



## Betulinic acid induces apoptosis in human chronic myelogenous leukemia (CML) cell line K-562 without altering the levels of Bcr-Abl

D.V. Raghuvar Gopal<sup>a</sup>, Archana A. Narkar<sup>a,\*</sup>, Y. Badrinath<sup>b</sup>,  
K.P. Mishra<sup>c</sup>, D.S. Joshi<sup>d</sup>

<sup>a</sup> *Laboratory Nuclear Medicine Section, Isotope Group, BARC, C/o Tata Memorial Hospital Annexe, Jorbari Wadia Road, Parel, Mumbai 400012, India*

<sup>b</sup> *Department of Medical Oncology, Tata Memorial Hospital, Parel, Mumbai 400012, India*

<sup>c</sup> *Radiation Biology and Health Sciences Division, BARC, Trombay, Mumbai 400085, India*

<sup>d</sup> *Molecular Biology and Agriculture Division, BARC, Trombay, Mumbai 400085, India*

Received 8 March 2004; received in revised form 23 June 2004; accepted 28 June 2004

Available online 8 December 2004

### Abstract

Betulinic acid (BA), a plant derived triterpenoid, isolated from various sources shows cytotoxicity in cell lines of melanoma, neuroectodermal and malignant brain tumors. Chronic myelogenous leukemia (CML) is characterized by Philadelphia chromosome (Bcr-Abl), a molecular abnormality leading to the intrinsic tyrosine kinase activity that provides growth and survival advantage to the cells. Present study describes the cytotoxicity of BA on human CML cell line K-562, positive for Bcr-Abl. The decrease in the viability of K-562 cells treated with BA at different concentrations and time intervals was assessed using MTT assay. Cell death induced by BA was determined to be apoptotic as measured by FACS analysis of PI stained nuclei, PS externalization by Annexin-V fluorescence and characteristic DNA fragmentation. DiOC<sub>6</sub>(3) fluorescent probe determined alterations in the mitochondrial membrane potential (MMP). RT-PCR confirmed the expression levels of Bcr-Abl in controls and K-562 cells treated with BA. The rapid loss of MMP of K-562 cells upon treatment with BA shows the direct activation of apoptosis at the level of mitochondria, overcoming the resistance of the high levels of expression of Bcr-Abl.

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**Keywords:** BA; CML; K-562; Cytotoxicity and apoptosis

### 1. Introduction

Many plant products are being evaluated for their chemotherapeutic potential for diverse diseases and

\* Corresponding author.

Tel.: +91 22 24135232/24130263/24130273; fax: +91 22 24157098.

E-mail address: [aanarkar@hotmail.com](mailto:aanarkar@hotmail.com) (A.A. Narkar).

their targets being validated (Dive et al., 1992; Dive and Hickman, 1999; Costantini et al., 2000). Betulinic acid (BA), a pentacyclic triterpene, isolated from various plant sources has shown therapeutic promise. It has manifested anti-inflammatory and anti-HIV activities (Zhu et al., 1996; Recio et al., 1995; Mukherjee et al., 1997; De Clercq, 2001). Studies have shown that BA is highly specific to melanoma with significant anti-tumor activity (Pisha et al., 1995). However, the cytotoxic activity of BA has been reported against the neuroectodermal tumors and malignant brain tumor cell lines (Fulda et al., 1999a, 1999b). Antiproliferative activity of BA on tumor cell lines, e.g. small and non-small cell lung carcinomas, ovarian and cervical carcinomas have been reported whereas the normal cell lines were found resistant (Zuco et al., 2002). The studies on human neuroblastoma cell line (SHEP) have revealed that BA acts on mitochondria without affecting cell surface receptor and induced apoptosis in cells (Fulda et al., 1999c). BA is known to cause changes in  $\Delta\psi_m$  of mitochondrial membrane, the release of cytochrome C which activates various down stream caspases, which is inhibited by over expression of Bcl2/BclXL (Fulda et al., 1998). In vitro studies on combination of betulinic acid with radiation have shown significant synergistic cytotoxic effects on human melanoma cell lines (Selzer et al., 2000). In addition low pH adapted melanoma cells show a decrease in the concentration of BA required to achieve the same cell killing further expanding its ability to be used as a combination therapy (Wachsberger et al., 2002). The antiangiogenic effect of BA has been attributed to the killing of endothelial cells possibly through action on mitochondria (Fulda and Debatin, 2000).

