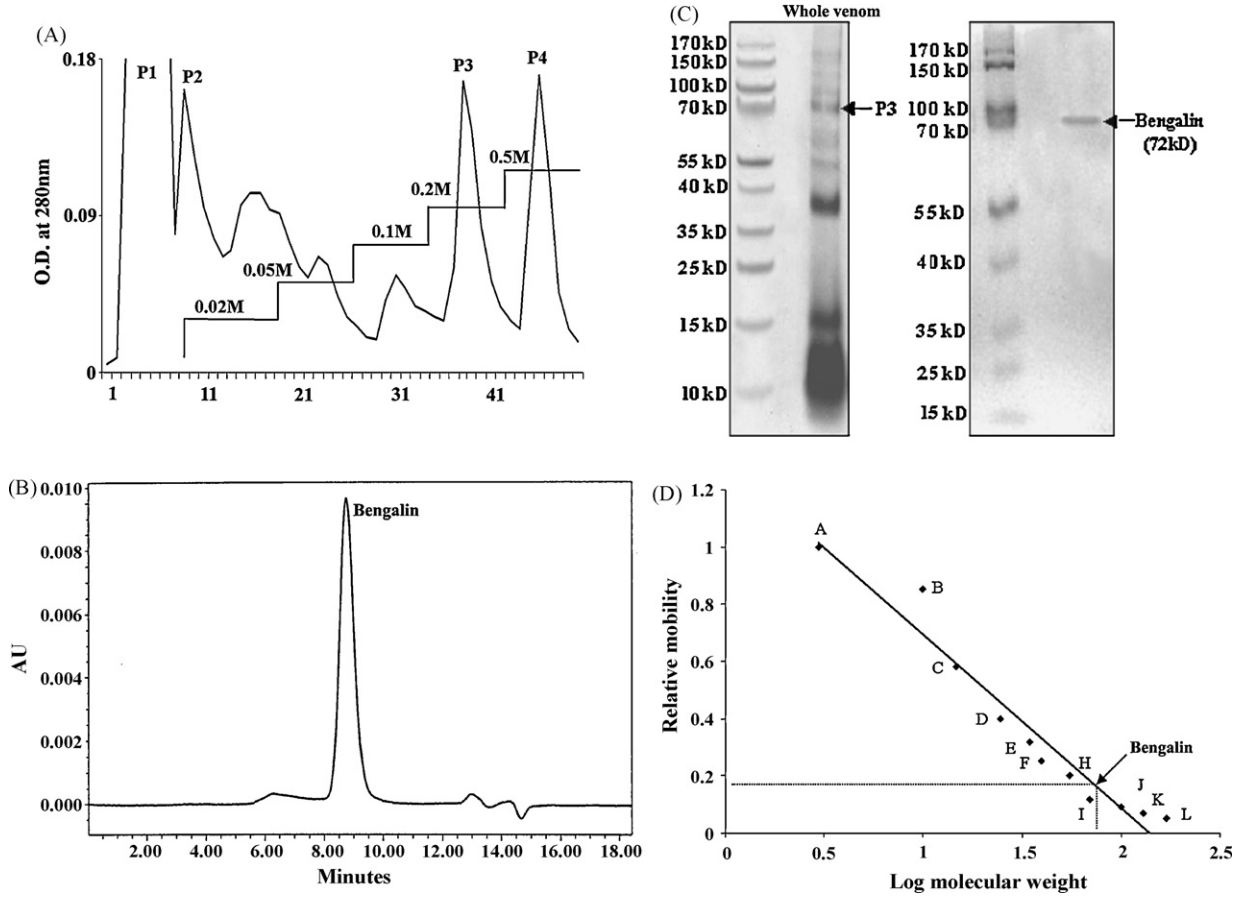

Fig. 1. Purification and molecular weight determination of Bengalin. (A) Ion exchange chromatography showing four different peaks. (B) HPLC peak of Bengalin at 280 nm with a retention time of 8.6 min. (C) SDS-PAGE profile of whole venom and Bengalin run against molecular weight marker with known molecular weights. (D) Graph showing the relative molecular weight calculation for determination of molecular weight of Bengalin: A (3 kDa), B (10 kDa), C (15 kDa), D (25 kDa), E (35 kDa), F (40 kDa), H (55 kDa), I (70 kDa), J (100 kDa), K (130 kDa), L (170 kDa).

3.4. Induction of cell cycle arrest and apoptosis in U937 and K562 cells after treatment of Bengalin

To evaluate the inhibition of cell proliferation by Bengalin in U937 and K562 cells, we studied cell cycle arrest and induction of apoptosis. To examine the effect of Bengalin on cell cycle progression, cells were treated with Bengalin at ½ IC_{50} and IC_{50} concentrations for 24 h. Flow cytometric analysis was conducted for cell cycle phase distribution. A dose dependent increase of sub G1 hypodiploid cell population was found in case of both cell lines (from 15% of control to 52.1% and 71.9% due to Bengalin treatment at ½ IC_{50} (1.85 μg/ml) and IC_{50} (3.7 μg/ml) concentrations for U937 and from 13.3% of control to 47.2% and 65.3% due to Bengalin treatment at ½ IC_{50} (2.05 μg/ml) and IC_{50} (4.1 μg/ml) concentrations for K562 cells), whereas the percentage of cells in G0/G1 phase (from 32.3% of control to 18.9% and 10.5% due to Bengalin treatment at ½ IC_{50} and IC_{50} concentrations for U937 and from 34% of control to 22.7% and 12.9% due to Bengalin treatment at ½ IC_{50} and IC_{50} concentrations for K562 cells) and S (from 24% of control to 10.8% and 5.4% due to Bengalin treatment at ½ IC_{50} and IC_{50} concentrations for U937 and from 26.4% of control to 13.1% and 7.9% due to Bengalin treatment at ½ IC_{50} and IC_{50} concentrations for K562 cells) phase decreased suggesting a clear arrest of the cell cycle in U937 and K562 cells due to Bengalin action (Fig. 3A).

Further we confirmed Bengalin induced apoptosis by means of AnnexinV-PI staining. Flow cytometric results revealed that Bengalin treatment at ½ IC_{50} and IC_{50} concentrations had effectively increased the percentage of early apoptotic cells thereby indicating an induction of apoptosis in a dose dependant manner (Fig. 3B). However Bengalin did not significantly induce apoptosis in normal human lymphocytes at respective IC_{50} values for U937 and K562 cells for 24 h treatment.

