JBUON 2018; 23(4): 965-970
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Antitumor effects of beta-amyrin in Hep-G2 liver carcinoma cells are mediated via apoptosis induction, cell cycle disruption and activation of JNK and P38 signalling pathways

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Summary

Purpose: *Liver cancer is one of the major causes of cancer related deaths throughout the world and the fifth most common type of malignancy in men and eighth in women. Beta-amyrin has been reported to exhibit significant pharmacological properties and in the present study we examined the anticancer and apoptotic effects of beta-amyrin against Hep-G2 liver cancer cells.*

Methods: *The antiproliferative activity of beta-amyrin was determined by MTT assay. Apoptosis was assessed by DAPI staining and DNA damage was checked by the comet assay. The cell cycle analysis was carried out by flow cytometry and protein expression was examined by western blotting.*

Results: *Beta-amyrin exhibited significant anticancer activity against Hep-G2 cancer with IC_{50} values of 25 μM. The anticancer effects were attributed to induction of apoptosis and G2/M cycle arrest in a dose-dependent manner. Moreover, beta-amyrin could also activate the p38 and JNK signalling pathways.*

Conclusion: *Based on the results of the current study, we propose that beta-amyrin may prove an important lead molecule for the treatment of liver cancer.*

Key words: *apoptosis, beta-amyrin, cell cycle, liver cancer, tritepenoids*

Introduction

Plants produce a diversity of secondary metabolites to adapt to the harsh environmental conditions and these metabolites have been observed to exhibit significant bioactivities in humans [1]. Terpenoids are also plant secondary metabolites that have been reported to exhibit a wide array of pharmacological properties [2]. Physiologically, they play vital roles in plants as they may help plants to lure pollinators to carry out the act of pollination or act as chemo-repellents to keep away the predators. Among these terpernoids, tritepenoids have been reported to posses anticancer, antimicrobial, antioxidant and several other properties [3,4]. The triterpenoids, such as ursolic acid, have been reported to inhibit the growth of human prostate cancer cells [5]. Similarly, many other triterpenoids of plant origin exhibit the capacity to prevent the growth and metastasis of cancer cells [6]. In the present study we evaluated the anticancer effects of beta-amyrin (Figure 1) against Hep-G2 liver cancer cells. Liver cancer is one of the major causes of cancer-related deaths throughout the world and the fifth most common type of malignancy in men and eighth in women. Every year around 400,000 men and more than 250,000 women are diagnosed with liver cancer [7]. Studies have shown that liver cancer is mostly detected before the age of 20 in high risk countries and around 50 years in low risk

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Received: 05/12/2017; Accepted: 03/01/2018

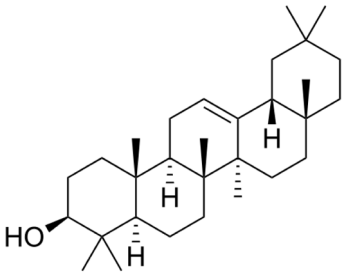

Figure 1. Chemical structure of beta-amyrin.

countries [8]. Previous reports have shown that the occurrence of liver cancer in men is twice as high compared to women [8]. Moreover, studies have reported that the incidence of liver cancer in developed countries is supposed to escalate in the future [9]. The increase in the incidence of liver cancer, the limited standard treatments and the side effects of the existing drugs demand exploration and identification of new bioactive molecules of plant origin.

In the present study we report that beta-amyrin exerts significant antiproliferative activity on Hep-G2 cancer cells by triggering apoptosis and cell cycle arrest in these cells. Moreover, beta-amyrin was also found to target the p53 and JNK signalling pathways. In conclusion beta-amyrin may prove to be a promising lead molecule for the treatment of liver cancer.

Methods

Chemicals, reagents and culture conditions

The following chemicals were used in the present study: DAPI, RNase. A triton X-100 dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Primary and secondary antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Fetal bovine serum (FBS), RPMI-1640 medium, L-glutamine, antibiotics were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Human liver cancer Hep-G2 and non-cancer FR-2 cell line were purchased from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI-1640 medium containing 10% FBS, penicillin and streptomycin (100 U/mL each) and maintained in a humidified atmosphere containing 5% CO_2.