Human chronic myelogenous leukemia (CML) is a hematological malignancy characterized by the balanced reciprocal translocation of c-abl protooncogene from chromosome 9 to the break point cluster region of Bcr gene on chromosome 22 (Maru, 2001). This leads to the formation of the Philadelphia chromosome (Ph chromosome). The human CML cell line, K-562, derived from the blast crisis stage is relatively resistant to apoptosis induced by a wide range of anticancer agents including etoposide and cisplatin. This is ascribed to Ph chromosome, which confers resistance to the CML cells to various antileukemic agents (Cotter, 1995; Di Bacco et al., 2000; Liu et al., 2002; Samali et al., 1997; Slupianek et al., 2000).

Ectopic expression of Bcr-Abl in HL60 cell line inhibited or delayed all the features of apoptosis pertaining to mitochondria including the activation of caspases, externalisation of phosphatidylserine (Gabriela et al., 2003). The cytotoxic effect of triterpenoids on K-562 cell line and their MDR derivatives has been studied by Hata and Fernandes (Hata et al., 2003; Fernandes et al., 2003). However, none of them deals with the levels of Bcr-Abl, and its relation to the initial mitochondrial event. The known inhibitory effects of Bcr-Abl on apoptosis at the level of mitochondria prompted us to study the cytotoxic activity of BA in K-562 cell line.

## 2. Materials and methods

### 2.1. Drugs and cell line

Betulinic acid (Sigma Chem. Co., St. Louis, USA), was dissolved at 5 mg/ml in DMSO and was diluted in complete IMDM before adding to the cells. Human erythroleukemic cell line K-562 obtained from NCCS, Pune India was maintained in complete IMDM at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere in air. Cells in log phase were used for the experiments. The  $2 \times 10^4$  cells/100  $\mu$ l were plated in each well of 96-well plates.

### 2.2. Assessment of viability of drug treated cells

Cells were treated with BA at a concentration ranging from 5 to 40  $\mu$ g/ml with a maximum vehicle concentration being 0.1%. Viability of BA treated cells was assessed at the end of 24, 48 or 72 h using MTT staining. The assay is based upon the reduction of soluble yellow salt 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) to purple formazan by viable cells. A high correlation between the viable cell number and the formazan product has been reported. The MTT assay was performed as previously described (Kano et al., 2001).

### 2.3. Apoptosis and cell cycle analysis

#### 2.3.1. FACS analysis of PI stained nuclei

The appearance of a sub diploid G1 peak characteristic of apoptosis was detected by FACS analysis of

PI stained nuclei using Becton Dickinson FACS scan. Briefly, cells were collected at the end of incubation periods and fixed in 70% ethanol and stored at 4 °C until use. After washing with PBS, the fixed cells were treated with RNase in PI buffer for 30 min and at the end of treatment PI (5 µg/ml) was added and cells were stained for 30 min in dark. Ten thousand events were acquired excluding the debris and analysed using the Cell Quest software for the detection of apoptosis. The Modfit software was used for the cell cycle analysis.

#### 2.3.2. Annexin-V FITC assay

The assay was performed according to the manufacturers instructions using Annexin-V FITC kit of Santacruz Biotech (USA). K-562 cells ( $5 \times 10^5$ ) were treated with 20 µg/ml of BA for time periods ranging from 4 to 32 h. At the end of the treatment cells were washed with PBS (pH 7.4) and resuspended in binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>). One microgram of Annexin-V was added and cells were incubated in dark for 15 min and 2.5 µg/ml PI was added. The cells were then gently vortexed and 10,000 events were acquired and analyzed using Becton Dickinson FACS calibur.