For quantification of the extent of apoptosis we measured DNA fragmentation from the cells labeled with fluorescent-tagged dUTP by flow cytometry. It is apparent from the figure that Bengalin treatment augmented statistically significant DNA fragmentation after 48 h in both U937 (from 6.3% of control to 48.7% and 57.3% due to Bengalin treatment at ½ IC_{50} and IC_{50} concentrations, respectively) and K562 cells (from 5.9% of control to 46.5% and 52.5% due to Bengalin treatment at ½ IC_{50} and IC_{50} concentrations, respectively) (Fig. 3C).

3.5. Effect of Bengalin treatment on telomerase activity

It is well known that cancer cells express high levels of telomerase. In our study, Bengalin treatment for 24 h, significantly decreased the telomerase activity by 69.1% and 51% at ½ IC_{50} and IC_{50} concentrations respectively in U937 and in K562 cells by 73% and 62.2% due to treatment at ½ IC_{50} and IC_{50} concentrations, respectively (Fig. 3D).

3.6. Effect of Bengalin treatment on MMP

Alteration of mitochondrial membrane integrity is one of the early events of apoptosis. Hence the effect of Bengalin treatment

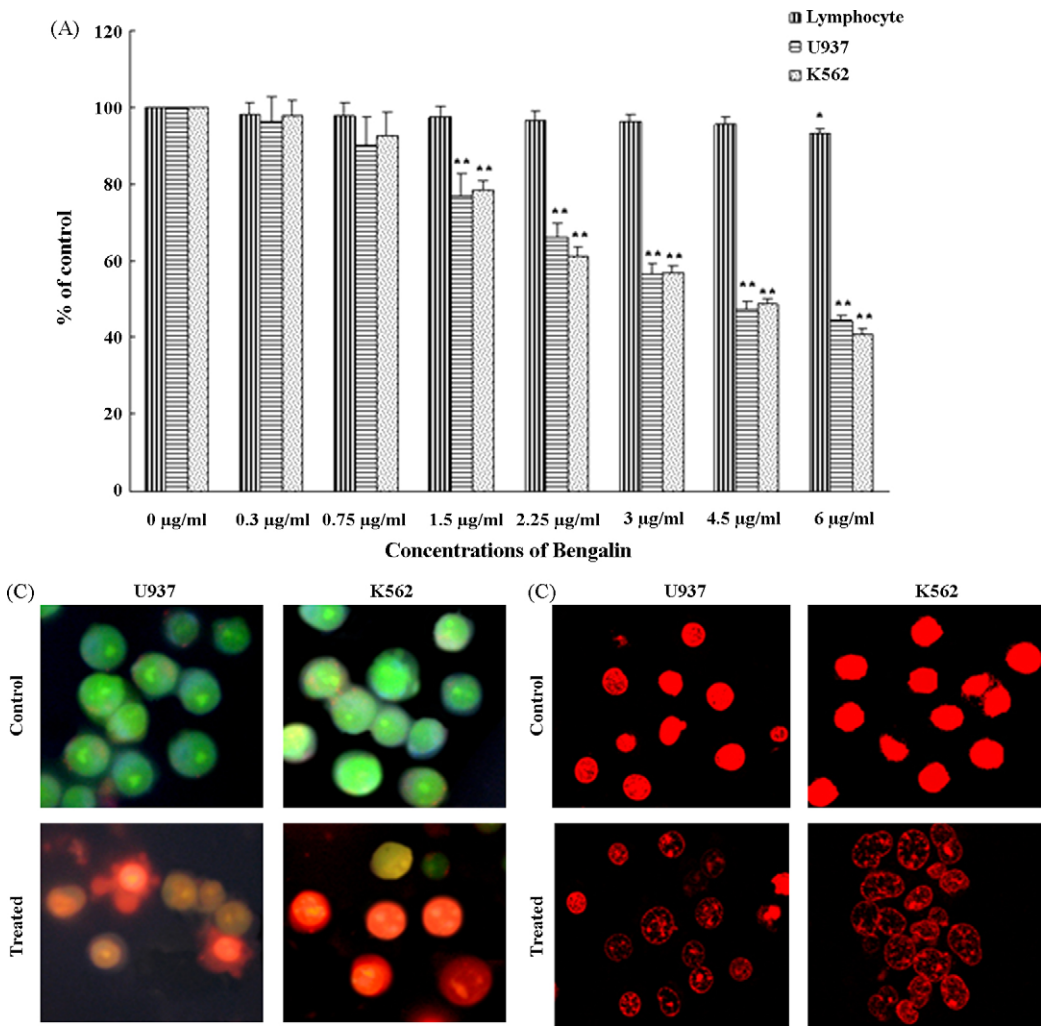

Fig. 2. Effects of Bengalin on the viability of normal human lymphocytes, U937 and K562 cells and representative fluorescence and confocal micrograph of Bengalin induced apoptotic cells. (A) Bengalin treatments were done for 48 h in U937 cells at 1.85 and 3.7 μg/ml while K562 cells at 2.05 and 4.1 μg/ml, respectively. Changes in cell viability was measured by MTT assay. Each value is expressed as mean ± S.D. ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ are the Bengalin treated groups compared to the control (0 μg/ml). (B) Bengalin treatments were done for 24 h in U937 cells at 1.85 and 3.7 μg/ml while K562 cells at 2.05 and 4.1 μg/ml respectively and morphology observed under fluorescence microscope using AO/EtBr (100× magnification). Representative figures are the U937 and K562 cells treated at 3.7 and 4.1 μg/ml of Bengalin, respectively. (C) After treatment with Bengalin at respective cells were seen under a confocal microscope using PI (1000× magnification). Representative figures are the U937 and K562 cells treated with Bengalin at 3.7 and 4.1 μg/ml, respectively. Data shown here are from one of the three repeated experiments with similar results.

on loss of MMP was quantified. A significant increase in fluorescence of JC-1 was observed due to Bengalin treatment (from 3.6% of control to 33.3% and 54.7% due to Bengalin treatment at ½ IC_{50} and IC_{50} concentrations in U937 and from 4.9% of control to 29.8% and 47.8% due to Bengalin treatment at ½ IC_{50} and IC_{50} concentrations, respectively). Our observations therefore signify an increased accumulation of cells with altered MMP (Fig. 4A).

