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ORIGINAL ARTICLE

Antitumor activity of Cedrelone in temozolomide-resistant human glioma cells is accompanied by mitochondrial mediated apoptosis, inhibition of angiogenesis, cell cycle disruption and modulation of ERK/MAPK signalling pathway

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Summary

Purpose: *Glioma is one of the most aggressive types of human cancers and responsible for considerable mortality across the globe. Moreover, the development of drug resistance and lack of efficient drug options form the major hurdle in the treatment of gliomas. Herein, the anticancer potential of Cedrelone was examined against temozolomide-resistant glioma cells.*

Methods: *The proliferation rate of malignant glioma cells was assessed by CCK-8 cell counting assay. Autophagy was detected by electron microscopy. Apoptotic cell death was revealed by propidium iodide (PI) staining. Cell cycle analysis was performed by flow cytometry. Protein expression was determined by immuno blotting.*

Results: *The results showed that Cedrelone could considerably inhibit the proliferation of glioma cells. The anticancer activity of Cedrelone against the U87 malignant glioma cells was found to be due to induction of apoptosis. The Cedrelone-triggered apoptosis was also linked with alteration in the apoptosis-related protein expression. It also caused increase of reactive oxygen species (ROS) and decline of mitochondrial membrane potential (MMP). Additionally, Cedrelone could also trigger G2/M cell cycle arrest of U87 cells. Furthermore, it was found that Cedrelone could inhibit the ERK/MAPK signalling pathway in the temozolomide-resistant malignant glioma cells.*

Conclusion: *These results indicate that Cedrelone could inhibit the growth of temozolomide-resistant malignant glioma in vitro and may be used for the development of chemotherapy against this disease.*

Key words: *autophagy, apoptosis, cell cycle, cedrelone, malignant glioma*

Introduction

Gliomas are responsible for significant mortality and morbidity across the world and are reported to be among the most aggressive types of human cancers [1]. They largely affect the central nervous system (CNS) and constitute about half of total primary malignant tumors of the CNS [2]. The treatment options for gliomas include multimodal therapies with surgery, radiotherapy finally followed by chemotherapy. However, the results are far from satisfactory and the overall survival of the patients is very low [3]. Recent studies have shown that molecules derived from edible plants can be used as safer anticancer drugs [4]. Plants form a ubiquitous source of novel and bioactive chemical scaffolds [5]. Pharmacological studies have shown that plant metabolites exhibit a number of bioactivities such as anticancer activity [6]. Several of the plant metabolites have been reported to halt the proliferation of cancer cells and thus may be considered essential for the development

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of systemic therapy for malignant gliomas [7]. Cedrelone is an important crystalline component of the heartwood of *Cedrela toona* and has been shown to inhibit the growth of several breast cancer cells [8]. However, the anticancer effects of Cedrelone against the temozolomide-resistant malignant glioma cells have not been investigated and the underlying mechanism is still not known. The main aim of the current study was to investigate the anticancer effects of Cedrelone against temozolomide-resistant glioma cells along with examining its effects on autophagy, apoptosis, cell cycle and ERK/MAPK signalling pathways.

Methods

Cell lines and culturing conditions

The temozolomide-resistant malignant glioma cell line U87 was procured from American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) in CO_2 incubator (Thermo Scientific, China) at 37°C with 98% humidity and 5% CO_2.

Assessment of cell viability

The viability of glioma U87 cells was examined by CCK-8 cell counting assay. In brief, 5×10^4 cells/well were seeded in 12-well plates and incubated for 24 h with different concentrations of Cedrelone. The aliquots of cells were then removed and counted in triplicate following Trypan blue staining. Cell morphology of the Cedrelone-treated cells was also examined by phase-contrast microscopy as described previously [13].

Acridine orange and ethidium bromide double staining for the detection of apoptosis

Firstly, the temozolomide-resistant glioma cells (0.6×10^6) were grown in 6-well plates. Following an incubation period of around 12 hours, the temozolomide-resistant glioma U87 cells were subjected to Cedrelone treatment for 24 h at 37°C. The cell cultures were then centrifuged and the pellets were washed with phosphate buffered saline (PBS). Thereafter, the cells were stained with a solution of acridine orange (AO) and ethidium bromide (EB), centrifuged and PBS-washed. Finally, the nuclear morphology of the stained cells was examined by fluorescence microscopy.

