β-Amyrin Modulates P38 MAPK and Jnk Pathway to Inhibit Cell Proliferation and Induce ROS-mediated Apoptosis in HeLa Cells

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It is aimed to investigate the effect of β-amyrin on p38 mitogen-activated protein kinase and Jun N-terminal kinase pathways and apoptosis in HeLa cells. HeLa treated cells were divided into 6 groups, group I- HeLa untreated cells as control, group II- dimethyl sulfoxide serve as vehicle control, group III- cisplatin as standard drug, group IV- β-amyrin-treated HeLa cells, group V- cells were pretreated with 100 μm N-acetyl-L-cystein for 1 hour and then treated with cisplatin and group VI- cells were pretreated with 100 μm N-acetyl-L-cystein for 1 hour and then treated with β-amyrin. The antiproliferative effect was measured using the MTT assay. Genotoxic effects were studied using micronucleus assay. Total reactive oxygen species, nitric oxide and caspase 3 level were determined on a spectrofluorimeter and colorimeter. Protein expression was analyzed by immunoblotting. β-Amyrin (10-200 μm) and cisplatin (0.01-100 μm) had an inhibitory effect on the proliferation of cancer cells in a dose-dependent manner, with the IC50 values at 100 μm and 10 μm for β-amyrin and cisplatin, respectively. Western blot analysis revealed expressions of apoptotic pathway related proteins, Bcl-2, caspase-3, caspase-9, phospho-p38 mitogen-activated protein kinase and phospho-Jun N-terminal kinase, growth arrest and deoxyribonucleic acid-damage-inducible, beta in all groups. Genotoxic effects were observed after treatment with β-amyrin as well as with cisplatin. It was observed that HeLa cells showed significant elevation of total reactive oxygen species after β-amyrin treatment. Protein expression analysis showed that the β-amyrin upregulated phospho-p38 mitogen-activated protein kinase, phospho-Jun N-terminal kinase and growth arrest and deoxyribonucleic acid-damage-inducible, beta on HeLa cells. Increased phospho-Jun N-terminal kinase directly activated caspases and decreased Bcl-2 in HeLa cells. These results indicated that β-amyrin induced the apoptosis through reactive oxygen species-mediated mechanism by activating p38 mitogen-activated protein kinase and Jun N-terminal kinase through transcriptional factor, GADD45β. In turn, activated Jun N-terminal kinase directly activated caspase-9 and caspase-3 and destined the HeLa cells to apoptosis.

Key words: β-Amyrin, HeLa, apoptosis, micronucleus, p38MAPK, JNK

Cancer is one of the most critical public health issues and a leading cause of human death worldwide[1-2]. Cervical cancer is generally caused by human papilloma virus (HPV) infection is the first prevalent gynecological cancers worldwide[3-4]. Recent data indicated that cervical cancer became the second ranked in morbidity and mortality in women after breast cancer[5]. Early diagnosis of cervical cancer is curable but metastasis is poor to prognosis. Approximately 50 % of cancer patients die at metastasis stage[6]. HeLa cell line is the one of the most important HPV type 18 infected-cervical cancer cells and widely used in various experimental studies of cervical cancer[7-8]. Recent reports have shown that the induction of

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Apoptosis is considered as a most promising approach to destroy cancer cells in the field of cancer biology. Apoptosis is a programmed cell death by which many intracellular events play a crucial role to remove the unwanted cells. Disorders in the apoptosis lead to many diseases including neurodegenerative diseases and autoimmune diseases. Cancer cells stop the apoptosis in order to metastasise but apoptosis mediated cell death eradicate the cancer cell without harming normal cells and without causing inflammatory response. Therefore, apoptosis targeting pathways in the treatment of cancer could be a useful therapeutic approach.

Reactive oxygen species (ROS) is secondary metabolites in aerobic metabolism and its low production in the cellular level regulates many signaling pathways including Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK) pathways. But its overproduction leads to oxidative stress. Apart from controlling the various signaling pathways ROS is also assumed to induce apoptosis pathways by promoting transcription of proapoptotic genes. Many anticancer agents including paclitaxel and tamoxifen are believed to kill cancer cells by apoptosis pathways. However, these compounds pose adverse side effects during cancer treatment, therefore it’s necessary to develop new drugs with little side effects or without side effects for managing this disease. It is strongly believed that plants are considered as an attractive natural sources with antitumor, antioxidant and antiinflammatory activities. More than 60 % of currently used anticancer drugs are derived from plants and marine sources and also many scientific studies have demonstrated the potentiality of plant-derived compounds against many cancers including cervical cancer.