Estimation of cell viability

The viability of liver Hep-G2 cancer cells was assessed by MTT assay. In brief, Hep-G2 cells were cultured in a 96-well plates at the density of 5×10^3 cells/well. Then the cells were incubated overnight and then the RPMI-1640 medium was removed and replaced with fresh RPMI-1640 with beta-amyrin separately at different concentrations (0-100 µM) for 24 hrs. Afterwards, MTT solution at 0.5 mg/ml concentration was added for the last 4 hrs of incubation and then the absorbance was measured at 570 nm.

Detection of apoptosis

Liver cancer Hep-G2 cells were seeded at a density of 2×10^5 cells/well in 6-well plates, exposed to 20 µM of beta-amyrin and incubated for 24 hrs. DAPI staining was carried by incubating the cells in 6-well plates with DAPI. The cells were then washed with PBS (phosphate buffered saline), fixed in formaldehyde (10%) and then again washed with PBS. The DAPI-stained cells were then examined by fluorescence microscope. For the estimation of the percentage of the apoptotic cells, FITC-Annexin V/PI (propidium iodide) Apoptosis detection kit was employed as per the instructions of the manufacturer.

Determination of mitochondrial membrane potential

Hep-G2 cells were seeded at a density of 2×10^5 cells/well in a 6-well plate, maintained for 24 hrs at 37°C and then treated with 0, 12.5, 25 and 50 µM beta-amyrin for 24 hrs at 37°C in incubator with 5% CO_2 and 95% air. Subsequently, cells from all samples were collected, washed twice with PBS and re-suspended in 500 µl 3'-dihexyloxacarbocyanine iodide (1 µmol/l) for mitochondrial membrane potential (MMP) analysis at 37°C in the dark for 30 min. The samples were then examined instantly using a flow cytometer.

Analysis of cell cycle phase distribution

For estimating the distribution of Hep-G2 liver cancer cells in different phases of cell cycle, the beta-amyrin-treated Hep-G2 cells were harvested and washed twice with PBS. Thereafter the cells were fixed with ethanol (70%) for about an hr and then washed again with PBS. The cells were finally resuspended in propidium iodide solution (50 µl/ml) and RNase1 (250 µg/ml). This was followed by incubation for 30 min at room temperature and fluorescence-activated cell sorting cater-plus cytometer using 10,000 cells/group.

Estimation of protein expression by western blotting

Total protein from cancer and normal cells was isolated in RIPA lysis buffer. Equal protein extracts from each group were run on SDS PAGE and then transferred to a polyvinylidene fluoride membrane. This was followed by blocking with 5% non-fat milk and incubation at room temperature for 1 hr. Thereafter, the membranes were incubated with a specific primary antibody at 4 °C overnight. This was followed by washing in washing buffer and incubation for 1 hr with the suitable secondary antibody. The protein bands of interest were visualised by an ECL Advanced Western Blot Detection Kit (Amersham, USA).

Statistics

The experiments were carried out in triplicate and presented as mean±SD. Statistically significant differences between groups were determined using one way

ANOVA and Tukey's *post hoc* test using GraphPad prism 7 software. A p value<0.01 was considered statistically significant.

Results

Beta-amyrin exerts antiproliferative effects on Hep-G2 cells

The antiproliferative effects of beta-amyrin were evaluated by MTT assay. The Hep-G2 cells were treated with beta-amyrin at varied concentrations. The results of MTT assay revealed that beta-amyrin exhibited considerable anticancer activity on the Hep-G2 cells and this acitivity was concentration-dependent (Figure 2). However, it was observer that the IC_{50} of beta-amyrin against Hep-G2 liver cancer cells was 25µM as compared to IC_{50} of 75 µM against non-cancer FR-2 cells.

