RESEARCH COMMUNICATION

Punarnavine Induces Apoptosis in B16F-10 Melanoma Cells by Inhibiting NF-kB Signaling

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Abstract

The objective of this study was to assess the effect of Punarnavine, an alkaloid isolated from *Boerhaavia diffusa*, on apoptosis in B16F-10 melanoma cells. Treatment of B16F-10 melanoma cells with nontoxic concentrations of Punarnvine resulted in the presence of apoptotic bodies and DNA fragmentation in a dose dependent manner. Cell cycle analysis and TUNEL assays also confirmed the observation. The apoptotic genes p53 and caspase-3 were found upregulated in Punarnavine treated cells, whereas the antiapoptotic gene Bcl-2 was downregulated. The inhibited nuclear translocation of NF-κBp65, NF-κBp50, NF-κB-c-Rel, c-Fos, ATF-2 and CREB-1 in Punarnavine treated B16 F-10 cells pointed to suppression of NF-κB signaling by Punarnavine. All these results demonstrate that Punarnavine induces apoptosis via activation of p53 induced caspase-3 mediated pro-apoptotic signaling and suppression of NF-κB induced Bcl-2 mediated survival signaling.

Key Words: Punarnavine - apoptosis induction - B16F-10 melanoma cells - NF-κB inhibition

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Introduction

Apoptosis is a process of programmed cell death which takes part in both embryonic tissue morphogenesis and adult tissue regulation by balancing homeostasis. Abnormalities in cell death control can contribute to a variety of diseases, including cancer, autoimmunity and degenerative disorders. The most evident morphological signs of apoptosis are cellular shrinkage, membrane blebbing, nuclear condensation and fragmentation, which are the final steps of consequential signaling cascades (Huppertz et al, 1999). The swelling of the outer mitochondrial membrane (Vander et al, 1997) and release of cytochrome c (Kluck et al., 1997; Yang et al., 1997) and apoptosis inducing factor, an oxidoreductase-related flavoprotein (Susin et al., 1999) from the mitochondrial intermembrane space are also reported during apoptosis. The morphological changes that we recognize as apoptosis and associated biochemical changes seen in a eukaryotic cell are caused by proteases. Specifically activation of a family of intracellular cysteine proteases which cleave their substrates at aspartic acid residues, known as caspases for cysteine aspartyl-specific proteases (Alnemri et al., 1996).

The p53 protein plays a central role in the cell. Cells that are insulted by oncogene expression, DNA damage or other forms of stress stabilize the p53 protein by phosphorylation or other modifications (Vogelstein et al., 2000; Xu, 2003). Once activated, p53 acts as a transcription factor and activates or represses a variety of genes involved in, for example, cell-cycle regulation, the induction of apoptosis, or senescence (e.g., BAX, NOXA, PUMA, BID, CD95, APAF-1, DR5, p53AIP1) (Miyashita and Reed, 1995; Wu et al., 1997; Muller et al., 1998; Oda et al., 2000; Moroni et al., 2001; Nakano and Vousden, 2001; Sax et al., 2002).

Bcl-2 is an integral membrane protein, even in healthy cells (Janiak et al., 1994), This prosurvival Bcl-2 family protein can prevent cytochrome c release, and hence activation of caspases. NF-κB has been implicated in carcinogenesis because it plays a critical role in cell survival, cell adhesion, inflammation, differentiation and cell growth. Cancer is a hyperproliferative disorder that results from tumor initiation and tumor promotion and ultimately produces tumor metastasis. Notably, several genes involved in cellular transformation, proliferation and apoptosis are regulated by NF-κB. Constitutive expression of NF-κB has been shown in cell lines derived from breast, ovarian, colon, pancreatic, thyroid, prostate, lung, head and neck, bladder and skin tumors (Rayet and Gelinas, 1999).