These plant compounds eliminate cancer cells using different channels but eventually all these compounds eradicate cancer cells via apoptosis and thus it is important to discover more specific targeted apoptosis inducing plant-derived compounds. Several studies reported that plant derived compounds selectively eliminate cancer cells through ROS metabolism. As ROS is a major attributed factor of regulating the apoptosis pathways and also involved in destroying cancer cells, it is important to unravel the ROS-mediated apoptosis mechanism in the cancer cells.

Triterpene compounds are well known for antiinflammatory, antiapoptotic activity in various cell lines. Many in vitro and in vivo studies have shown that oleanolic acid and other oleanane triterpenoids modulate intracellular signaling pathway and exert antitumor activity against various cancer cell lines. β-Amyrin is a pentacyclic triterpenes found in many plants and barks of trees such as Protium kleinii, Protium hepta phylum and Moldenhawera Nutans. Previous studies showed that β-amyrin possess different pharmacological activities such as antiinflammatory, antifibrotic and antidiabetic activities. Several studies reported that pentacyclic triterpenes such as ursolic acid and oleanolic acid which are structurally similar to β-amyrin can suppress the cancer development and metastasis through the inhibition of angiogenesis. However, no studies analyzed the effect of β-amyrin on ROS mediated signaling that induce apoptosis. Therefore, we investigated the effect of β-amyrin role in controlling cervical cancer through ROS-mediated apoptosis.

**MATERIALS AND METHODS**

HeLa cells were obtained from National Center for Cell Science (Pune, India) was maintained and grown in a humidified incubator at 37° with 5 % CO₂. Cells were grown as a monolayer in plastic tissue culture flasks in Dulbecco’s modified Eagle’s medium (DMEM) Gibco, USA). The medium was supplemented with 10 % fetal bovine serum (FBS) and antibiotics, penicillin 50 IU/ml, streptomycin 3.5 μg/ml and gentamycin 2.5 μg/ml. All these were procured from Gibco, Grand Island, NY, USA. Triton X-100 (Cat No. 93443), 0.1 mg/ml RNase (R4642) and 40 μg/ml propidium iodide (PI, P4170) were all procured from Sigma-Aldrich.

**Cell viability assay:**

Cells were seeded in 96-well plates at a density of 5×10³ cells/well in 200 μl DMEM containing 10 % FBS overnight. Non-adherent cells were removed by gentle washing after 24 h. Cells were replaced with serum-free medium with varying concentrations of β-amyrin (10-200 μm) and cisplatin (0.01-100 μm). A negative control containing serum-free medium with dimethyl sulfoxide (DMSO) was also evaluated. After 72 h of treatment, the plates were incubated with 20 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) for 3 h at 37°. The formazan was dissolved in 150 μl/well DMSO and the absorbance was detected at 590 nm using a microplate reader (Bio-Rad, USA). Cell viability was expressed as percentage of untreated cells, which served as the negative control group and was designated as 100 %. The results were expressed in percentage of the negative control. The median inhibitory concentration...
(IC\textsubscript{50}, defined as the drug concentration at which cell growth was inhibited by 50 \%) was assessed from the dose-response curves\cite{23}.

**Experimental groups:**

The experiment was divided into six groups, group I- HeLa untreated cells served as control, group II- DMSO-treated HeLa cells served as vehicle control, group III- 10 μm cisplatin-treated HeLa cells, group IV- 100 μm β-amyrin-treated HeLa cells, group V- cells were pretreated with 100 μm N-acetyl-L-cystein (NAC) for 1 h and then treated with 10 μm of cisplatin and group VI- cells were pretreated with 100 μm NAC for 1 h and then treated with 100 μm of β-amyrin.

**Micronuclei assay by flow cytometry:**

Flow cytometry provides a convenient research tool to determine the frequency of micronuclei. The \textit{in vitro} flow cytometry-based micronucleus (MN) assay\cite{24} provides an alternative to the traditional MN genotoxicity assay that has potential use as a rapid screening assay.