3.7. Cytochrome c release assay due to Bengalin treatment

Loss of mitochondrial membrane potential leads to release of downstream cytochrome *c* in cytosol. Hence cytochrome *c* release from mitochondria was quantified in Bengalin treated U937 and K562 cells. Bengalin treatment increased cytosolic cytochrome *c* release by 2.9- and 4.9-fold at ½ IC_{50} and IC_{50} concentrations respectively in U937 and in K562 cells by 2.1- and 3.9-fold due to treatment at ½ IC_{50} and IC_{50} concentrations respectively as compared to their control counterparts. However Bengalin had reduced the mitochondrial cytochrome *c* levels in both the cell lines (Fig. 4B).

3.8. Analysis of caspase-9, caspase-3 and caspase-8 generation in U937 and K562 cells after treatment with Bengalin

Caspase-8 production is a late consequence of death receptor mediated apoptosis which was not elevated in both U937 and K562 cells at significant levels upon treatment with ½ IC_{50} and IC_{50} concentrations of Bengalin (Fig. 5A). Since cytochrome *c* release helps in activation of caspase-9 which in turn activates downstream caspase-3, therefore we evaluated the effect of Bengalin on caspase activities. It is evident from our results that a significant increase in caspase-9 (in U937 cells by 2.3- and 3.9-fold for ½ IC_{50} and IC_{50} concentrations respectively while in K562 cells by 2.1- and 3.5-folds due to treatment at ½ IC_{50} and IC_{50} concentrations, respectively) (Fig. 5B) and caspase-3 (in U937 cells by 1.2- and 1.9-fold for ½ IC_{50} and IC_{50} concentrations respectively while in K562 cells by 1.3- and 1.9-fold due to treatment at ½ IC_{50} and IC_{50} concentrations, respec-