Punarnavine is an alkaloid found in the plant *Boerhaavia diffusa* (family-Nyctaginaceae), a perennial herb ascribed the Sanskrit name punarnava (Punah punarnava bhawati iti, which translates as "that which becomes fresh again and again . . .") a drug known since long in the indigenous system of medicine in India. It is widely distributed in the tropics and subtropics (CSIR, 1988). The chemical formula of Punarnavine is $C_{17}H_{22}N_2O$, having a melting point of 236-237°C

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(Agarwal and Dutt, 1935; Basu and Sharma, 1947; Surange and Pendse, 1972). It is also considered as the active principle in the plant extract (Sethi and Zafar, 2003). We have already reported antimetastatic (Manu and Kuttan, 2009b) and immunomodulatory (Manu and Kuttan, 2007, 2009a) activities of Punarnavine. Here in the present study, we analyzed the effect of Punarnavine on induction of apoptosis in B16F-10 melanoma cells.

Materials and Methods

Materials

B16F-10 melanoma cells were obtained from National Centre for Cell Science, Pune, India. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics. Dulbecco's modified Eagle's medium was purchased from Himedia Laboratory, Mumbai, India. Cells-cDNA kit was purchased from Ambion Inc (Austin Tex, USA). Oligonucleotide primer sequences were purchased from Maxim Biotech Inc (San Francisco, Calif, USA) (Table 1). All other reagents used were of analytical reagent grade. The alkaloid Punaranavine was isolated from the plant *Boerhaavia dffusa* Linn. as per the protocol (Agarwal and Dutt, 1935; Manu and Kuttan, 2007; 2009a,b). Authenticated *B.diffusa*. Linn. was obtained from the Amala Ayurvedic centre Pharmacy, Thrissur, India. The overall yield of Punarnavine was 0.01%. Melting point of crystallized Punarnavine is 236°C. The isolated compound gave positive results for the alkaloid Punarnavine giving a green color with $FeCl_3$, a greenish yellow color with concentrated H_2SO_4, red color with HNO_3, no color with HCl, a black precipitate with KI_3, a blue color followed by a blue precipitate with phosphomolybdic acid, a grayish white precipitate with phosphotungstic acid, and a brown precipitate with Dragendorff's reagent.

Determination of the cytotoxic effect of Punarnavine on B16F-10 melanoma cells

Nontoxic concentrations of Punarnavine against B16F-10 melanoma cells, was determined. B16F-10 cells (5000 cells/well) were plated in a 96-well flat-bottom titer plate and incubated at 37°C in 5% CO2 atmosphere. After 24 hours, escalating concentrations of Punarnavine (1-500 µg/ml) were added and the incubation was continued for 48 hours under the same conditions. Cell viability was determined by the MTT assay.

Determination of the effect of Punarnavine on proliferation of B16F-10 melanoma cells

B16F-10 melanoma cells (5000 cells/well) were seeded in a 96-well culture plate and incubated at 37 °C in 5% CO_2 atmosphere. After 24 h, various nontoxic concentrations of Punarnavine based on the MTT assay (1, 5 and 10µg/ml) were added and further incubated for 48 h. ^{3}H-thymidine was added to each well (1µCi/well) and incubation was continued for additional 18 h. After completing incubation, the plates were centrifuged and the culture supernatant was removed, the cells were washed three times with PBS and then treated with ice cold PCA for 15 min. The resulting precipitate was dissolved in 0.5 N NaOH and was added to the scintillation fluid and the radioactivity was counted using a Rack Beta liquid scintillation counter.

Determination of the effect of Punarnavine on cell cycling of B16F-10 melanoma cells

One million B16F-10 cells -suspended in DMEM containing 10% FCS and antibitotics-100 units/ ml penicillin, 100 µg/ml streptomycin -were seeded in a culture flask and incubated for 10 hours at 37˚C in CO_2 atmosphere with and without Punarnavine (10µg/ml). After incubation the cells were washed in PBS and analyzed for cell cycle changes in flow cytometer using Becton-Dickson kit as per the manufacture's protocol.

Determination of the effect Punarnavine on the apoptosis of B16F-10 cells.