**Sample preparation for MN analysis:**

Cells were seeded at 1×10^5 in 60 mm dishes. After removing non-adherent cells by gentle wash, the cells were treated with 10 μm cisplatin and 100 μm β-amyrin, respectively. To arrest the cells at cytokinesis stage at 37\(^\circ\), cytochalasin-B (3 μg/ml) was added. The cells were harvested and fixed by adding ice-cold 70 % ethanol after 72 h. For the experiment, the samples were centrifuged for 10 min at 1500 rpm. After discarding the supernatant, the pellets were resuspended in PBS until ethanol removed completely. Following this, the cells were then resuspended in PBS (500 μl) containing NaCl (584 mg/l), sodium citrate (1000 mg/l), 0.5% Triton X-100, 0.1 mg/ml RNase and 40 μg/ml PI in a dark room. Triton X-100 and RNase were added to permeable the cell membrane and eliminate RNA. Samples were protected from light and allowed to equilibrate to room temperature before flow cytometric analysis was performed. After 45 min incubation at 37\(^\circ\), the cells were transferred to a FCM tube and analyzed on a flow cytometer (FACS Calibur, Becton Dickinson Biosciences), equipped with an air cooled argon laser providing 15 mW at 488 nm with standard filter setup and 100 000 events were collected.

Bivariate dot plots displayed on logarithmic scales were used to register signals of DNA (FSC vs. FL2-H or SSC vs. FL2-H). Nuclei and MN were discriminated from non-specific debris using the electronic gates. The percentage of MN frequency was calculated based on the acquisition of 100 000 events using the formula, \% of MN=(total MN/total N)×100, MN indicates total micronuclei and N indicates total nuclei.

**Determination of total ROS and nitric oxide (NO):**

Cells were washed with PBS and loaded with CM-H2DCFDA for 15 min. Fluorescence of the H2DCFDA was read at 480 nm excitation/520 nm emission in a Hitachi F2000 spectrofluorimeter. The results were expressed as nM/mg of protein. Nitrite (NO\textsubscript{2}) and nitrate (NO\textsubscript{3}) in HeLa cells were measured using NO assay kit (ab65328, Abcam) according to manufacturer protocol.

**Determination of caspase-3 for colorimetric assay:**

Caspase-3 activity was determined using a colorimetric method in which the presence of caspase-3 lysate produces p-nitroaniline, which generates a yellow color. Cells plated in a sterile 24-well plate at a concentration of 1×10^5 cells/ml and were incubated at 37\(^\circ\) in 5 % CO\textsubscript{2} for 72 h. After this period, the determination of caspase-3 was performed according to the manufacturer’s specification (Sigma-Aldrich, St. Louis, MO) using an ELISA reader. The analyses were the average of three replicates, and the results were expressed in ΔmOD405 nm/min\cite{25}.

**Western blotting:**

Cells were seeded in 24-well plates at a density of 5×10^4 cells/well in 1 ml of medium containing 10 % FBS overnight. Non-adherent cells were removed by gentle washing after 24 h. Cells were lysed by the addition of cold RIPA buffer (150 mM NaCl, 50 mM Tris HCL, 0.1 % SDS, 1 % Triton X-100, 1 mM PMSF, 2 mM NaF, Na\textsubscript{2}VO\textsubscript{4}, β-glycerophosphate and 2 mM EDTA, and fresh protease inhibitor cocktail (Cat No. P8340, Sigma Aldrich), and the cell lysate was centrifuged at 14 000 rpm at 4\(^\circ\) for 20 min. The supernatant was harvested and analyzed for protein content using the BCA method (Cat No.23227, Pierce, USA). Protein was denatured in sample buffer, then separated on 12 % SDS-PAGE, and transferred to polyvinylidene difluoride membranes (semidyry transblot system). The blots were blocked for 1 h at room temperature with Tris-buffered saline (TBS, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 5 % non-fat milk. The blots were washed three times with TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.02 % Tween 20) and incubated with Bcl-2, caspase-3, caspase-9, phospho-p38 MAPK,
and phospho-JNK, GADD45β (Santa Cruz, CA, 1:1000 dilutions) antibodies at 4°C overnight. The blots were incubated for 1 h at room temperature with secondary antibody (1:5000 dilutions) and detected by ECL detection reagent. To ensure that an equal amount of sample protein was applied for electrophoresis, β-actin was used as an internal control. Densitometric analysis was done using ImageJ software.