#### 2.3.3. Cytofluorimetric analysis of mitochondrial membrane potential

The change in mitochondrial membrane potential, characteristic of apoptosis was studied using the cationic lipophilic dye dihexyloxocarbocyanine iodide (3) {DiOC<sub>6</sub>(3)}. K-562 cells were treated with BA (20 µg/ml) for time periods ranging from 1 to 18 h. At the end of incubation period, cells were washed and resuspended in PBS. Cells were then loaded with 40 nM DiOC<sub>6</sub>(3) and further incubated for 15 min at 37 °C. Cells were then placed on ice and 10,000 cells were immediately acquired without delay on Becton Dickinson FACS calibur with FL-1 on abscissa for DiOC<sub>6</sub>(3).

#### 2.3.4. Agarose gel electrophoresis of DNA

The degradation of DNA into 185 bp oligonucleosomal fragments was analyzed using agarose gel electrophoresis. DNA from the cells was extracted using the phenol chloroform:isoamyl alcohol method. Ten micrograms of DNA was loaded on 2% agarose gel containing 2 µg/ml ethidium bromide. The electrophoresis was performed at 2 v/cm for 16 h. The degradation pattern was assessed using UV transilluminator.

#### 2.4. RT-PCR amplification of Bcr-Abl

RT-PCR for Bcr-Abl was performed as described earlier (Bedi et al., 1994). Briefly RNA was extracted from K-562 cells treated with 5–20 µg/ml for 24 h using Trizol (Gibco BRL USA). First strand cDNA synthesis was performed using cDNA synthesis kit (MBI fermentas, USA) using random hexamer primers. Aliquot of the cDNA product was used for PCR using the CML specific primers. The PCR was performed in the logarithmic scale of amplification and the conditions were as follows: 95 °C for 3 min followed by 30 cycles of 95 °C for 30s, 62 °C for 20 s, 72 °C for 1 min followed by a final extension of 10 min at 72 °C. As a control the housekeeping gene cABL was amplified using the same samples. PCR products were analyzed by 2% agarose gel electrophoresis and visualised using UV transilluminator.

#### 2.5. Statistical analysis

All results are expressed as mean ± S.D. of three experiments with triplicates for each sample.

### 3. Results

#### 3.1. Effect of BA on the viability of K-562 cells

Experiments were conducted to determine the in vitro effects of BA and it was found to display significant antiproliferative activity on K-562 cells. Fig. 1 shows the viability of the K-562 cells as a function of BA concentration (5–40 µg/ml) after 24, 48 and 72 h of incubation time. It can be seen that the viability of treated cells decreased with the increasing concentration of BA, which was also dependent on the period of incubation. The IC<sub>50</sub> of BA was found to be 12.5 µg/ml after interaction for 48 h.

#### 3.2. Detection and analysis of apoptosis

##### 3.2.1. FACS analysis of PI stained nuclei

The FACS analysis of PI stained nuclei of K-562 cells treated with BA is shown in Fig. 2. The appearance of sub diploid G1 peak reveals apoptotic cell death. The percent cells in the sub diploid G1 peak increased to