1) Morphological analysis. B16F-10 melanoma cells (5,000 cells/ well) suspended in DMEM supplemented with 10% FCS, 100µg/ml streptomycin and penicillin and 2mmol/l glutamine were plated in 96-well flat bottom titer plate and incubated for 24 h at 37°C in 5% CO_2 atmosphere. After 24 h, different nontoxic concentrations of Punarnavine (1, 5 and 10 µg/ml) were added to the cells and incubated further for 48 h under the same conditions. The cells were then washed twice with PBS (pH 7.4), fixed with 5% formalin and stained using haematoxylin and eosin, observed under phase contrast microscope and photographs were taken. Apoptosis was characterized by examining the morphological changes such as chromatin condensation, nuclear condensation, membrane blebbing or presence of apoptotic bodies.

2) DNA fragmentation analysis. One million B16F-10 melanoma cells were treated with different nontoxic concentrations of Punarnavine (1, 5 and 10 µg/ml) for 48 hrs. After incubation, the cells were treated with 0.1ml lysis buffer (100 mmol/L Tris-HCl, pH 8.0, containing 0.2% Triton-X 100, and 1mmol/L EDTA) for 10 minutes at -20°C. DNA was extracted using phenol-chloroform method, precipitated with chilled ethanol and resuspended in Tris/EDTA buffer (10 mmol/L Tris-HCl, pH 8.0 and 1mmol/L EDTA). DNA samples were separated by electrophoresis in 1% agarose gels. DNA was stained with ethidium bromide and photographed under UV light.

3) TUNEL assay.Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was done to detect apoptosis via DNA fragmentation using Apoptag Peroxidase in situ Apoptosis detection kit, CHEMICON International, Inc.

B16F-10 melanoma cells (5,000 cells/ well) suspended in DMEM supplemented with 10% FCS, 100µg/ml streptomycin and penicillin and 2mmol/l glutamine were plated in 96-well flat bottom titer plateds and incubated for 24 h at 37°C in a 5% CO_2 atmosphere. After 24 h, 10µg/ml Punarnavine was added to the cells, which were then incubated further for 48 h under the same conditions. The cells were washed in PBS and stained as per manufacturer's protocol.

Table 1. Primer Sequences for Different Genes

Gene	Primer sequences
p53	
Forward	5'-CGGAGGTCGTGAGACGCTG-3'
Reverse	5'CACATGTACTTGTAGTGGATGGTGG-3'
Caspase -3	
Forward	5'-GAGCACTGGAATGTCATCTCGCTCTG-3'
Reverse	5' TACAGGAAGTCAGCCTCCACCGGTATC-3'
BCl-2	
Forward	5'-CAGCTGCACCTGACGCCCTT-3'
Reverse	5' CCCAGCCTCCGTTATTCTGGA-3'
GAPDH	
Forward	5' CGTCCCGTAGACAAAATGGT 3'
Reverse	5' CCTTCCACAATGCCAAAGTT 3'

Determination of the effects of Punarnavine on the expression of p53, caspase-3 and Bcl-2 genes in B16F-10 cells.

RT-PCR and Gene expression studies. B16F-10 melanoma cells (2x10 cells) suspended in serum free DMEM (250µl) were seeded in 96-well titre plate and incubated for 24 h at 37°C in 5% CO2 atmosphere. Punarnavine (10 µg/ ml) were added to well and incubation was continued for another 4 h. After 4 h, cDNA was synthesised using cells to cDNA kit (Ambion Inc.). Cells were washed with phosphate buffered saline and heated in cell lysis buffer (provided in the kit) to release the RNA into the solution. This was followed by a heating step to inactivate endogenous RNases. The genomic DNA was further degraded by treating with DNase followed by inactivation of DNase by heating at 70°C. Reverse transcription was performed at 42°C for 50 minutes using Moloney murine leukemia virus RT (supplied along with the kit). Gene expression analysis was done by PCR. The mouse bcl-2, caspases-3 and p53genes (Table 1) were amplified against GAPDH standard. The cycling conditions used were as follows: 1 minute at 94°C, 1 minute at 58°C and 1 minute at 72°C for 40 cycles, followed by a 10-minute extension at 72°C. Amplified PCR products were subjected to electrophoresis on a 1.8% agarose gel and stained with ethidium bromide and photographed under UV light.