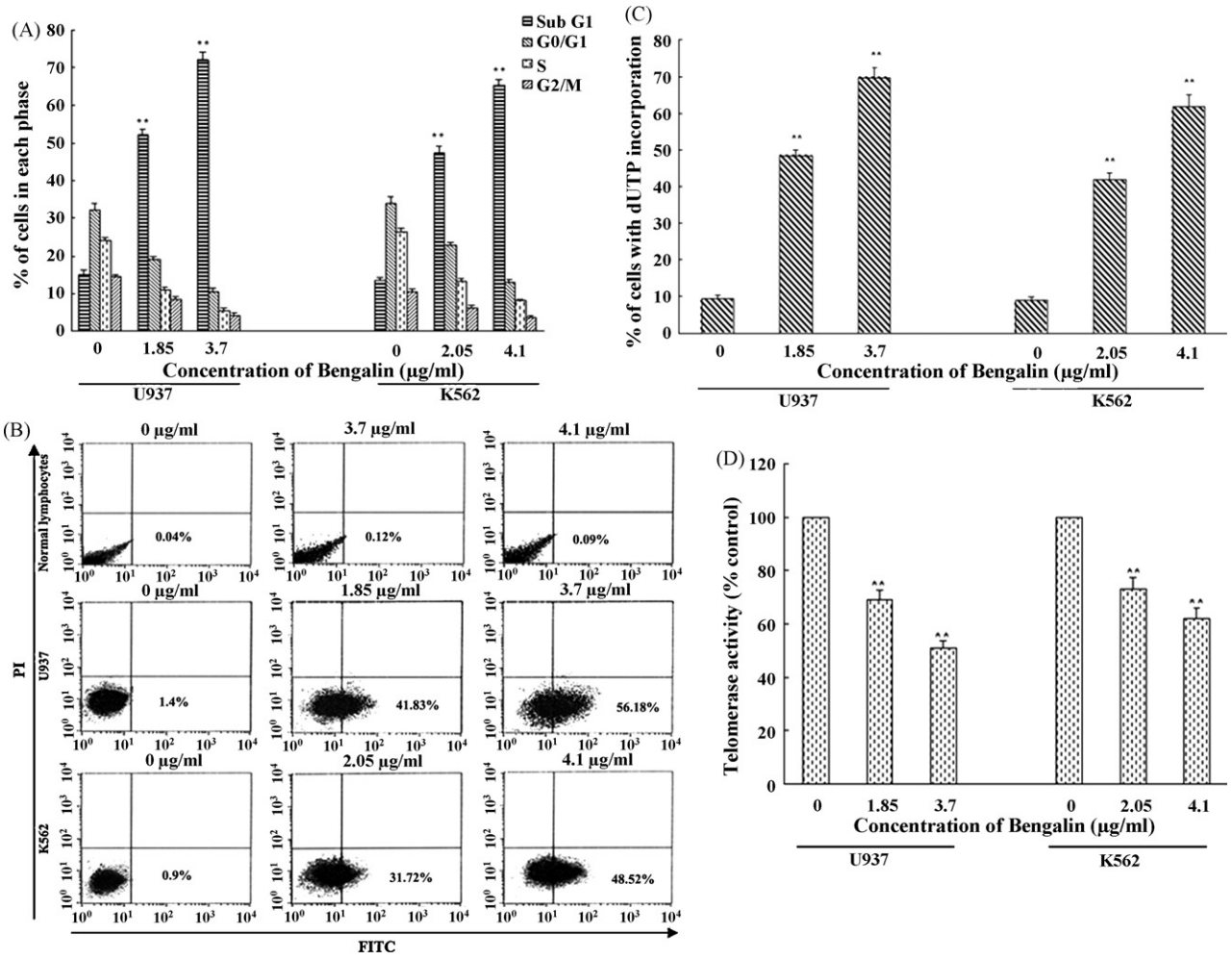

Fig. 3. Effect of Bengalin induced apoptosis in U937 and K562 cells. (A) U937 cells were treated with Bengalin at 1.85 and 3.7 μg/ml while K562 cells were treated with Bengalin at 2.05 and 4.1 μg/ml respectively for 24 h. Nuclear DNA was labeled by PI and determined by single label flow cytometry. Histogram showing the percentages of cells at various phases of cell cycle. Each value is expressed as mean ± S.D. ($n = 3$). $^{**}p < 0.01$ are the Bengalin treated groups compared to the control (0 μg/ml of Bengalin). (B) U937 cells were incubated with Bengalin at 1.85 and 3.7 μg/ml while K562 cells were incubated with Bengalin at 2.05 and 4.1 μg/ml respectively for 24 h. Normal human lymphocytes were also incubated with Bengalin at 3.7 and 4.1 μg/ml for 24 h. Control (0 μg/ml of Bengalin) and treated cells were labeled with PI and AnnexinV tagged-FITC and then fixed and analyzed on a flow cytometer. Dual parameter dot plot of FITC-fluorescence (*x*-axis) vs. PI fluorescence (*y*-axis) has been shown in logarithmic fluorescence intensity. *Quadrants*: lower left, live cells (−FITC); lower right, apoptotic cells (+FITC); upper right, necrotic cells or late phase of apoptotic cells (+PI, +FITC). All the experiments were done in triplicates and repeated three times. (C) Measurement of DNA fragmentation by TUNEL assay. U937 cells were treated with Bengalin at 1.85 and 3.7 μg/ml while K562 cells were treated with Bengalin at 2.05 and 4.1 μg/ml respectively for 48 h and then stained with FITC-conjugated dUTP and PI. Events were analyzed by a flow cytometer. Each value is expressed as mean ± S.D. ($n = 3$). $^{**}p < 0.01$ are the Bengalin treated groups compared with the control (0 μg/ml of Bengalin). (D) Estimation of telomerase activity. U937 cells were treated with Bengalin at 1.85 and 3.7 μg/ml while K562 cells were treated with Bengalin at 2.05 and 4.1 μg/ml respectively for 24 h and telomerase activity from cell extracts was evaluated. Percentage of telomerase activity in control (0 μg/ml of Bengalin) are shown as mean ± S.D. ($n = 3$). $^{**}p < 0.01$ are the Bengalin treated groups compared with the control.

tively) (Fig. 5C) activities occurred due to 24 h of incubation with Bengalin.

3.9. Expression of Bax, Bcl2, HSP70, HSP90, Apaf-1 and PARP after Bengalin treatment

To further evaluate the expression levels of various intracellular proteins related to apoptosis, the cells were treated with Bengalin for 24 h and this induced dose dependant changes in several intracellular proteins involved in the apoptotic cascade in both U937 and K562 cells. The upregulation of proapoptotic Bax, Apaf-1, cleaved PARP (85 kDa) and the downregulation of the antiapoptotic protein Bcl2 was seen in U937 (Fig. 6A) and K562 (Fig. 6B) cells. However, Bengalin downregulated HSP70 and HSP90 in a dose dependant manner.

3.10. Effect of Bengalin on leukemic cells in presence of HSP70 and HSP90 inhibitors

To evaluate the role of HSPs inhibition in apoptosis induction we further performed experiments in presence of HSP70 and HSP90 inhibitors. Co-treatment of Bengalin with HSP70 and HSP90 inhibitors (KNK437 at 100 μM and 17AAG at 500 nM concentrations, respectively) downregulated HSP70 and HSP90 expressions in U937 (Fig. 6C) and K562 (Fig. 6D) cells. Although inhibition of HSP70 and HSP90 upregulated Apaf-1, however co-treatment of respective HSP inhibitors with Bengalin additionally enhanced Apaf-1 in the treated U937 (Fig. 6C) and K562 (Fig. 6D) cells. Decrease in cell viability due to this co-treatment was also reflected by MTT assays (Fig. 6E and F). To investigate the relationship between HSPs and caspase-3 activity, we conducted caspase-3

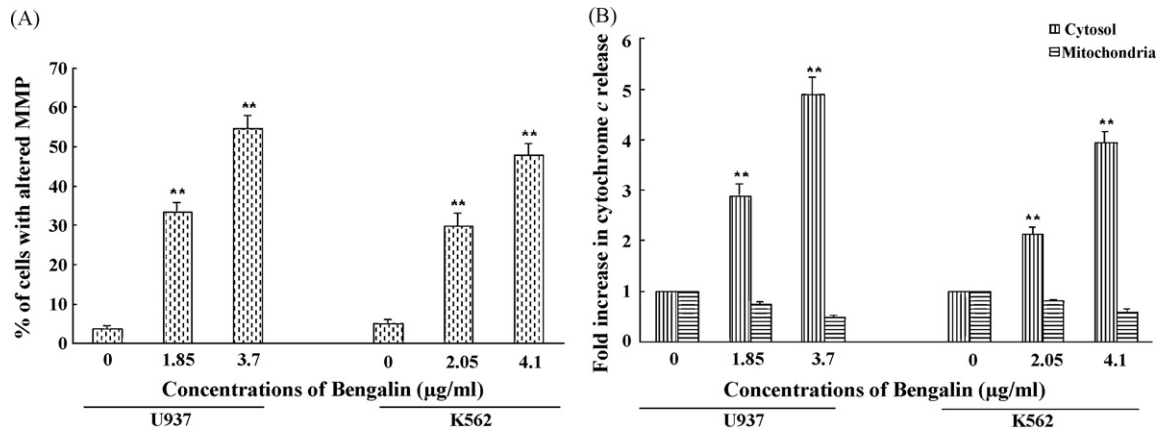

Fig. 4. Effect of different concentrations of Bengalin on mitochondrial membrane potential (MMP) and cytochrome *c* release in U937 and K562 cells. (A) Effect of Bengalin on MMP in U937 and K562 cells. U937 cells were treated with Bengalin at 1.85 and 3.7 μg/ml while K562 cells were treated with Bengalin at 2.05 and 4.1 μg/ml respectively for 24 h. Bengalin induced cells were labeled with JC-1 probe and the altered MMP was measured flow cytometrically. Each value is expressed as mean ± S.D. ($n = 3$). $^{**}p < 0.01$ are the Bengalin treated groups compared to the control (0 μg/ml of Bengalin). (B) Release of cytochrome *c* in cytosol from mitochondria in U937 and K562 cells after treatment with Bengalin in a dose dependent manner. U937 cells were treated with Bengalin at 1.85 and 3.7 μg/ml while K562 cells were treated with Bengalin at 2.05 and 4.1 μg/ml respectively for 24 h. Cells were harvested and cytochrome *c* level in cytosolic and mitochondrial fractions was determined by ELISA. Value of each experiment of cytochrome *c* was quantified and represented here as fold change. The cytochrome *c* level in cytosol and mitochondria in control (0 μg/ml of Bengalin) was considered as 1-fold. Each value is expressed as mean ± S.D. ($n = 3$). $^{**}p < 0.01$ are the Bengalin treated groups compared to the control.