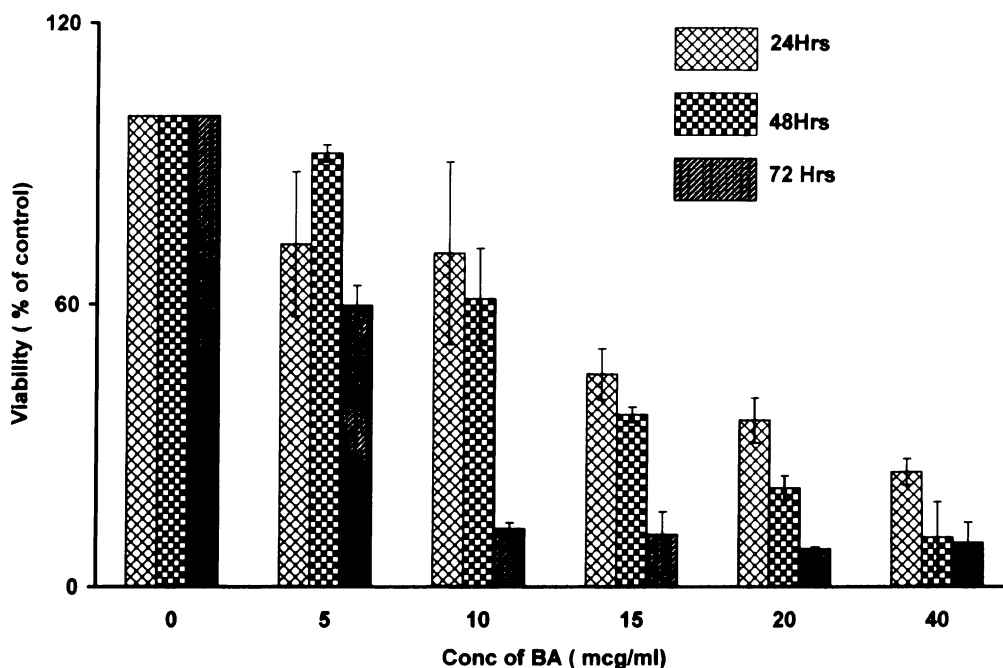


Fig. 1. Viability of K-562 cells treated with BA. K-562 cells were treated with BA as described in the text. The viability of the cells was estimated using MTT assay. Results are expressed as the mean  $\pm$  S.D. of the samples.

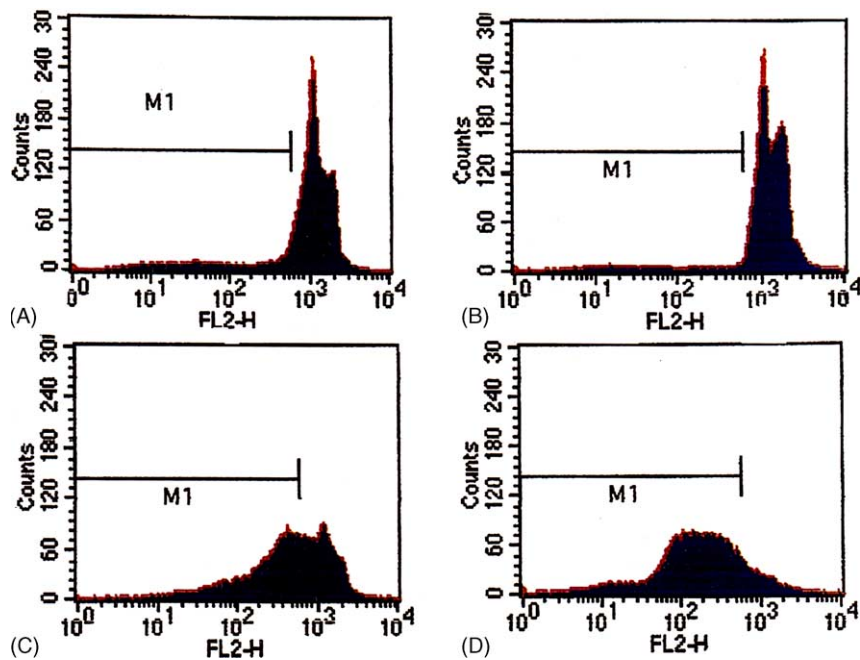


Fig. 2. DNA fragmentation analysis of K-562 cells treated with BA. K-562 cells were treated with BA 10  $\mu$ g/ml (B), 20  $\mu$ g/ml (C) and 40  $\mu$ g/ml (D) with control (A) and stained with PI. Ten thousand events were acquired on Becton Dickinson FACS caliber and analyzed using the cell quest software.