Determination of the effect Punarnavine on transcription factor profile.

Preparation of nuclear extracts: Nuclear extracts were prepared by the previously published method (Dignam, 1983). B16F-10 cells were exposed to Punarnavine (10µg/ ml) for 2 hr, washed with PBS twice and then treated with TNF-α for 30 minutes and lysed using lysis buffer for 15 minutes on ice. The cell suspension was centrifuged and disrupted using a syringe and centrifuged at 10,000-11,000 x g for 20 minutes. The crude nuclear pellet obtained is suspended in nuclear extraction buffer. Nuclei were disrupted by using a fresh syringe, centrifuged and the supernatant was collected. Protein concentrations of the nuclear extracts were estimated using standard Bradford method and stored at -70˚C.

Transcription factor profiling: This was done with the BD MercuryTM Transfactor kit obtained from BD Biosciences (Palo Alto, Calif). This kit provided rapid, high-throughput detection of specific transcription factors, namely NF-κBp65, NF-κBp50, NF-κBc-Rel, c-Fos, ATF-2 and CREB-1 in the nuclear extract. Using ELISA-based format, the transfactor kit detected the DNA-bound transcription factors (Shen et al., 2002) by specific primary antibody towards NF-κBp65, NF-κBp50, NF-κB c-Rel, c-Fos, ATF-2 and CREB-1. A horse radish peroxidase conjugated secondary antibody was then used to detect the bound primary antibody. The enzymatic product was measured with standard microtitre plate reader at 655nm. Percentage inhibition was calculated by the formula, 100-((OD of treated / OD of control) X 100).

Results

Cytotoxic effect Punarnavine on B16F-10 melanoma cells

Punarnavine was found to be 19%, 12.1%, 11% , 8.8%,7% toxic to B16F-10 melanoma cells at the concentrations of 500, 250, 100, 50, 25 µg/ ml respectively. The concentrations 10, 5 and 1 µg/ml were nontoxic to the cells.

Effect of Punarnavine on proliferaton of B16F-10 melanoma cells.

The effect of Punarnavine on the rate of proliferation of B16F-10 cells was determined by ^{3}H-thymidine incorporation assay. Control B16F-10 cells showed very high rate of proliferation (5,171.7±167 cpm). Administration of Punarnavine at a concentration of 10µg/ ml significantly inhibited proliferation of B16F-10 cells. Considerable inhibition in the rate of proliferation of B16F-10 cells was also observed when Punarnavine was administered at concentrations of 5µg/ml and 1µg/ml (Table 2).

Effect of Punarnavine on cell cycling of B16F-10 melanoma cells.

In untreated control, 46.6% of the cells were in G0/ G1-phase, 17.9% in G2/M-phase and 13.3% in S-phase. The percentage of cells with fragmented DNA (sub G0) were only 2%. Treatment with 10µg/ml Punarnavine could increase the percentage of cells with fragmented DNA (subG0/G1) to 71%. But the cells in G0/G1 phase, S-phase and G2/M phase decreased considerably (14.6%, 4.27%, 9.44% respectively for Punarnavine.

Effect of Punarnavine on inducing Apoptosis in B16F-10 melanoma cells

The presence of apoptotic bodies in cells treated with

Table 2. Effect of Punarnavine on Proliferation of B16F-10 Melanoma Cells

Treatment		Counts Per Minute	% Inhibition
Control		5,171.7±167	
Punarnavine	(10µg/ml)	2,398.7±15*	53.6
	(5 µg/ml)	3,057.6±59*	40.9
	(1 µg/ml)	4,062.7±60*	21.4

B16F-10 (5000 cells/well) plated in 96-well flat bottom titre plate and incubated at 37°C in 5% CO_2 atmosphere with and without ursolic acid and rate of proliferation was checked by H^3-thymidine incorporation assay. The statistical analysis was done by using Dunnett's test. *p<0.001

cytotoxic agents has been considered as a marker of cell death by apoptosis and has been linked to DNA fragmentation associated with apoptosis. Microscopic evaluation of B16F-10 cells treated with different doses of Punarnavine confirmed the presence of apoptotic bodies (Figure 1a). The enhanced DNA fragmentation in Punarnavine treated cells compared to the untreated control also support the above observation (Figure 1b).