**Statistical analysis:**

Data were presented as mean±SEM. Each value is the mean of at least three separate experiments. Statistical evaluation was performed using an unpaired Student’s t test. p values <0.05 were considered to be statistically significant. Data were analyzed using SPSS software (version 16.0).

**RESULTS AND DISCUSSION**

The effects of β-amyrin and cisplatin on the proliferation of HeLa cells were examined. Fig. 1A and B showed the percentage of viable cells obtained in the MTT assay with varying concentrations of β-amyrin (5-200 µm) and cisplatin (0.01-100 µm). The significantly dose-dependent reduction on the cell proliferation was observed in cells treated with both β-amyrin and cisplatin. After 72 h of incubation, the IC50 values for β-amyrin and cisplatin were 100 µm and 10 µm, respectively. Interestingly cell viability slightly but not significantly increased with increasing β-amyrin concentration. Moreover, there is a time- and dose-dependent increase in cell death of HeLa cells, reaching approximately 60 % of cells after 36 h of treatment with 100 µm β-amyrin. The doses of 150 and 200 µm showed an increased cell death of approximately 90 % after 36 h treatment (fig. 1C).

The effect of β-amyrin on total ROS level in HeLa was determined using spectrofluorimetric method (fig. 2A). It was observed that HeLa cells showed significant elevation of total ROS after cisplatin and β-amyrin treatment (p<0.001) compared to control and vehicle groups, but there is no significant differences were observed between cisplatin and β-amyrin-treated HeLa cells. β-amyrin ROS elevating action was further confirmed by using NAC. NAC pretreatment to cisplatin and β-amyrin group showed significant (p<0.001) decrease in ROS level compared to cisplatin and β-amyrin-treated HeLa cells. However pretreatment of cells with 3 mM of NAC reversed ROS accumulation close to the untreated control levels. This indicated that ROS could be the major factor for the drug-induced
apoptosis. Fig. 2B shows the effect of β-amyrin on nitrate-nitrite levels in HeLa cells. Results showed that levels of nitrite-nitrate were significantly higher in control groups and their levels decreased significantly (p<0.001) after cisplatin and β-amyrin treatment.

Fig. 3 showed the Gating image of the MN frequencies. Baseline micronuclei frequency obtained in untreated control cells were 0.86±0.09. There were no significant differences between the DMSO and the control group. There was a significant increase (p<0.001) in MN frequencies of cisplatin-treated cells when compared to both HeLa cells and DMSO+HeLa cells groups. A significant (p<0.001) frequency of MN was obtained in cells treated with β-amyrin 3.25±0.53 when compared to both HeLa cells and DMSO+HeLa cells groups. There is no significant changes observed between cisplatin and β-amyrin-treated HeLa cells (fig. 4).

The activity of cleaved caspase-3 in β-amyrin and cisplatin-treated HeLa cells were determined colorimetrically at ΔmnOD405 nm/min (fig. 5). The activity of caspase-3 were observed to be decreased in controls groups, whereas β-amyrin and cisplatin treatment significantly (p<0.001) increased the caspase-3 activity compared to both control groups.

The ROS-mediated apoptosis on HeLa cells was induced by β-amyrin through activating various proteins involved in p38 MAPK pathway in order to cause programmed cell death of cancer cells. The expressions of various proteins like phospho-p38 MAPK, phospho-JNK, GADD45β, Bcl-2, caspase-9 and caspase3 were done by using immunoblotting (fig. 6A). The densities of bands corresponding to all these proteins were normalized on the basis of β actin and analyzed relative to that of the normal control group, as shown in fig. 6B. The phosphorylated kinases like p38 MAPK, JNK and transcription factor GADD45β involved in the ROS-mediated apoptosis expression were downregulated in untreated HeLa cells as well as DMSO-treated HeLa cells, no significant differences were found between these groups. HeLa cells treated with β-amyrin and cisplatin showed significant (p<0.001) upregulation of all proteins compared to both control groups but did not show any significant differences among drug-treated cells.