activity assays after co-treatment of Bengalin with HSP70 (Fig. 7A) or HSP90 (Fig. 7B) inhibitors in both the cell lines. We observed an increase in caspase-3 activity due to inhibition of HSP70 (by 2.3- and 2.6-fold in U937 while 2.1- and 2.5-fold in K562 cells due to co-treatment of KNK437 with Bengalin at ½ IC_{50} or IC_{50} concentrations, respectively) and HSP90 (by 2.4- and 2.5-fold in for U937 while 2.3- and 2.6-fold for K562 cells due to co-treatment of 17AAG with Bengalin at ½ IC_{50} or IC_{50} concentrations, respectively) (Fig. 7A and B). We showed an increase in apoptosis upon co-treatment of Bengalin with KNK437 (48.3% and 59.6% for U937

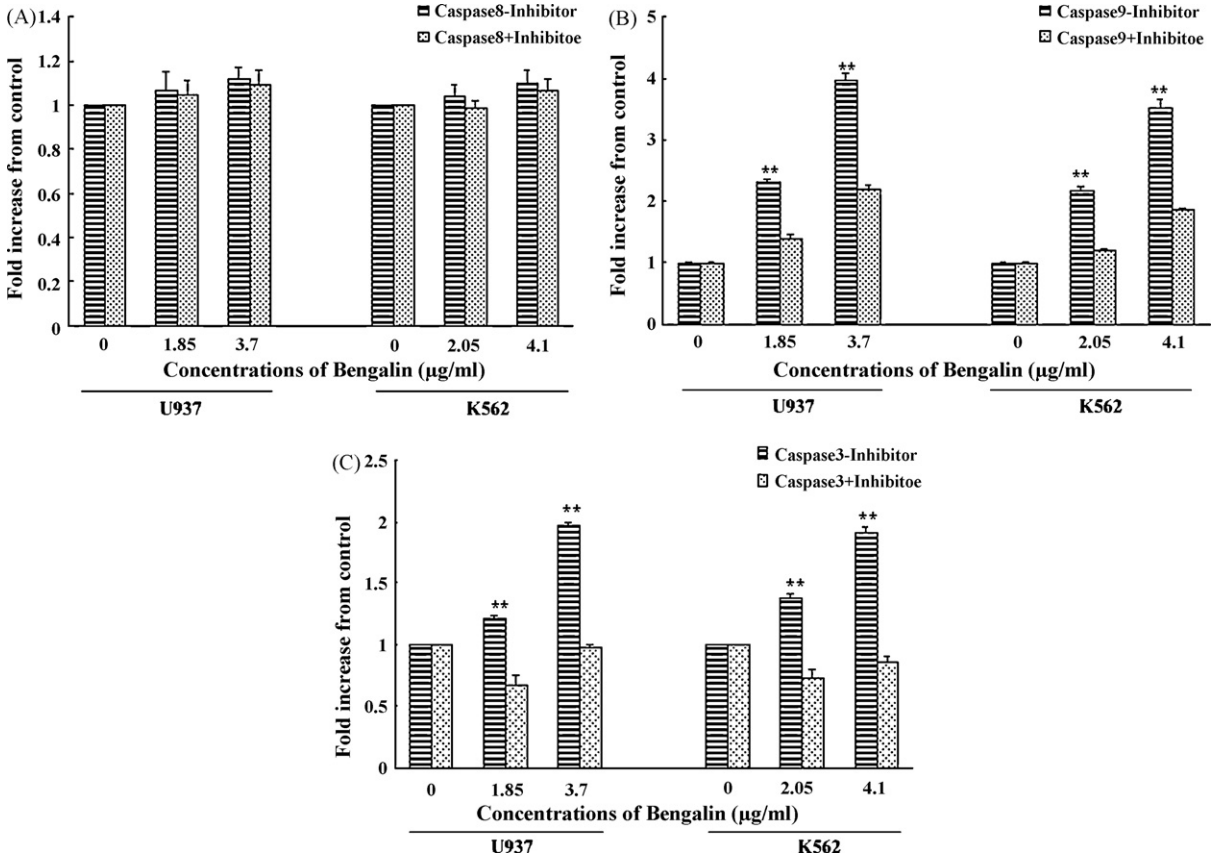

Fig. 5. Effect of Bengalin treatment on modulation of caspase-8, caspase-9, caspase-3 and proteins associated with apoptosis in U937 and K562 cells. Bengalin treatment was done for 24 h in U937 cells at 1.85 and 3.7 μg/ml and K562 cells at 2.05 and 4.1 μg/ml. They were then subjected to measurement of caspase activity by means of cleavage of color substrate (A) Ac-IETD-pNA for caspase-8, (B) LEHD-pNA for caspase-9, (C) Ac-DEVD-pNA for caspase-3. Effects of different inhibitors on the activation of casapses in Bengalin treated cells were also estimated in a co-culture. U937 and K562 cells were preincubated with respective caspase inhibitor for 2 h followed by Bengalin treatment for 24 h. Value of each experiment of caspase activity (with or without inhibitors) was quantified and represented here as fold change. The caspase activity in control (no treatment) was considered as 1-fold. Each value is expressed as mean ± S.D. ($n = 3$). $^{**}p < 0.01$ are the Bengalin treated groups as compared to control (0 μg/ml of Bengalin).