Cell cycle analysis, ROS and MMP levels

To analyse the effect of Cedrelone on the distribution of the U87 glioma cells in different cycle phases, flow cytometry after PI staining was performed as described in literature [13]. In brief, the U87 cells were grown in 6-well plates and treated with Cedrelone for 24 h. Then the cells were collected and PBS-washed, followed by fixation in ethanol (70%). After overnight incubation at 4°C, the cells were subjected to PI staining and to flow cytometry. The ROS and MMP levels were determined as described previously [14].

In vitro angiogenesis assay

The *in vitro* angiogenesis assay was carried out by using an Angiogenesis kit (Kurabo, Japan). In brief, human umbilical vein endothelial cells (HUVECs) and fibroblasts were co-cultured in 24-well plates and U87 cells were cultured in the upper chamber separated from lower chamber by a membrane. The upper chamber was removed after shifting to lower chamber after 7 days and HUVECs were stained with rabbit anti-human, mouse CD31 monoclonal antibodies. Formation of the tubes was measured by counting 10 random fields.

Western blotting

To determine the expression of the selected proteins in the Cedrelone-treated temozolomide-resistant glioma cells, the cells were subjected to lysis with RIPA buffer and the protein content of each lysate was estimated by bicinchoninic acid assay (BCA) assay. The samples were then loaded on the SDS-PAGE. The gels were then transferred to nitrocellulose membranes and subjected to treatment with Brd U primary antibody at 4°C for a period of 24 h. After this, the membranes were incubated with HRP-conjugated secondary antibody for 50 min at 25°C. Enhanced chemi-luminescence reagent was used to visualise the protein bands.

Statistics

SPSS statistical software package was used for analyses.

Data are shown as mean ± SD. Statistical analysis was done using Students *t*-test with GraphPad prism 7 software. Values of $p<0.05$ were taken as indicative of significant difference.

Results

Growth inhibitory effects of Cedrelone in temozolomide-resistant glioma cells

To confirm the antiproliferative effects of Cedrelone (Figure 1), the CCK-8 cell counting assay was performed. Cedrelone showed significant antiproliferative effects on the temozolomide-resistant U87 glioma cells which were found to be dose-

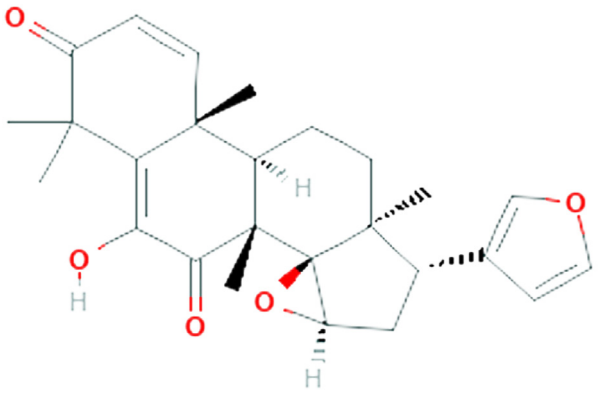

Figure 1. The chemical structure of Cedrelone.

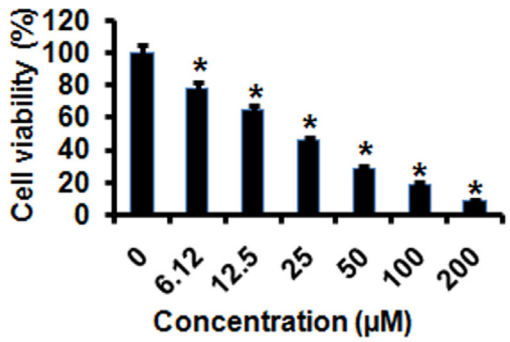

Figure 2. Effect of Cedrelone on the viability of the U87 cells as determined by CCK-8 cell counting assay. The experiments were performed in triplicate and shown as mean ± SD (*p<0.01).