Beta-amyrin triggers apoptosis in Hep-G2 cells

The Hep-G2 liver cancer cells were first treated with beta-amyrin at different concentrations, and then subjected to DAPI staining and observed under fluorescence microscope (Figure 3). It was observed that beta-amyrin induced apoptosis in Hep-G2 cells as evidenced from the increased number of cells with white color nuclei. The results of annexin V/PI further revealed that the apoptotic cell populations increased from 3.7% in the control to 56.7% at 50 µM beta-amyrin concentration (Fig-

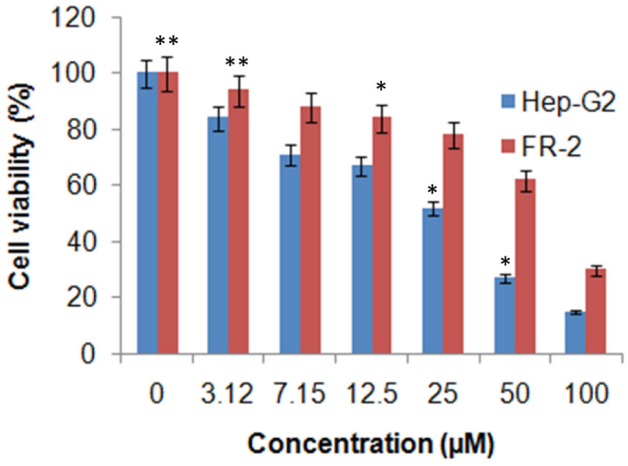

Figure 2. Assessment of cell viability of Hep-G2 and FR-2 cells at indicated concentrations of beta-amyrin. The experiments were carried out in triplicate and expressed as mean ± SD. *p<0.01, **p<0.001.

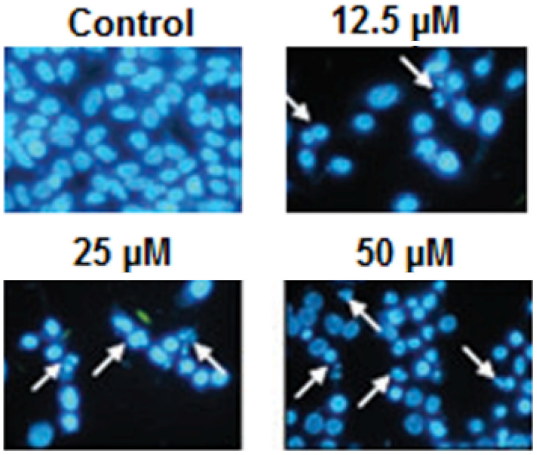

Figure 3. Induction of apoptosis in Hep-G2 cells at indicated concentrations of beta-amyrin with DAPI staining. The experiments were carried out in triplicate and expressed as mean ± SD. Arrows indicate the apoptotic cells after beta-amyrin treatment.

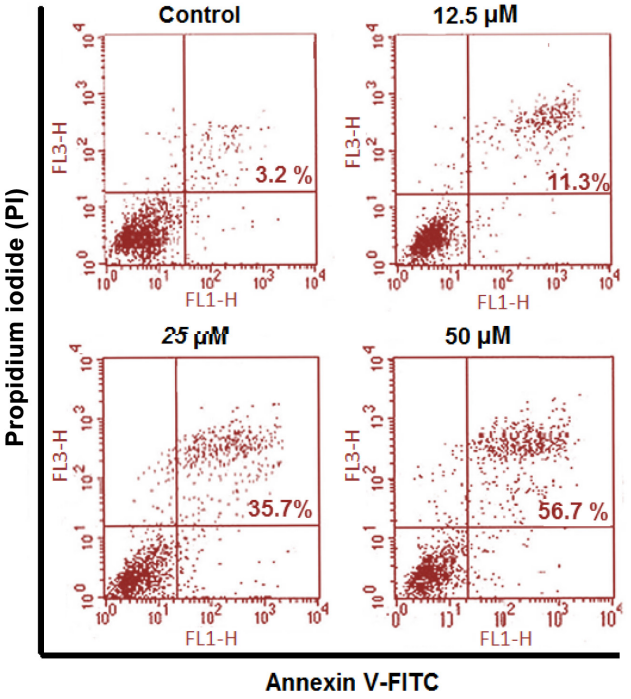

Figure 4. Induction of apoptosis as revealed by Annexin-V FITC assay. The cells were treated with increasing doses of the drug and it was seen that the fraction of cells with apoptosis increased noticeably.