TUNEL assay also confirmed the presence of apoptotic bodies by staining free 3'-OH termini enzymatically labeled with modified nucleotides of the Punarnavine treated B16F-10 cells which support the above observation (Figure 1c). These new DNA ends that are generated upon DNA fragmentation are typically localized in morphologically identifiable nuclei and apoptotic bodies. In contrast, the normal B16F-10 cells (control) which have relatively insignificant numbers of DNA 3'-OH ends didn't stain in above experiment.

Effect of Punarnavine on the expression of p53, casapse-3 and bcl-2 genes.

Agarose gel electrophoresis of the amplified samples show (Figure 2) that the expression of p53 and caspase-3 were upregulated in Punarnavine (10µg/ml) treated cells compared to the nontreated control cells. Bcl-2 gene was expressed in control cells but showed diminished expression in Punarnavine treated cells.

Effect of Punarnavine on NF-κB, c-fos, ATF-2 and CREB-1.

The DNA bound transcription factor was analyzed by ELISA using correspoding primary antibody which was detected using horseradish peroxidase-conjugated secondary antibody. The enzymatic product was measured spectrophotometrically. Punarnavine could significantly ($p<0.001$) inhibit the translocatin of NF-κB subunits such as p65 (71.8%), p50 (78.6%) and c-Rel (80.2%). The nuclear translocation of c-fos (75.8%), ATF-2 (68.9%) and CREB-1 (73.7%) was also inhibited by the treatment with Punarnavine.

Discussion

Cancer cells are characterized by a failure of cell cycle control which results in their over proliferation (Griffiths et al, 2002). Apoptosis or programmed cell death, plays an important role in many physiologic and pathologic processes (Thompson, 1995). Major function of apoptosis is the elimination of damaged cells. For example, cells with genetic damage caused by exposure to carcinogens may be deleted by undergoing apoptosis, thereby preventing their replication and the accumulation of clones of abnormal cells. There is increasing evidence to support the hypothesis that failure of apoptosis may be an important factor in the evolution of cancer and its poor response to chemotherapy and radiation (reviewed by Watson (Watson, 1995). In the present study, Punarnavine treatment could induce apoptosis in B16F-10 melanoma cells in dose dependent manner. Deregulated cell proliferation together with suppressed apoptosis constitutes the minimal common platform upon which all