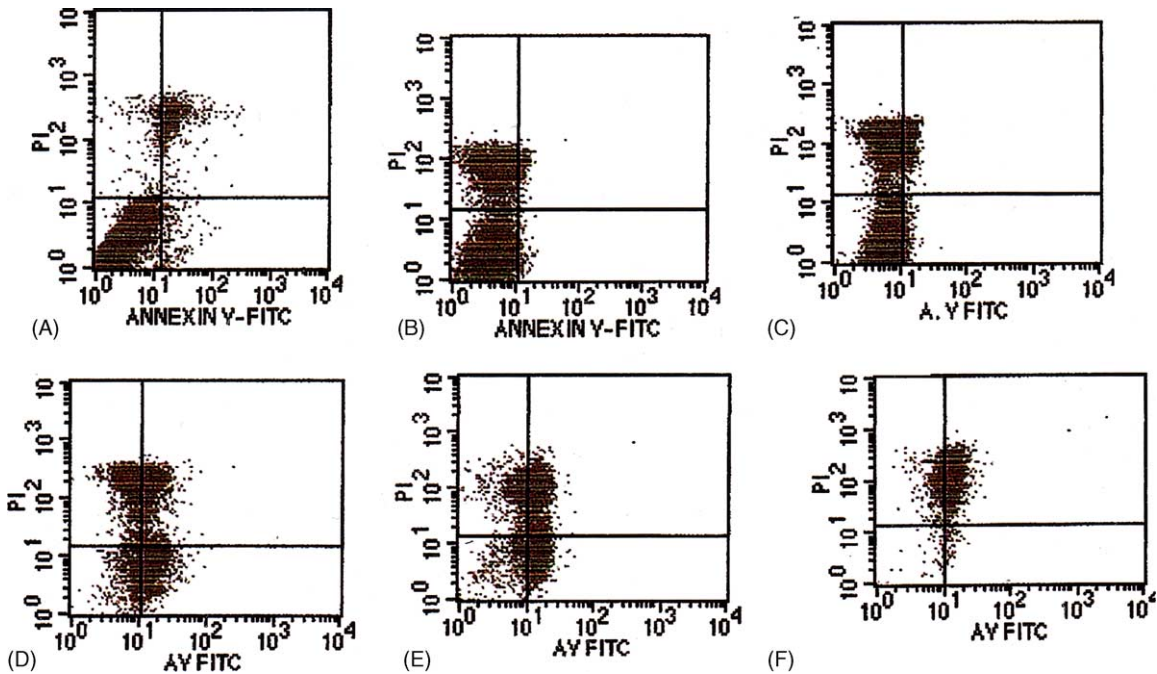


Fig. 3. Annexin-V FITC staining of K-562 cells. Cells treated with 20  $\mu\text{g/ml}$  BA for 2 h (B), 4 h (C), 8 h (D), 18 h (E) 24 h (F) and control cells (A) stained with Annexin-V FITC and PI as described in the text. Cells were acquired for FITC in FL-1 and PI in FL-3 in Becton Dickinson FACS scan and analyzed. Data representative of triplicates of three independent experiments.

58% upon treatment with 20  $\mu\text{g/ml}$  BA for 48 h. Further increase in the concentration of BA to 40  $\mu\text{g/ml}$  increased the percent cells to 88% at the same time interval.

### 3.2.2. Annexin-V FITC assay

Bivariate analysis of FITC fluorescence (FL1) and PI fluorescence (FL3) gave different cell populations wherein FITC negative and PI negative cells were designated as viable cells, FITC positive and PI negative were apoptotic and FITC positive and PI positive were necrotic. As is evident from Fig. 3, K-562 cells treated with 20  $\mu\text{g/ml}$  BA showed Annexin-V positivity which increased non linearly with time showing both apoptotic as well as necrotic populations. An increase was also noted in the PI positive population in the early stages of treatment, which clearly documents that BA induced the changes in cell membrane permeability. At 32 h time period almost all the cells had undergone secondary necrosis and were positive for Annexin-V as well as PI.