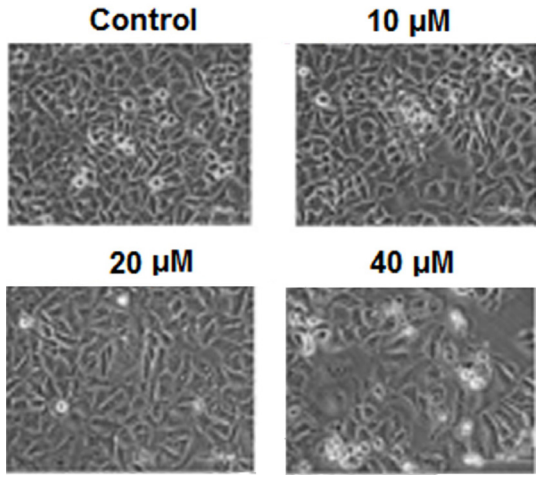

Figure 3. Effect of Cedrelone on the morphology of glioma cells. Cedrelone caused significant changes in cellular morphology including cell shrinkage. The experiments were performed in triplicate.

dependent (Figure 2). The IC_{50} of Cedrelone against the U87 cells was 20 μM. In addition, it was found that the anticancer effects of Cedrelone on the glioma cells were concentration-dependent. Furthermore, it was observed that Cedrelone caused nuclear condensation, membrane shrinkage and blebbing of the U87 cells (Figure 3).

Induction of apoptosis in temozolomide-resistant glioma cells

The fact that Cedrelone also induces apoptosis was validated by AO/EB staining which showed apparent changes in the nuclear morphology of the U87 cells, characteristic of apoptosis (Figure 4).The increased expression of Bax, Caspase 3 and 9 and decreased expression of the Bcl-2 in U87 cells further confirmed the activation of programmed cell death (Figure 5). To confirm the mitochondrial apoptosis, we checked the levels of the ROS and MMP and found that Cedrelone caused considerable increase in the levels of ROS which was accompanied with decrease in the levels of MMP (Figure 6).

Cedrelone causes arrest of the temozolomide-resistant malignant glioma cells in G2/M phase

The effects of Cedrelone on the distribution of U87 cells in various cell cycle phases were assessed by flow cytometry. It was found that Cedrelone caused noteworthy increase in the percentage of U87 cells in the G2 phase of the cell cycle. The percentage of U87 cells in the G2 phase increased from 19.7 to 40.7% upon treatment with Cedrelone (Figure 7). These results clearly indicate that Cedrelone induces G2/M cell cycle arrest of glioma cells.

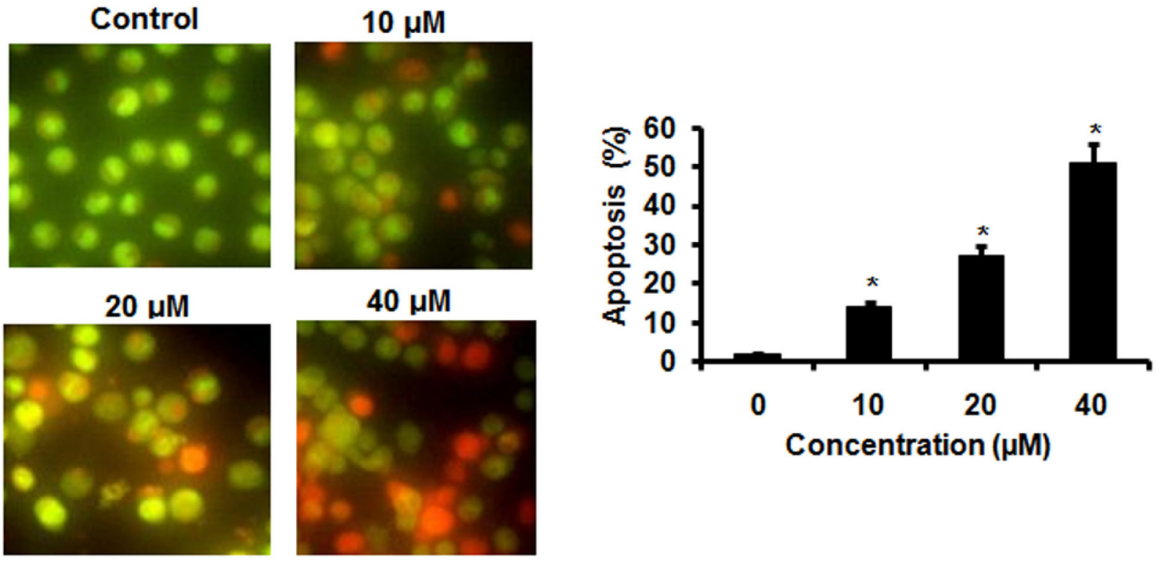

Figure 4. AO/EB staining images showing induction of apoptosis by Cedrelone on the U87 cells. Orange fluorescence indicates the onset of apoptosis in glioma cells with increasing dose of Cedrelone. The experiments were performed in triplicate (*p<0.01).

