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ORIGINAL ARTICLE

In vitro anticancer effects of levopimaric acid in cisplatin-resistant human lung carcinoma are mediated via autophagy, ROS-mediated mitochondrial dysfunction, cell apoptosis and modulation of ERK/MAPK/JNK signalling pathway

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Summary

Purpose: *Lung cancer is one of the deadly diseases with limited treatment options available. The main aim of the current study was to investigate the antitumor effects of levopimaric acid - a naturally occurring diterpene, against cisplatin-resistant non-small cell lung carcinoma cells A-549 and normal MRC5 cells. Effects of levopimaric acid on autophagy, reactive oxygen species (ROS), apoptosis and ERK/MAPK signalling pathways were also investigated in the current study.*

Methods: *Proliferation rate was monitored by MTS assay. Apoptosis was detected by DAPI staining as well as western blot assay. Electron microscopy was used to investigate the autophagic effects of levopimaric acid. Effects on ROS and mitochondrial membrane potential (MMP) were evaluated by flow cytometry. Protein expression was examined by western blotting.*

Results: *It was found that levopimaric acid exerts potent antiproliferative effects against the cisplatin-resistant lung cancer cells and exhibited an IC_{50} of 15 μM. However, the toxic effects of levopimaric acid were seen to be insignificant against the normal cells. The anticancer effects of levopimaric acid were due to induction of apoptosis which was also associated with modulation of apoptosis-related proteins (Bax and Bcl-2). Levopimaric acid also induced autophagy which was also associated with alterations of autophagy-related protein expressions (LC3I, II, and p62). Levopimaric acid caused ROS-mediated alterations in the MMP. It was also found that the molecule could induce drug-resistant lung cancer cell death by activating p38 MAPK and JNK signalling pathways while inhibiting ERK pathway.*

Conclusion: *The current results strongly indicate that levopimaric acid may prove to be a potential anticancer drug candidate provided further in depth studies are carried out.*

Key words: *levopimaric acid, apoptosis, autophagy, lung cancer, flow cytometry*

Introduction

Lung cancer (LC) is the major frequent reason of cancer related deaths across the globe. This lethal malignancy takes the lives of about 1,6 million people every year [1]. Nearly 85% of lung cancer are non-small cell carcinoma (NSCLC). Among the subtypes of NSCLC, LUSC (squamous cell carcinoma) and LUAD (lung adenocarcinoma) are most frequent subtypes [2]. Around 80% of the cases of lung carcinoma are due to the most common aetiological reason i.e. tobacco smoking. The countries where smoking is common, lung cancer remains predominant like in United States [3]. While the key histological subtype NSCLC along with small cell lung carcinoma (SCLC) are linked to smok-

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ing, the link is stronger with SCLC and LUSC than LUAD, the latter being more frequently seen in non-smokers [4]. The reason for lung cancer in non-smokers is mostly due to environmental exposure to occupational carcinogens, second-hand smoking, pollution, and by means of congenital genetic susceptibility [5,6]. Elimination of tobacco use and tobacco related products is the main target of the global fight against cancer and it needs a more comprehensive methodology. Alternative delivery of nicotine, without co-administration of carcinogens present in cigarettes, remains the primary prevention effort to target nicotine addiction like e-cigarettes [7]. Use of varenicline (a partial substitute of the nicotinic acetylcholine receptor) is another approach to tackle nicotine addiction [8]. This fatal disease has been found more prevalent in males than in females and besides this, women have also shown a higher potential of better prognosis, higher survival rate and better treatment responses [9-11]. Despite this, there is only 18% of 5-year survival chances for lung cancer, suggestive of the need of more efficient drugs and more comprehensive approach to tackle this malignancy. Pharmacological studies have revealed that plants are potential carriers of compounds with anticancer properties, that can be utilized to fight cancer [12]. One such compound is levopimaric acid, a diterpene, and the major constituent of Pine Oleoresin has revealed various biological activities like antioxidant, antibacterial and cardiovascular [13,14]. Herein, an *in vitro* study was performed to evaluate/examine the anticancer effects of levopimaric acid in cisplatin-resistant human lung carcinoma cells which showed that these effects are mediated via autophagy, ROS-mediated mitochondrial dysfunction, cell apoptosis and modulation of ERK/MAPK/JNK signalling pathway. Different experiments were carried out to collect the data of anticancer effects of levopimaric acid in cisplatin-resistant human lung carcinoma cells.