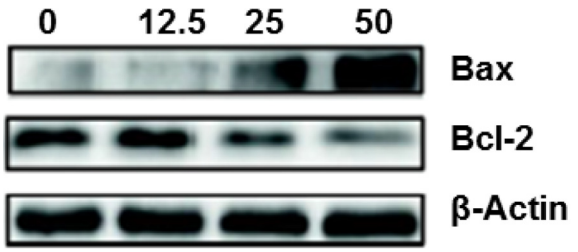

Figure 5. Expression of Bax and Bcl-2 in Hep-G2 cells at indicated concentrations of beta-amyrin (western blotting). The experiments were carried out in triplicate. Beta-amyrin increased the expression of Bax and decreased the Bcl-2 proteins, indicative of mitochondrial apoptosis.

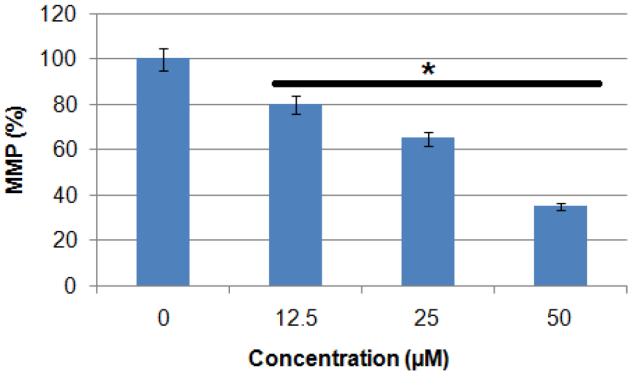

Figure 6. Effect of beta-amyrin on the mitochondrial membrane potential loss in Hep-G2 cells at varying doses. The experiments were carried out in triplicate. Increasing dosage of the drug led to increase in mitochondrial membrane potential loss. *p<0.01

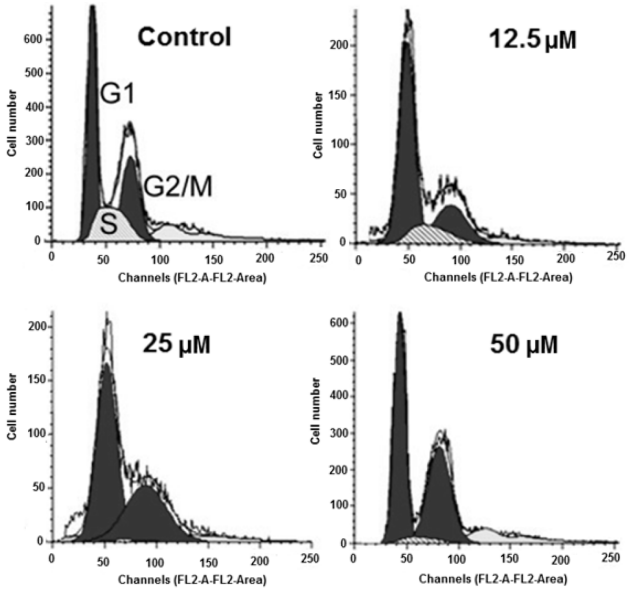

Figure 7. Induction of cell cycle arrest in Hep-G2 cells at IC_{50} concentrations of beta-amyrin as indicated by flow cytometry. The experiments were carried out in triplicate. Beta-amyrin led to accumulation of Hep-G2 liver cancer cells in G2/M phase of the cell cycle, triggering G2/M cell cycle arrest.

ure 4). To further examine whether beta-amyrin induced apoptosis followed the mitochondrial pathway we checked the expression of Bax and Bcl-2 proteins (Figure 5). The results revealed that beta-amyrin increased the expression of Bax and decreased the Bcl-2 proteins, indicative of mitochondrial apoptosis. This was further confirmed by the concentration-dependent decrease in the MMP (Figure 6).