### 3.2.3. Assessment of mitochondrial membrane potential

Human K-562 cells treated with BA 20  $\mu\text{g/ml}$  showed a decrease in the mitochondrial potential measured by DiOC<sub>6</sub>(3) retention. K-562 cells showed a decrease in the MMP in the first hour of treatment with BA, which further decreased with time. Within the time period of 1–18 h there was complete loss of MMP as shown in Fig. 4. The loss of MMP in the early stages of treatment of BA suggests the direct effect of BA on the mitochondria. Interestingly the decrease was evident in the cell line positive for Bcr-Abl.

### 3.2.4. Agarose gel electrophoresis

Agarose gel electrophoresis of DNA extracted from the K-562 cells treated with BA showed a characteristic ladder of 180–200 bp. The degradation of DNA was concentration dependent as shown in Fig. 5. DNA fragmentation occurred in cells treated with 5  $\mu\text{g/ml}$  BA at 48 h but not to completion whereas 20  $\mu\text{g/ml}$  showed a typical fragmented ladder characteristic of apoptosis.

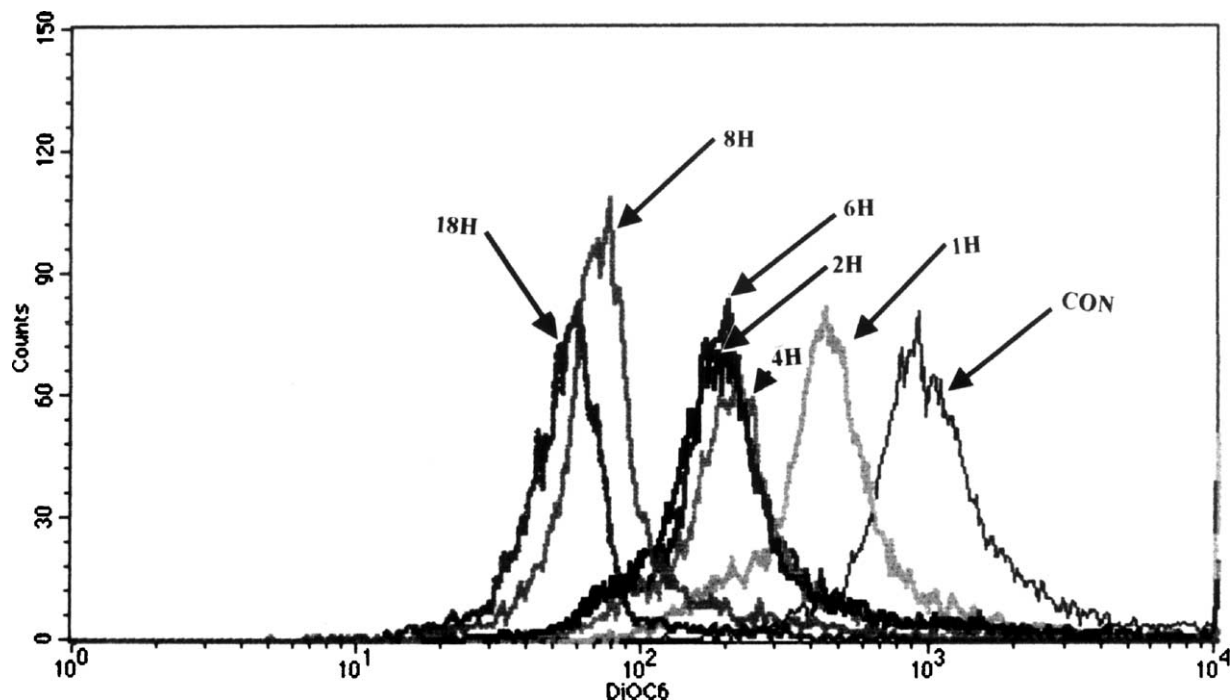


Fig. 4. Mitochondrial membrane potential of K-562 cells treated with BA. K-562 cells were treated with BA (20  $\mu\text{g/ml}$ ), for time periods of 1, 2, 4, 6, 8, 18 h and the loss of mitochondrial membrane potential was assessed by the FACS analysis of the retention of lipophilic cationic dye DiOC<sub>6</sub>(3). Peaks show the loss with time.