As observed earlier, the activity of caspase-3 was further confirmed by immunoblotting. Expression of apoptotic proteins, caspase-9 and caspase-3 decreased in the untreated HeLa cells, DMSO-treated HeLa cells, while in cisplatin- and β-amyrin-treated cells the expression was significantly (p<0.001) increased compared to both control groups. But there is no significant difference was observed between cisplatin- and β-amyrin-treated HeLa cells.

Expression of antiapoptotic protein Bcl-2 in HeLa cells was analysed using the Western blot. Bcl-2 expression was upregulated in HeLa cells as well as in DMSO-treated cells. Treatment of HeLa cells with β-amyrin
Fig. 5: Colorimetric analysis of caspase-3 activity on HeLa cells before and after β-amyrin with references to NAC treatment

Fig. 6: Immunoblotting analysis of ROS induced various proteins on β-amyrin-treated HeLa cells

and cisplatin significantly downregulated Bcl-2 protein expression, when compared to the normal control group. Also, Bcl-2 protein expression in cisplatin-treated HeLa cells showed no significant differences compared to β-amyrin-treated HeLa cells.

β-Amyrin impact on ROS-induced apoptosis is further confirmed by the NAC pretreated group. p38, JNK, GADD 45β, caspase-9, caspase-3 and Bcl-2 showed no significant changes in NAC-pretreated cisplatin and β-amyrin groups compared to HeLa cells and DMSO+HeLa cells.

Metastasis and proliferation are the main pathological factors of cancer. It mainly occurs due to abnormality in apoptotic molecular machinery, which leads to survival of metastatic cells in cancer. To minimize or prevent the proliferation of cancer cells, the therapeutic agents are designed to induce various apoptosis signal transduction to destroy the cancer cells. Many reports have shown antiproliferative properties of triterpenes against multiple tumor cells. In fact, to improve their activities, synthetic analogs of oleanolic acid have been developed and are under clinical evaluation as antitumor agents for hematologic malignancies[26-29]. Based on the previous evidences, chosen β-amyrin, a natural triterpene with oleanonic structure was chosen to study on HeLa cells.

The appearance of MN is closely linked to DNA damage process and genome instability. Monitoring the frequency of micronuclei is therefore widely used to assess the environmental or endogenous stresses that damage the genome[30]. The increased formation of micronuclei is usually an indication of increased DNA damage or mutation. It is characteristically found in all types of cancer cells. Increased frequency of micronuclei in the cisplatin- and β-amyrin-treated groups was observed. Cisplatin exhibited growth inhibition and cytotoxicity in a dose-dependent manner, with an IC50 value of 10 μM on HeLa cells. Cisplatin forms highly reactive, charged, platinum complexes which bind to nucleophilic groups such as GC-rich sites in DNA, inducing intra and inter strand DNA cross-links, as well as DNA-protein cross-links. These cross-links result in cell growth inhibition and apoptosis. Studies reported that the inhibition of transcription and replication due to cisplatin-induced DNA lesions and the subsequent generation of DNA strand breaks activate the ATR and ATM kinases as well as the p38 MAPK/MK2 pathway[31,32]. Present study proved for the first time that β-amyrin is a potent clastogenic, which produced a large number of DNA lesions. Increased ROS production in HeLa cells may occur due to the prooxidative shift in the redox state and impaired glucose clearance in mitochondria, which directly causes mitochondrial oxidative stress. Prooxidative shift observed in all cancers is mainly mediated by an increased availability of mitochondrial energy substrate and inflammatory oxidative conditions. Chronic inflammatory tissue contains large amounts of NO and derivatives ROS. NO and ROS generally cause direct and indirect damage to DNA and other genetic material[33]. The significant balance and interaction between NO and ROS has a foremost role in the etiology of a tumor[34]. All these conditions involved with pathological changes are indicative of a dysregulation of signal cascades or gene
expression that leads to increase ROS production in cells\textsuperscript{35}.