Inhibition of ERK/MAPK1 signalling pathway by Cedrelone

Cedrelone-treated cells were also used for investigation of the effect of Cedrelone on the ERK/MAPK1 signalling pathway. What was found was that Cedrelone treatment resulted in dose-dependent reduction in the phosphorylation of p-ERK1/2, p-p38 and p-AKT, while no obvious effect was seen on the expression of ERK and p38 (Figure 8). Taken together, the results suggest that Cedrelone inhibits the ERK/MAPK1 signalling pathway in U87 cells.

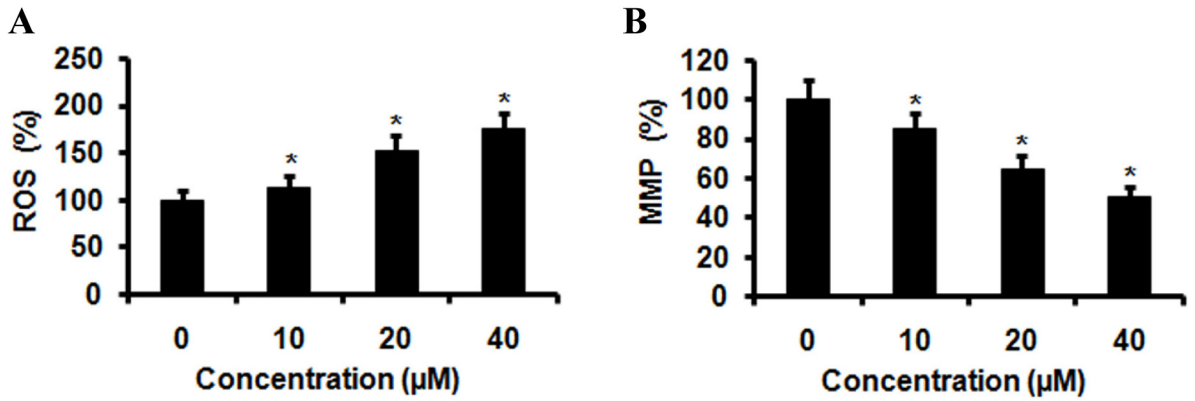

Figure 5. Cedrelone caused increase of ROS **(A)** and decrease of MMP **(B)** in U87 glioma cells in a dose-dependent manner. The experiments were performed in triplicate and shown as mean ± SD (*p<0.01).