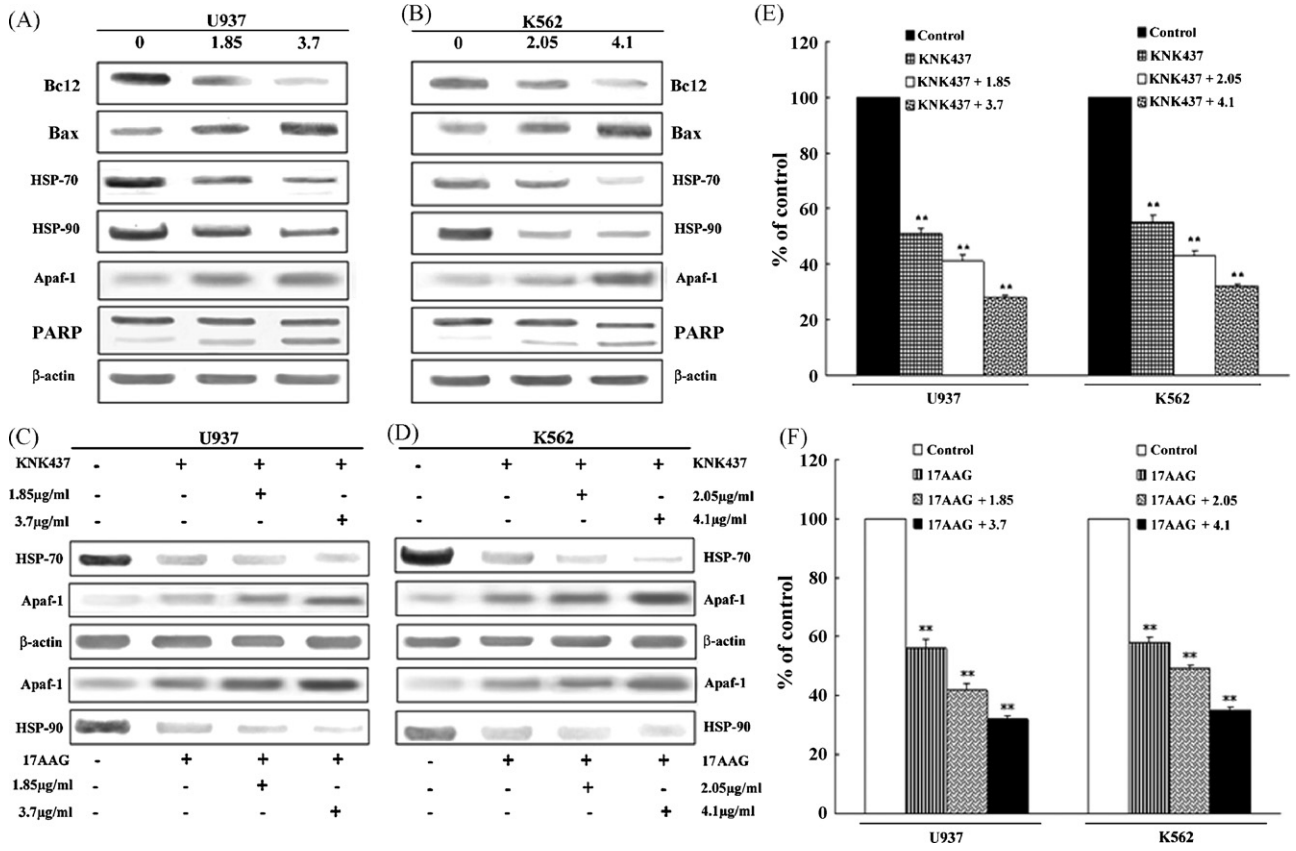

Fig. 6. Effect of Bengalin on apoptosis associated proteins and cell proliferations in presence or absence of HSP inhibitors. (A and B) Bengalin treatment was done for 24 h in U937 cells at 1.85 and 3.7 μg/ml and K562 cells at 2.05 and 4.1 μg/ml. Protein from the total cell lysate was subjected to SDS-PAGE and western blot using Bcl2, Bax, PARP, HSP70, HSP90, Apaf-1 and β-actin antibody. Representative blots from three independent experiments gave identical results. (C and D) U937 and K562 cells were incubated with KNK437 (100 μM) or 17AAG (500 nM) alone or in combination with Bengalin for 24 h. The blot was incubated with primary HSP70, HSP90 and β-actin antibodies. Representative blots from three different experiments showed identical results. The relative intensity of each band was measured after normalization with the intensity of β-actin in a blot (given below each Western blot). Here control served as group with no Bengalin treatment. (E and F) U937 and K562 cells were treated with KNK437 or 17AAG alone or in combination with Bengalin for 48 h. MTT assay was performed to detect the inhibition of cell proliferation. Each value is expressed as mean ± S.D ($n = 3$). $^{**}p < 0.01$ are the Bengalin treated groups as compared to control (0 μg/ml of Bengalin).

and 50.2% and 67.8% for K562 due to co-treatment with ½ IC_{50} or IC_{50} Bengalin concentrations, respectively) and 17AAG (42.3% and 51.6% for U937 and 45.6% and 55.9% for K562 due to co-treatment with ½ IC_{50} or IC_{50} Bengalin concentrations, respectively) (Fig. 7C and D). These findings were further supported by the TUNEL assay where co-treatment further increased the percentage of DNA fragmentation in both the cell lines (59.8% and 81.2% for U937 while 50.5% and 71.2% TUNEL positive K562 cells due to co-treatment of KNK437 with ½ IC_{50} or IC_{50} concentrations of Bengalin, respectively (Fig. 7E); again 55.6% and 75.6% for U937 while 46.4% and 64.7% TUNEL positive K562 cells due to co-treatment of 17AAG with ½ IC_{50} or IC_{50} concentrations of Bengalin, respectively) (Fig. 7F).

4. Discussion

Scorpion venom consists of several components of which proteins and peptides are the chief contributors for general functioning of the venom like inhibition of growth and proliferation of various tumors. Venom from *L. quinquestriatus* inhibited growth of breast and prostate cancer cells *in vitro* [26] while crude venom from *B. martensii* Karsch induced apoptosis in Raji and Jurkat cells by upregulating PTEN [27]. Antitumor peptides have been isolated from venom of *B. martensii* which inhibited growth of ehrlich ascites carcinoma, S-180 fibrosarcoma cells *in vivo* [2] and human glioma cells SHG-44 *in vitro* [5]. However there is no report of any anticancer proteins or peptides from scorpion venom having antileukemic activity or their molecular mechanism of action. In our previous study, we had established the antileukemic potential of the crude venom of Indian black scorpion *H. bengalensis* Koch [20]. Here we have isolated and purified a protein Bengalin, from the venom of Indian black scorpion *H. bengalensis* Koch having anticancer activity against human leukemic U937 and K562 cells and elucidated its probable molecular mechanism involved in leukemic cell death.