Methods

Cell viability assay

Cell proliferation of MRC5 normal lung cells and A549 human lung carcinoma cells was established by MTS analysis (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). MTS analysis is a cell titer 96 aqueous one solution cell proliferation/multiplication assay. At a density of 2×10^6 cells per well, both normal (MRC5) as well as cancer cells (A549) were seeded in 96-well plates separately. These cell types were incubated overnight prior to their exposure to target molecule (levopimaric acid) at different concentrations e.g., 0, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µM. These doses were added to the plates at different time intervals followed by further incubation. Afterwards, considering the manufacturer's guiding principle (MGP), MTS solution was added and finally the absorbance was calculated at a wavelength of 490nm using an ELISA plate reader (ELX 800; Bio-Tek Instruments, Inc., Winooski, VT, USA).

Clonogenic assay

The A549 human lung cancer cells were harvested at the exponential phase of growth and numbered by using hemocytometer. Seeding was done at a density of 360 cells/well, followed by incubation for 2 days so that the cells got attached. Afterwards, these cells were exposed to varying doses of the target molecule i.e. 0, 6, 12, and 24 µM. Treated cells were then uninterruptedly incubated for 96 h at 37°C. After incubation, the cells were washed with phosphate-buffered saline (PBS) and then methanol was used for colony fixation. Later, the cell colonies were stained using crystal violet for 1 h before being investigated under a light microscope.

Electron microscopy

The effect of autophagy induction by levopimaric acid on A549 human lung carcinoma cell line was done by transmission electron microscopy (TEM). Briefly, A549 cell line was exposed to levopimaric acid at 12 µM for 48 h. Afterwards, trypsinization was performed for cell assembling followed by washing with phosphate buffered saline (PBS). After washing, the treated cells were fixed with 2% glutaraldehyde in 0.1M phosphate buffer, followed by post fixation with 1% osmium tetroxide (OsO_4). Thereafter, the target cells were subjected to treatment with ethanol, embedded in resin and then thin sections were cut using ultramicrotome. Finally, the thin sections were observed under electron microscope.

Determination of ROS and MMP

For the determination of ROS and MMP, A549 human lung cancer cells were cultured at 37°C for 24 h. Then, the A549 cells were exposed to the target molecule at varying concentrations i.e. 0, 6, 12, and 24 µM for 24 h. Afterwards, the Dulbecco's modified Eagle's medium (DMEM) was totally drawn off and sampling of the cells was performed at 1000 cells per sample. For ROS determination cells were subjected to 5 µM DCH-DA treatment and data was collected by using flow cytometry. MMP was estimated by adding a fluorescent dye rhodamine 123 (Rh123) to the samples and hence MMP was determined by flow cytometry.

Apoptosis detection by DAPI

For the purpose of apoptosis detection the A549 human lung cancer cells were cultured using 6-well plates, followed by incubation at 37°C for 24 h. Thereafter, A549 cells were subjected to levopimaric acid treatment at varying doses, i.e. 0, 6, 12, and 24 µM for 24 h at 37°C. When the target cells started to cast-off, about 20 µM of cast cells was obtained and put on a glass slide. Furthernore, these cells were stained with DAPI (4',6-diamidino-2-phenylindole) which is a fluorescent dye. Finally the slides were cover-slipped and studied under a fluorescent microscope.

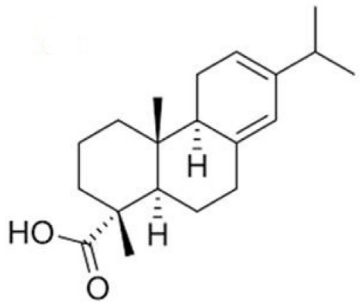

Figure 1. Chemical structure of levopimaric acid.