In this study, increased total ROS level and NO level were found in HeLa cells and DMSO-treated HeLa cells. Cisplatin and β-amyrin treatment on HeLa cells increased total ROS further and decreased NO level, which was confirmed by NAC pretreatment to these cells. The increased ROS and NO in HeLa cells might be ROS derived from NO•, which are released from inflammatory cells and act on neighboring dividing cells leading to somatic mutations in critical cancer-causing genes. NO• produced by inducible nitric oxide synthase in solid tumors has been implicated in enhanced vascular permeability and increased tumor blood flow and hence sustained tumor growth\textsuperscript{35}. Chronic inflammation has also been considered to be a risk factor for a variety of human malignant tumors, particularly cervical carcinoma. Even though increased ROS production were observed in all types of cancer cells still these cancer cells does not undergone ROS-mediated apoptosis, this might be due to the increased NO level. Generally, increased NO have the direct opposing effect on ROS-mediated apoptosis by inhibiting heat shock protein (HSP)\textsuperscript{36,37}. Recent studies using triterpenoids supported the results that β-amyrin increased further total ROS levels in HeLa cells\textsuperscript{38-40}. As stated earlier, β-amyrin, a triterpenes with oleanolic compound structure, which increased ROS production further and induces ROS-mediated apoptosis. This action might occur due to its antiinflammatory property. This property cause’s decreased production of inflammatory mediators thereby reducing the NO level which might lead to activate HSP thereby induces ROS-mediated apoptosis of HeLa cells. Apart from controlling inflammatory oxidative condition, β-amyrin also causes mitochondrial oxidative stress to induce ROS-mediated apoptosis by activating p38 and JNK signaling pathway and these results were discussed further in this study. JNK and p38 MAPK family members function in a cell context-specific and cell type-specific manner to integrate signals that affect proliferation, differentiation, survival and migration. Consistent with the importance of these events in tumorigenesis, JNK and p38 MAPK signalling is associated with cancers in humans and mice\textsuperscript{41}. In the present study, β-amyrin was found to activate/ phosphorylate p38 MAPK, JNK signaling in HeLa, which in turn activated the transcription factor GADD β45 and β-amyrin also activated p38 and JNK pathway to induce apoptosis of HeLa cells. Many studies have shown that these two pathways induced apoptosis in cancer cells\textsuperscript{42-43}. Several factors are involved in the activation of these pathways. ROS is considered as a one of the most important factor that activate these signaling pathway to induce cancer cell death\textsuperscript{44}. Many synthetic and plant-derived triterpenoids have potential anticancer activities\textsuperscript{45-49}. Recent studies showed that triterpenoid mainly induced apoptosis by targeting ROS-induced activation of JNK and p38 MAPK, which further activate caspases\textsuperscript{50}. Recent reports have provided evidence that JNK activated caspases\textsuperscript{51}. Consistent with these findings, in the present study β-amyrin activated p38 MAPK and JNK pathways in a caspase-dependent manner. Activation of transcription factor GADD45β by JNK further leads to cleave the procaspase 9 and increased caspase 9 and caspase 3 expressions to induce ROS-mediated apoptosis. Activation of these signaling pathways by β-amyrin not only cleaved the caspase 3 but also activated antiapoptotic protein Bcl-2 by phosphorylation. Several studies have reported that the antiapoptotic function of Bcl-2 depended on its phosphorylation\textsuperscript{52}. p38 MAPK and JNK are involved in phosphorylation of Bcl-2, resulting in increased apoptosis. Stress kinase p38 was shown to play a proapoptotic role after various insults and was identified as one of kinases able to directly phosphorylate Bcl-2\textsuperscript{53}. Antiapoptotic function of Bcl-2 has been disturbed due to its phosphorylation in the cells treated with taxol or other anticancer agents\textsuperscript{54}. Consistent with these findings, it was found that β-amyrin and cisplatin treatment decreased the levels of Bcl-2 in HeLa cells.

In conclusion, ROS play an important role in β-amyrin-induced apoptosis in human cervical adenocarcinoma (HeLa) cell line. The mechanism by which β-amyrin induced apoptosis involved the following steps. Chronic inflammation and mitochondrial oxidative stress induce rapid generation of ROS, which is required for subsequent activation of apoptosis by β-amyrin. ROS generation by β-amyrin occurs mainly in the mitochondria. Apoptosis induced by β-amyrin is mediated through the activation of p38, JNK and activated GADD45β, which in turn activates mitochondrion-dependent caspase activation pathway, which is negatively regulated by the antiapoptotic Bcl-2 protein.

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**Conflict of Interest:**

No conflict of interest between any of the authors.

**REFERENCES**