P3 and P4 purified by ion exchange chromatography, showed cytotoxic activities against human leukemic cells. Since P3 showed a single band and higher cytotoxicity than P4, which showed two distinct bands in SDS PAGE, hence it was selected for further purification and anticancer studies. N-terminal amino acid sequence of Bengalin showed partial resemblance with 5′ nucleotidase [28]. However the sequence does not match with any existing proteins or peptides from the scorpion toxin database. The cytotoxic effect of Bengalin was more pronounced for U937 than K562 suggesting that U937 cells are more susceptible to Bengalin induced death. In comparison with the whole venom [20] the IC_{50} value of Bengalin is 11.22- and 21.54-fold lower for U937 and K562 cells, respectively. However using the IC_{50} concentration of Bengalin for either of the cell lines, there was no significant death of normal human lymphocytes. Since *H. bengalensis* venom induced apoptosis in both U937 and K562 cells [20], we decided to examine whether Bengalin induced apoptosis in these leukemic cells. Therefore we observed the morphology of the treated cells using AO and EtBr. It is reported that AO penetrates living cells, causing these cells to fluoresce green, while EtBr enters dead cells causing them to fluoresce red [29]. In our experiments, cells incubated with

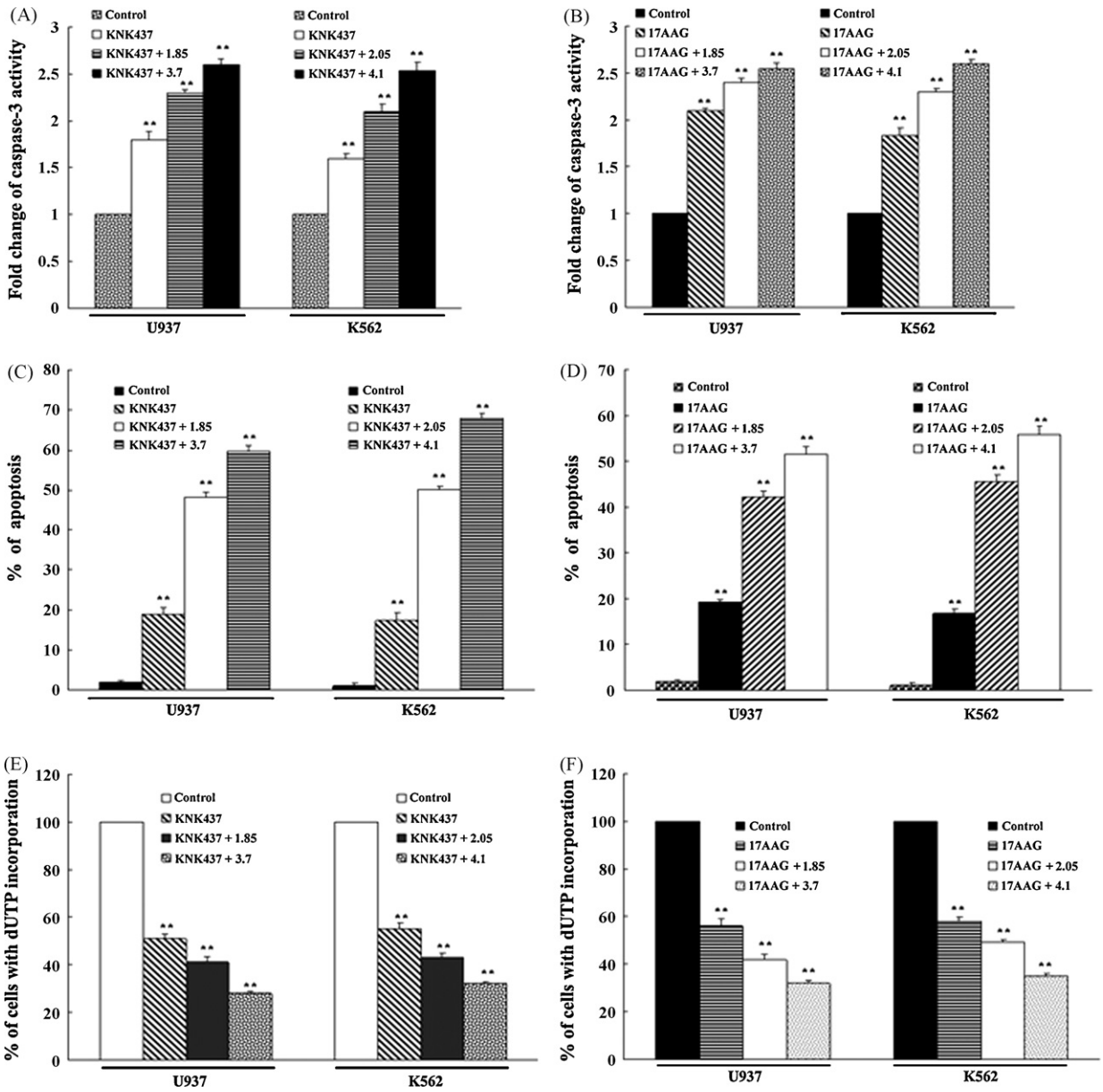

Fig. 7. Modulation of the effect of Bengalin on caspase-3 activity, apoptosis and DNA fragmentation in presence of respective HSP inhibitors (KNK437, inhibitor of HSP70 at a concentration of 100 μM or 17AAG, inhibitor of HSP90 at a concentration of 500 nM). (A and B) Caspase-3 activity was measured in U937 and K562 cells after incubation with KNK437 or 17AAG alone or in combination with Bengalin for 24 h. Results are representative of three independent experiments. Value of each experiment of caspase activity was quantified and represented here as fold change. The caspase activity in control (0 μg/ml of Bengalin) was considered as 1-fold. Each value is expressed as mean ± S.D. Data represented as relative caspase-3 activities. $^{**}p < 0.01$ are the Bengalin treated groups as compared to control (0 μg/ml of Bengalin). (C and D) U937 and K562 cells were incubated with KNK437 or 17AAG alone or in combination with Bengalin for 24 h. Apoptosis was determined by flowcytometry. Each value is expressed as mean ± S.D ($n = 3$). $^{**}p < 0.01$ are the Bengalin treated groups as compared to control (0 μg/ml of Bengalin). (E and F) Estimation of DNA fragmentation by TUNEL assay in U937 and K562 cells after incubation with KNK437 (inhibitor of HSP70) or 17AAG (inhibitor of HSP90) alone or in combination with Bengalin for 48 h. Each value is expressed as mean ± S.D ($n = 3$). $^{**}p < 0.01$ are the Bengalin treated groups as compared to control (0 μg/ml of Bengalin).