### 3.3. RT-PCR amplification of *Bcr-Abl*

As shown in Fig. 6a, RT-PCR amplification of the cDNA from K-562 cells with CML specific primers showed no change in the levels of *Bcr-Abl* upon treatment with BA at concentrations ranging from 5 to 20  $\mu\text{g/ml}$  for 24 h. Hence, it can be observed that the apoptotic effects of BA were not mediated by the decrease in the levels of *Bcr-Abl*. However, as shown in Fig. 6b, the expression of the housekeeping gene was uniform at all the concentrations studied.

## 4. Discussion

The present report describes the cytotoxic activity of BA on a human CML cell line K-562. Among the various factors that govern the outcome of drug treatment, the expression of anti-apoptotic molecules has been widely discussed and is of clinical significance. The most studied anti-apoptotic molecules are members of

the *bcl2* family like *bcl2*, *bclX<sub>L</sub>*. *Bcr-Abl* is known to phosphorylate *stat5*, a transcription factor directly activating *Bcl X<sub>L</sub>*, which acts at the level of mitochondria in the inhibition of apoptosis. The anti-apoptotic effects of *Bcr-Abl*, *Bcl2* and *BclX<sub>L</sub>* transfected into HL60 cell lines showed that upon apoptogenic stimulus with a wide variety of agents, HL60. *Bcr-Abl* cells were the most resistant to the induction of apoptosis (Gabriela et al., 2003).

In the present study, we show that BA induces apoptosis in K-562 cell line with an  $\text{IC}_{50}$  of 12.5  $\mu\text{g/ml}$ . K-562 cells treated with BA showed all the characters of apoptosis at 24 h period of treatment. The expression levels of *Bcr-Abl* were consistent throughout the study period. BA induced apoptosis in K-562 cell line without any effect on the expression levels of *Bcr-Abl*, and induces mitochondrial perturbations, which has a significant practical implication. The early loss of MMP in BA treated cells shows the robustness in its ability to target mitochondria and induce apoptotic cell death. This is of important implication since *Bcr-*



Fig. 5. Agarose gel electrophoresis of DNA (10  $\mu$ g) extracted from K5-62 cells treated with BA at different concentrations for 48 h: Lane (1).  $\lambda$  DNA Hind III marker (2) 100 bp ladder (3) control K-562 cells (4) K-562 cells treated with 5  $\mu$ g/ml BA (5) K-562 cells treated with 20  $\mu$ g/ml BA.

Abl expressing cells show resistance primarily at the level of mitochondria. Eventhough no change in the levels of Bcr-Abl was noticed at the mRNA level it is quite possible that the tyrosine kinase activity of the same may be effected. Since the PCR was performed along with the samples of CML patients under Gleevec therapy which showed considerable decrease in the levels of mRNA (data not shown), we concluded that BA does not induce any changes in Bcr-Abl at mRNA level.

Changes in the membrane of the cells treated with BA by double staining with Annexin-V and PI confirmed the apoptotic status of the K-562 cells. The increase in the population of PI stained cells showed that BA induced apoptosis is also associated with changes in the membrane permeability, partly governed by the steroidal nature of BA. The structure of BA resem-

bles that of the cholesterol with modifications in the steroidal ring structure. Cholesterol inserts itself into the membrane and effects the fluidity of both natural and artificial membranes and hence the permeability of the membrane. BA has been shown to insert itself into the membrane of erythrocytes and cause significant changes in the morphology of the RBCs, which render them resistant to plasmodial infections (Ziegler et al., 2004). Hence, it is quite possible that a fraction of BA may insert into the membrane of the tumor cell and cause changes in the permeability of the membrane.