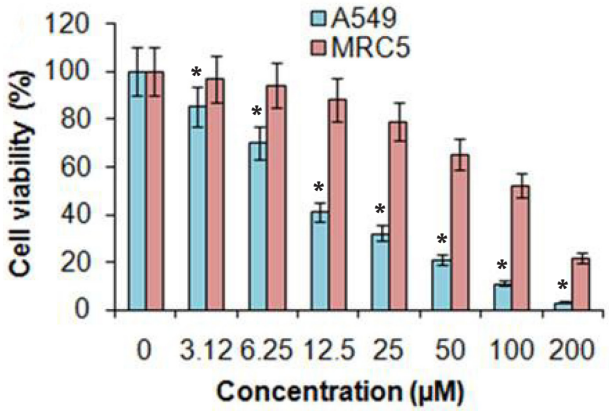

Figure 2. MTS assay indicating the effects of levopimaric acid on the viability of the A-549 human non-small cell lung cancer cells and MCR-5 normal lung cells. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05)

Western blotting analysis for protein expression

Levopimaric acid-treated cells (at different concentrations i.e. 0, 6, 12, and 24 μM) were lysed with RIPA buffer and bicinchoninic acid (BCA) assay was performed for estimation of protein content inside each lysate. Samples were loaded on SDS-PAGE followed by transfer of gels to nitrocellulose membranes. Afterwards the membranes were referred to primary antibody treatment at a temperature of 4°C and for a time period of 24 h. After the completion of primary antibody treatment, the membranes were subjected to secondary antibody treatment with HRP-conjugation for a time interval of 1 h at room temperature. For visualization and development of protein bands advanced chemi-luminescence reagent was used.

Statistics

Data was presented as mean ± standard deviation (SD) from at least three independent experiments. Statistically important difference was taken with $p<0.05$. One-way ANOVA with graph pad prism 7 software was used to investigate the differences among groups.

Results

Cell cytotoxicity of levopimaric acid and inhibition of colony formation

To decipher the cytotoxic effects of Levopimaric acid (Figure 1) on A549 and MRC5 cells, the cell proliferation was analysed by MTS assay. All treated cell collections showed a significant drop

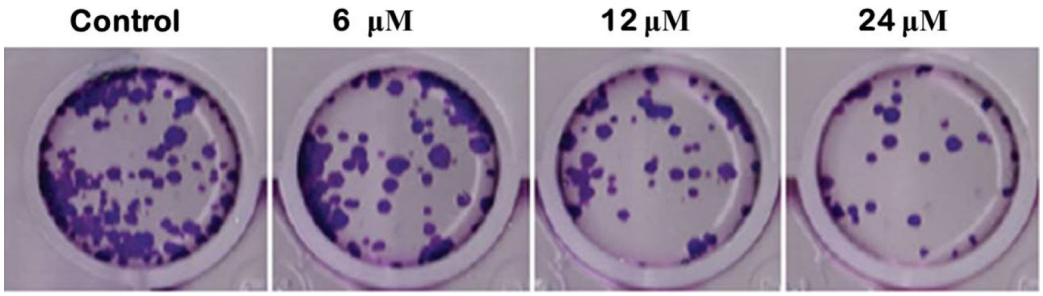

Figure 3. Clonogenic assay showing the effect of levopimaric acid at different concentrations on the cancer cell colony formation tendency of A-549 human lung cancer cells. The experiments were repeated thrice