Bengalin showed red fluorescence indicating dead cells. Confocal microscopy of treated cells also revealed chromatin condensation along with highly disintegrated nuclear material in contrast to the intact nuclei of untreated cells.

Externalization of phosphatidylserine is an early apoptotic event and serves as a binding site for AnnexinV-FITC. Counterstaining with PI enables the distinction between the apoptotic and necrotic cells. A significant number of Bengalin treated U937 and K562 cells were present in the AnnexinV-FITC positive quadrant during flow cytometry, indicating early apoptosis. But Bengalin treatment did not induce any significant apoptosis in normal human lymphocytes. A characteristic feature of apoptotic cell death is fragmentation of nuclear DNA and this was clearly visible in our dose dependant experiments with Bengalin treated leukemic cells. A marked increase in the hypodiploid DNA content was also observed in the sub G1 phase of the treated cells stained with PI, signifying an arrest in the cell cycle. These observations prompted us to further study whether the early apoptotic events were mitochondria mediated. The extrinsic pathway signal is carried downstream from death receptors to procaspase-8 [30], while caspase-8 produced by autoproteolytic cleavage of procaspase-8 and then subsequently activates caspase-3 to initiate apoptotic events. The mitochondria associated intrinsic apoptotic pathway is mediated by caspase-9. Therefore we evaluated the activity of these two caspases to distinguish whether cells are dying through extrinsic or intrinsic pathway of apoptosis [31]. Bengalin treat-

ment of both U937 and K562 cells failed to significantly elevate caspase-8 levels but it did elevate the levels of caspase-9. Hence it is assumed that Bengalin induced apoptosis may be mediated by mitochondria. Disruption of MMP is a common occurrence during apoptosis [32]. This disruption results from opening of mitochondrial membrane pores and Bcl2 prevents their opening [33]. Bax facilitates the opening of pores [34] and plays a crucial role in bringing about changes in MMP along with the liberation of cytochrome *c* to the cytosol. This phenomenon is prevented by the antiapoptotic protein Bcl2 [8] thus an increase in the Bax:Bcl2 ratio is observed during apoptosis [35]. A significant number of U937 and K562 cells with altered MMP were observed due to Bengalin treatment. Upregulation of Bax along with downregulation of Bcl2 was also apparent from our Western blot experiments. These observations confirmed the possibility that the apoptosis due to Bengalin was mediated by the intrinsic pathway. Increase in mitochondrial membrane permeability augments the release of cytochrome *c* and the subsequent activation of downstream caspases [8,13,36]. This released cytochrome *c* then associates Apaf-1 to form apoptosome [37] which in turn activates caspase-9 and downstream caspase-3 [38]. In our studies a significant number of treated cells showed altered mitochondrial membrane potential. Cytochrome *c* levels were elevated along with a significant increase in the caspase-9 and caspase-3 levels due to Bengalin action. Hence our results reflected a number of characteristics of the mitochondria mediated intrinsic apoptotic pathway. There are several reports which support the hypothesis that telomere function and telomere length also play important roles in cell survival and evasion of apoptosis [39]. Our results showed that Bengalin also significantly inhibited telomerase activity in the human leukemic cells and this may contribute to its apoptotic activity.

Heat shock proteins belong to a family of highly conserved molecular chaperones which are expressed during various stress conditions [40]. Among them, HSP70 and HSP90 play crucial roles in cell survival and are therefore considered to be antiapoptotic [41]. HSP70 acts downstream of cytochrome *c* release but it also works upstream of caspase-3 [42]. More specifically HSP70 functions by blocking the attachment of procaspase-9 to the Apaf-1 of the apoptosome complex thereby preventing cellular apoptosis [16]. HSP90 also performs downstream of cytochrome *c* release but it prevents Apaf-1 oligomerization and the association of procaspase-9 [43]. In our study, Bengalin showed a dose dependant reduction in expression of HSP70 and HSP90 which may therefore contribute to its apoptotic activity. We also evaluated a dose dependent increase of Apaf-1. Thus our results suggest that Bengalin induced human leukemic cells apoptosis may be partially due to the consequences of downregulation of HSP70, HSP90 and upregulation of Apaf-1. In addition DNA damage activates intact PARP molecule (116 kDa), which is cleaved by caspase-3 to yield a comparatively low molecular weight (85 kDa) fragment, which is a considerable marker of apoptosis [29]. In our studies Bengalin augmented cleavage of PARP in both the cell lines.

We also investigated the effect of inhibitors (KNK437 for HSP70 and 17AAG for HSP90) on HSP70 and HSP90 alone, as well as with co-treatment of Bengalin on both cell lines. Although the inhibitors could reduce expression levels of HSPs, co-treatment with Bengalin further reduced of expression of both the HSPs in U937 and K562 cells. Additionally co-treatment of HSPs inhibitors with Bengalin further enhanced the expression of Apaf-1 when compared with Bengalin alone. Since co-treatment of Bengalin with HSP inhibitors also upregulated caspase-3, as well as reduced cell viability and induced apoptosis hence it can be postulated that HSPs might play a crucial role in apoptosis by modulating caspase-3 activities in both U937 and K562 cells. This fact was further substantiated by our results which demonstrated a significant increase in the percentage of apoptotic U937 and K562 cells upon co-treatment of Bengalin with HSP inhibitors. By using TUNEL assay, we also showed that co-treatment of Bengalin and HSP inhibitors further increased DNA fragmentations.

To conclude we can say that the Indian black scorpion venom fraction Bengalin induced U937 and K562 cell death through induction of apoptosis, which is predominantly mediated by the mitochondrial pathway with the participation of pro and antiapoptotic proteins. Our experiments also showed that the inhibition of HSP70 as well as HSP90 might be important features in Bengalin mediated apoptosis in U937 and K562 cells. Moreover suppression of telomerase activitiy and initiation of DNA damage were evidenced in Bengalin mediated apoptosis of human leukemic cells. The present study may hold clues to future anticancer drug development from scorpion venom and its mechanism of action; however, a more detailed study needs be carried out to discover the structure and the site(s) of activity of this isolated protein. To our knowledge, this is the first report of apoptosis induction in human leukemic cells and its mechanism induced by any pure protein isolated from scorpion venom.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors are thankful to the Department of Science and Technology, Government of India for partial financial support and Council of Scientific and Industrial Research, Government of India for providing Senior Research Fellowship to Shubho Das Gupta.

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