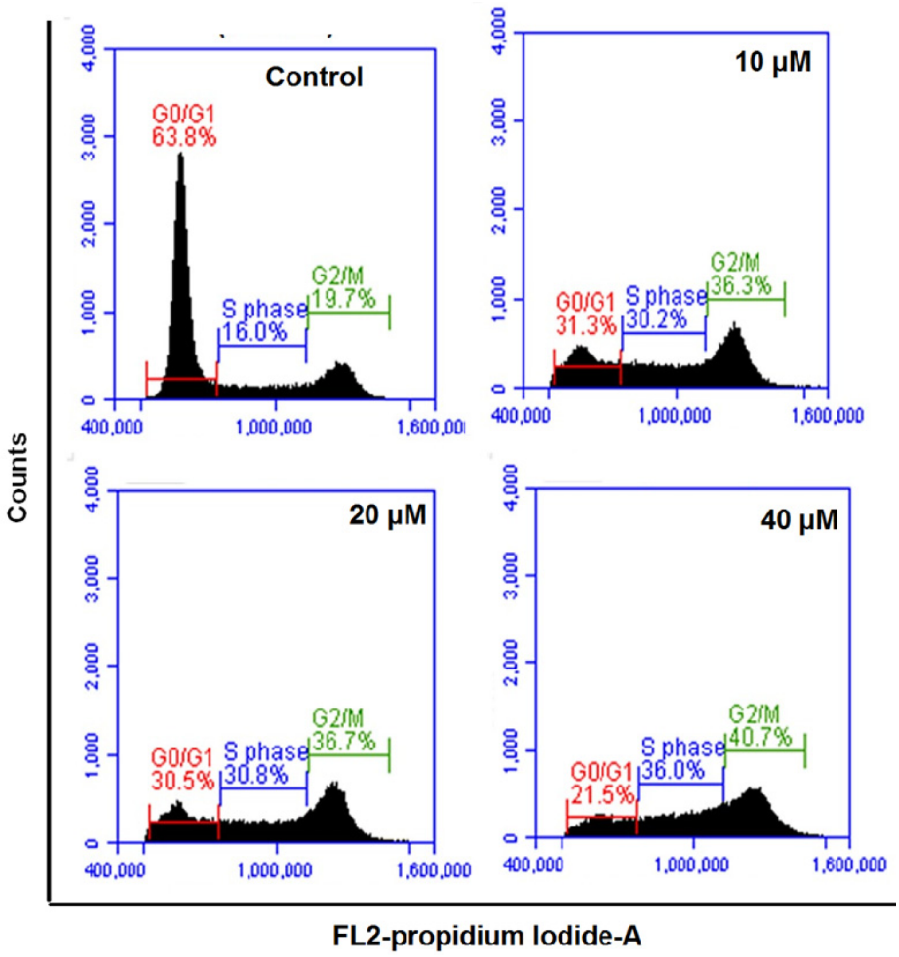

Figure 6. Induction of G2/M arrest of U87 glioma cells by Cedrelone as depicted by flow cytometry. The percentage of G2/M phase cells increased significantly in a dose-dependent manner. The experiments were performed in triplicate.

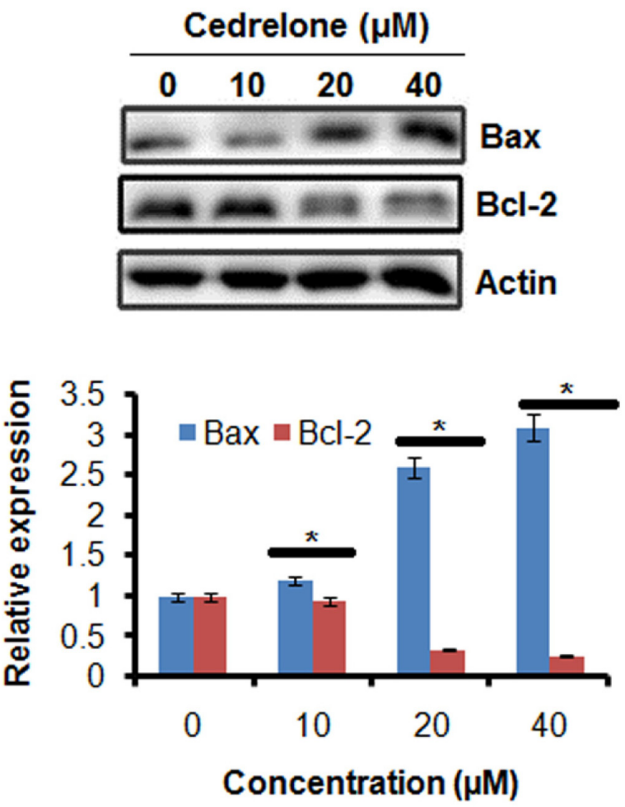

Figure 7. Effect of Cedrelone on the expression of Bax and Bcl-2 proteins as depicted by western blot analysis. Bax expression was increased while Bcl-2 expression was decreased in a concentration-dependent manner. The experiments were repeated in triplicate (*p<0.01).

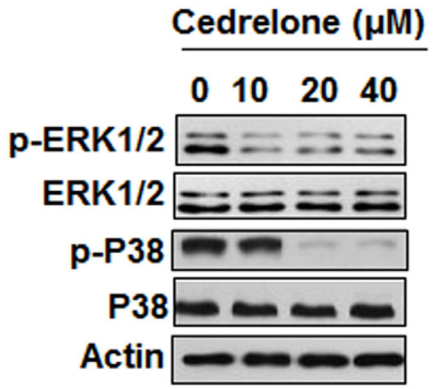

Figure 8. Effect of Cedrelone on the ERK/MAPK signalling pathway as depicted by western blot analysis. Cedrelone led to dose-dependent reduction in the phosphorylation of P-ERK1/2, p-p38 and p-AKT, while no obvious effect was seen on the expression of ERK and p38. The experiments were performed in triplicate.