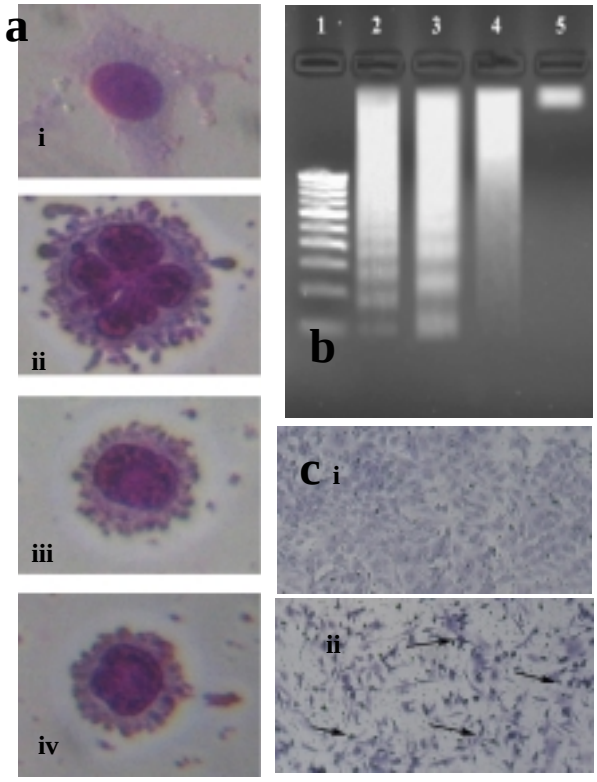

Figure 1. Effects of Punarnavine on B16F-10 Melanoma cells. a) Morphology. i) Untreated control; ii) 10µg/ml; iii) 5 µg/ml; iv) 1 µg/ml; b) DNA integrity. Lanes, 1, molecular weight markers; 2, DNA from Punarnavine (10µg/ml) treated cells; 3 (5 µg/ml); 4 (1 µg/ml); 5untreated control cells; c) TUNEL assay; i) Control; ii) Punarnavine (10 µg/ml)

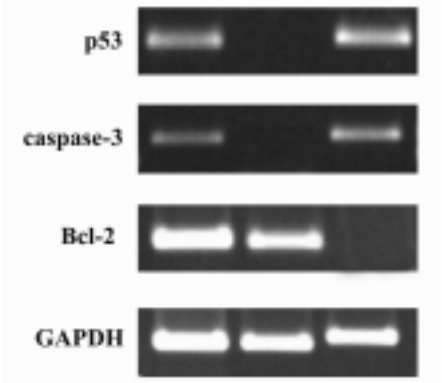

Figure 2. Effects of Punarnavine on Expression of p53, caspase-3, bcl-2 and GAPDH in B16F-10 Melanoma Cells. B16F-10 cells ($5x10^4$) were treated with or without Punarnavine for 4 h, and cDNA was synthesized and amplified with appropriate primers for p53, caspase-3, bcl-2 and house keeping gene GAPDH. Left, positive control; Middle, Control; Right, Punarnavine treated (10 µg/ml)

neoplastic evolution occurs. The H-3 thymidine incorporation assay showed significantly reduced rate of proliferation of B16F-10 melanoma cells after the treatment with Punarnavine.

The p53-dependent apoptosis pathway is of particular interest in cancer therapy since it can be used to eliminate tumors. The involvement of p53 in multiple biological pathways suggests that its loss may have dramatic consequence for the cell and that it may be an important event in cancer development. The study of p53 gene knockout mice (Donehower et al, 1992) and the analysis

of the status of the p53 gene in human tumors show that the loss of p53 activity is a key event in cancer development. Here in this study we found a clear upregulation of the p53 gene in B16F-10 melanoma cells after the treatment with Punarnavine.

Apoptosis is characterized by the activation of a specific family of cystein proteases, the caspases, followed by a series of caspase mediated morphological changes such as shrinkage of the cells, the condensation of chromatin and membrane blebbing (Kerr et al., 1972; Clarke and Clarke, 1995). The presence of cells with apoptotic bodies in cultured cells has been considered as a marker of cell death by apoptosis and has been linked to DNA fragmentation. Here the cells after treatment of Punarnavine showed chromatin condensation, apoptotic body formation and membrane blebbing in a dose dependent manner.

Caspase-3 is an effector caspase which once activated, cleaves many substrate proteins and structural proteins to generate the characteristic apoptotic morphology (Kothakota et al., 1997). The data from the reverse transcription PCR and agarose gel electrophoresis showed clear band of caspase-3. In cancerous cells, the expression of caspase-3 is much suppressed and in the present study the untreated control B16F-10 melanoma cells did not exhibit its expression. Caspase-3 is a most likely candidate to mediate Punarnavine induced apoptosis in B16F-10 melanoma cells as evidenced by increased expression of this gene upon treatment with Punarnavine. Cleavage of ICAD by caspase-3 leads to the activation of CAD, which in turn cleaves genomic DNA within internucleosomal regions and generates multimers of nucleosomal domain-sized (ladder like) fragments (Sakahira et al., 1998). DNA extracts from B16F-10 melanoma cells incubated with Punarnavine displayed characteristic ladder pattern of discontinuous DNA fragments which support the above results. The increased sub G0 population of B16F-10 melanoma cells in cell cycle analysis and increased number of stained cells in the TUNEL assay also support Punarnavine induced apoptosis and DNA fragmentation in Punarnavine treated cells.