In conclusion, our results show that anti-tumor activity of BA is not restricted to only melanoma and neuroectodermal tumors. Our observations clearly suggest that BA induces apoptosis in K-562 cells without altering the levels of Bcr-Abl. Direct targeting of mitochon-

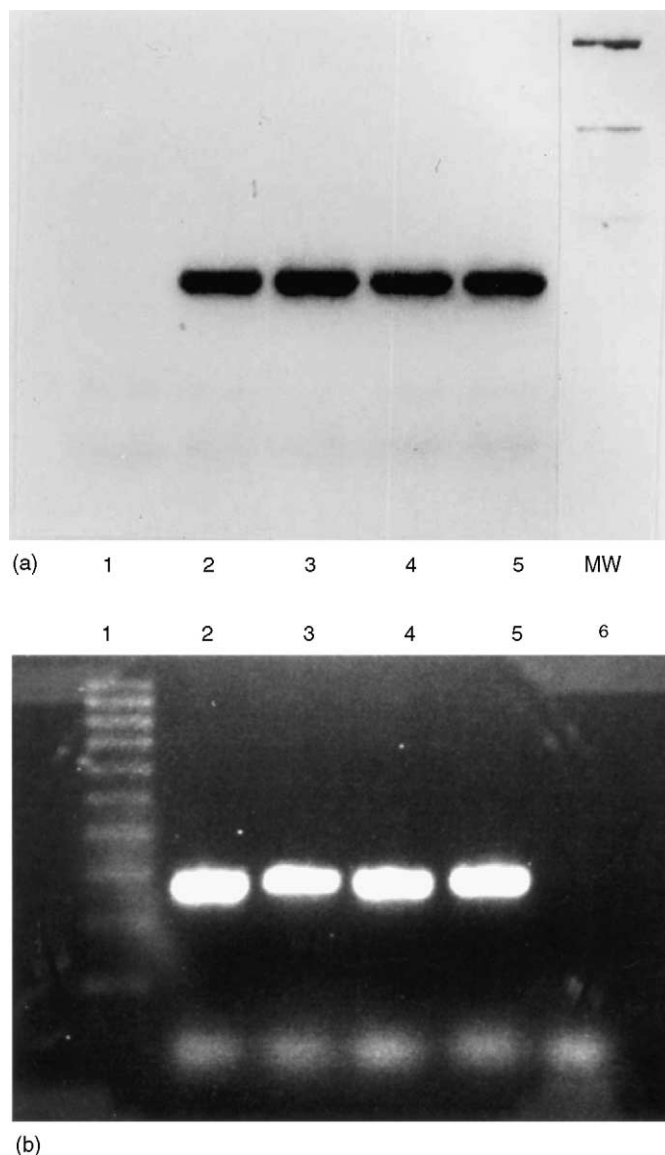


Fig. 6. (a) RT-PCR of Bcr-Abl. Lane (1) negative control, lane (2) K-562 control, lanes (3–5) are 5, 10, and 20 µg/ml BA and MW-molecular weight marker. (b) cABL housekeeping gene expression at the same concentrations studied as in (a). Lane (1) 100 bp ladder, lane (2) K-562 control, lanes (3–5) are 5, 10, and 20 µg/ml BA, respectively and lane (6) negative control.

dria by BA in the presence of Bcr-Abl also suggests that it overcomes the resistance of Bcr-Abl and acts by independent mechanisms. Furthermore, the induction of apoptosis also shows an alteration in membrane permeability, encompassing the role of membrane damage in BA induced apoptosis, attributed to the steroidal

nature of BA. The synergistic effect of BA in combination with irradiation as demonstrated in melanoma may further potentiate its use as a single agent or in combination in CML patients. However, further animal studies are required to validate the anti-tumor activity of BA.

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