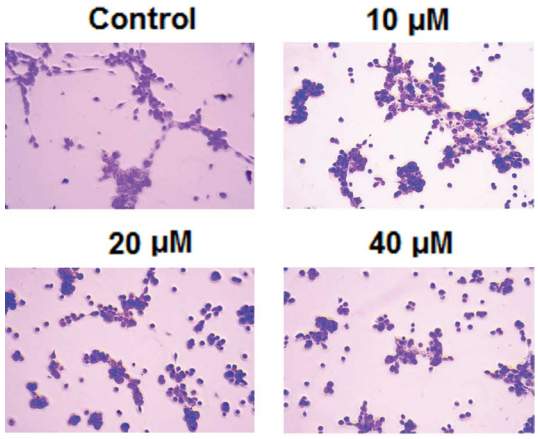

Figure 9. Cedrelone exerted anti-angiogenesis effects by suppressing the tube formation of the HUVECs cells in a concentration-dependent manner.

Cedrelone exhibits anti-angiogenesis effects

The anti-angiogenesis potential of Cedrelone was investigated using the Angiogenesis kit. The results revealed that Cedrelone suppressed the tube formation of the HUVECs cells in a concentration-dependent manner, suggestive of the anti-angiogenesis effects of Cedrelone (Figure 9)

Discussion

Gliomas are a destructive type of cancer [15]. Because of the inefficient treatment options and diagnosis at advanced stages, the incidence of malignant glioma is increasing [1]. The anticancer drugs that are being used for the management of malignant glioma have serious side effects affecting the patient quality of life [2]. Compounds derived from plants have gained tremendous attention in the recent past due to their comparatively lower toxic effects. Hence, the spotlight of the researchers across the globe is to identify and screen natural products against cancer cells for developing efficient drugs to target gliomas [16]. In the present study, the antiproliferative effects of Cedrelone were evaluated against temozolomide-resistant glioma cells and found that this compound resulted in decline in their proliferation rate. In the studies carried out previously, plant metabolites have also been reported to inhibit the growth of cancer cells by triggering apoptosis [17]. Apoptosis is a process that maintains the tissues homeostasis and help in the elimination of unwanted, harmful or cancer cells [18]. Herein, it was found that Cedrelone activated the mitochondrial apoptotic cell death of the U87 glioma cells. This was also associated with changes in the expression of apoptosis-related protein expression. Previous studies have indicated that Cedrelone induces apoptosis of breast cancer cells [8]. ROS generation and reduction of the MMP have also been linked to the induction of apoptosis [19]. Our investigation on whether Cedrelone has any effect on the ROS and MMP levels

showed that Cedrelone treatment caused increase in ROS levels and decline in the MMP levels in the U87 cells.

Cell cycle arrest causes inhibition of the cell growth as the cells are unable to enter mitosis and complete the division [20]. A number of anticancer molecules cause arrest of the cells at various cell cycle checkpoints [21]. Herein, we observed that Cedrelone caused arrest of the U87 cells in the G2/M phase of the cell cycle. Furthermore, Cedrelone also suppressed the angiogenesis of the U87 cells concentration-dependently.

In cancer tissues/cells several of the signalling pathways are aberrantly activated and ERK/MAPK is one such pathway that has been shown to play essential role in the proliferation of cancer cells [11,12]. In this study we found that Cedrelone could inhibit the expression of p-ERK1/2, and p-p38 in U87 cells concentration-dependently, suggestive of its potent anticancer potential.

Conclusions

In conclusion, Cedrelone exerts growth inhibitory effects on the temozolomide-resistant glioma cells by induction of mitochondrial-mediated apoptotic cell death. In addition, it also suppressed the ERK/MAPK signalling pathway. Cedrelone also caused arrest of the U87 cells in the G2/M phase of the cell cycle. Hence, Cedrelone may prove to be a potential therapeutic agent and merits further investigation.

Conflict of interests

The authors declare no conflict of interests.

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