A central player in the cellular genetic program, and the link between apoptosis and cancer, emerged when B-cell lymphoma 2 (BCL-2), the gene that is linked to an immunoglobulin locus by chromosome translocation in the follicular lymphoma, was found to inhibit cell death, rather than promote proliferation (Vaux et al., 1988). Bcl-2 is an integral membrane protein, even in healthy cells (Janiak et al., 1994) whereas Bcl-w and Bcl-xL only become tightly associated with the membrane after a cytotoxic signal by an induced conformational change. These prosurvival Bcl-2 family proteins can prevent cytochrome c release, and hence activation of caspases. It is becoming increasingly evident that every nucleated cell requires protection by at least one Bcl-2 homologue and that the abundance of these 'guardians' regulate tissue homoeostasis. Bcl-2 over expression in haematopoetic lineages yield excess B,T and myeloid cells that are refractory to diverse cytotoxic insults. (McDonnell et al., 1989; Sentman et al, 1991; Strasser, 1991; Strasser et al., 1991; Ogilvy et al., 1999). Conversely, inactivation of Bcl-2 homologous genes augments apoptosis in specific cell types, presumably because the concentrations of other guardians are too low to compensate. In the present study the RT-PCR analysis showed clear bands of Bcl-2 in untreated B16F-10 cells which were downregulated after the treatment with Punarnavine.

NF-kB is a family of more than a dozen transcription factors that are comprised of homo- and heterodimers of five proteins: p50, p52, c-Rel, RelA/p65 and RelB, encoded by the nfkb1, nfkb2, rel, rela and relb genes (Gilmore, 2006). In many cell types such as cancer cells, NF-κB activation inhibits cell apoptosis (Banki et al., 1999) by inhibitng expression of caspase-3, caspase-9 (Kawamura et al., 2003), and caspase-8 (Matta et al., 2002) proteins. Certain cell cycle regulatory proteins such as cyclin D1, which is required for transition of cells from the G1 to S phase, are also regulated by NF-kB (Mukhopadhyay et al., 2002). Transcription factor AP-1 consists of homodimers of Jun (v-Jun, c-Jun, Jun-B and Jun-D), Fos (c-Fos, v-Fos, FosB, Fra 1 and Fra-2), or activating transcription factor (ATF-2, ATF-3 and B-ATF) proteins (Karin and Delhase, 2000). Similar to NF-κB, Ap-1 and its components regulating gene expression have been shown to play an important role in cell proliferation, cell cycle regulation and tumor promotion. (Silverman and Maniatis, 2001). Fos proteins form heteromeric dimmers with members of the Jun family to generate AP-1 complexes with transcriptional activity. Activation of AP-1 protein is required for the preneoplastic to neoplastic progression of different cancers such as prostate cancer and epithelial cancer (Huang et al., 2002; Zerbini et al., 2003), and nuclear translocation of c-Fos and ATF-2 has been reported in different cancer cells (Huang et al, 1998; Angel et al, 1991). CREB is a cyclic AMP response element-binding protein, which is activated through protein kinase A and mediates phosphorylation in signaling cascade; CREB binds to the cyclic AMP response element sequence and promotes the specific gene transcription (Zhong et al., 1998). In present study treatment with Punarnavine found to inhibit the nuclear translocation of NF- κBp50, NF- κBp65, NF-κBc-Rel, c-Fos, ATF-2 and CREB significantly in B16F-10 melanoma cells.

The above data clearly demonstrate that Punarnavine could induce apoptosis in B16F-10 melanoma cells by activation of p53 induced caspase-3 mediated apoptotic signaling and suppression of NF-κB induced Bcl-2 mediated survival signaling.

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