Beta-amyrin causes G2/M cell cycle arrest in Hep-G2 cells

The distribution of Hep-G2 cells in the different cell cycle phases after treatment with beta-amyrin

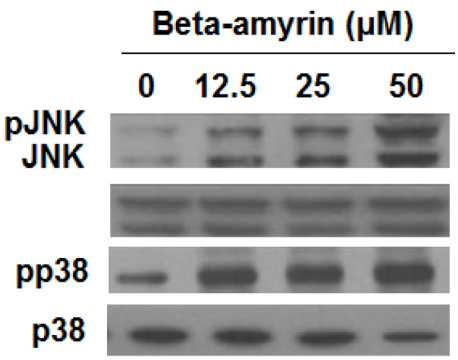

Figure 8. Effect of beta-amyrin on the phosphorylation of JNK and p38 as indicated by western blotting. The experiments were carried out in triplicate. Administration of beta-amyrin at increasing concentrations increased the phosphorylation of both JNK and p38 in a concentration-dependent manner.

was determined at varied concentrations. The results showed that beta-amyrin led to accumulation of Hep-G2 liver cancer cells in G2/M phase of the cell cycle, triggering G2/M cell cycle arrest (Figure 7).

Beta-amyrin activates JNK and P38

The effect of beta-amyrin on the phosphorylation of JNK and p38 was examined and the results indicated that the administration of beta-amyrin at varied concentrations increased the phosphorylation of both JNK and p38 in a concentration-dependent manner (Figure 8).

Discussion

Over the years, triterpenoids have attained considerable attention as bioactive molecules. They have been shown to possess anticancer activities against a range of cancer types such as breast, skin and liver [2,3]. Liver cancer, being one of the common reasons of cancer-related deaths [7-9], demands exploration of novel compounds that would prove effective and exhibit lower toxicity. In this context, the present study was carried out to investigate the anticancer effects of beta-amyrin against Hep-G2 liver cancer cells. The results showed that beta-amyrin exhibits considerable anticancer activity with an IC_{50} of 25 μM. To further unveil the reasons behind the anticancer effects of beta-amyrin we carried out DAPI staining and observed that beta-amyrin exerted anticancer effects via induction of apoptosis. Moreover, the apoptotic effects of beta-amyrin were concentration-dependent and the apoptotic cell populations increased with increase in the concentration of the beta-amyrin. Apoptosis is a series of consecutive steps that lead to cell's death without releasing any

harmful chemicals. It an important mechanism by which several of the chemotherapeutic drugs exert their antiproliferative effects [10-12]. The results of the present study are well supported with previous studies wherein triterpenes, such as such as ursolic acid, have been reported to trigger apoptosis in cancer cells [5]. Next, to explore whether the beta-amyrin-induced apoptosis follows the mitochondrial pathway, we estimated the expression of Bax and Bcl-2 proteins. The results of the western blotting revealed that the expression of Bax was increased and that of Bcl-2 was decreased in response to the beta-amyrin treatment. Another important mechanism that has been reported to contribute to the anticancer effects of many well known drugs is cell cycle arrest [13,14]. Some anticancer drugs halt the progression of the cells from the one phase of the cell cycle to the other by targeting specific proteins leading to the accumulation of cancer cells at a particular phase. Arrest of the cell cycle prevents the cancer cells to develop into tumors and to spread to other parts of the body [15]. Consistent with this, we observed that the triterpenoid beta-amyrin caused G2/M cell cycle arrest of Hep-G2 cancer cells in a concentration-dependent manner. Previously it was reported that many anticancer molecules trigger apoptosis by activating JNK and p38 signalling [16]. Therefore, we investigated the effect of beta-amyrin on the phosphorylation of JNK and p38 and observed that beta-amyrin enhanced the phosphorylation of both JNK and p38 in a concentration-dependent manner. Taken together these results suggest that beta-amyrin is a promising lead molecule for the treatment of liver cancer.

Conclusion

From the results of this study we conclude that beta-amyrin exhibits significant anticancer activity against liver cancer cells. The anticancer activity can be attributed to the ability to trigger apoptosis, DNA damage, cell cycle arrest and activation of p38 and JNK signalling pathways Our results suggest that beta-amyrin may prove a new promising lead molecule for the development of systemic cancer therapy and deserves further investigation.

Acknowledgements

This study was supported by the following institutions:

1. National Natural Science Foundation of China (#81401460).
2. Natural Science Foundation of Jiangxi Province (#20151522070010, #20151122070030).
3. Health and Family Planning of Jiangxi Province (#20155294).
4. Health and Family Planning of Zhejiang Province (# 2018261835).

Conflict of interests

The authors declare no conflict of interests.

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