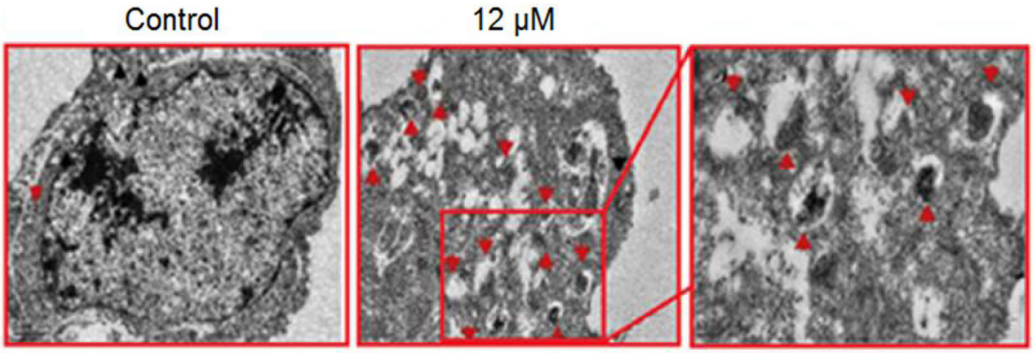

Figure 4. Electron microscopic analysis exhibiting the effects of levopimaric acid on the autophagy induction in A-549 human lung cancer cells. Zoomed area indicates presence of autophagosomes. The experiments were repeated thrice.

in cell proliferation after 48h exposure. The IC_{50} of the levopimaric acid was 12 μM in A549 cells and 75 μM in normal lung cells MRC-5 (Figure 2). The MTS assay determined that levopimaric acid significantly prevented the proliferation of A549 and MRC5 cells (cancer and normal lung cells respectively) in a dose-dependent manner (ranging from 0–200 μM), with normal cells showing lesser toxicity. Furthermore, to determine the impact of the test molecule on colony formation, a clonogenic assay was performed. When the cells were observed under light microscope, levopimaric acid revealed tremendous retardation on colony formation in A549 human lung cancer cells in a dose-dependent manner (Figure 3).

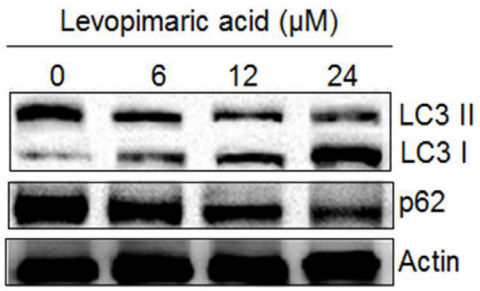

Figure 5. Effects of levopimaric acid on the expression of LC3-1, LC3-II and p62 in the A-549 human lung cancer cells as shown by western blot analysis. The Figure shows that levopimaric acid increases LC3 II expression and decreases p62 dose-dependently. The experiments were repeated thrice.

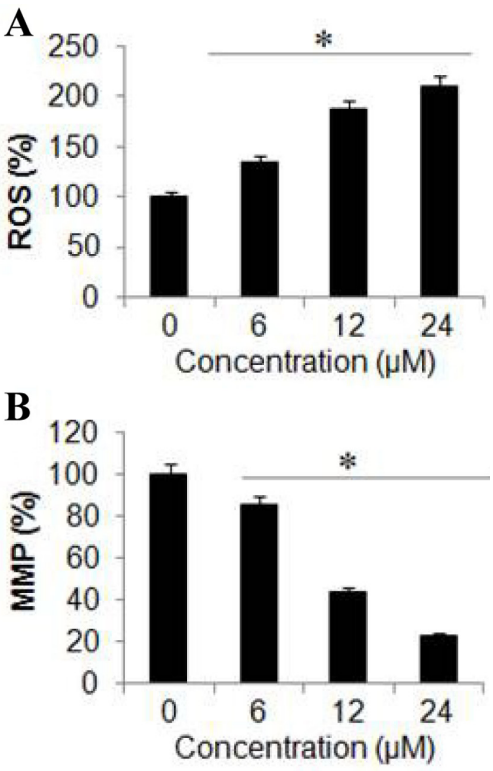

Figure 6. Effect of levopimaric acid on the reactive oxygen species (ROS) production **(A)** and mitochondrial membrane potential (MMP) **(B)** in A-549 human non-small cell lung cancer cells. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05).

Autophagy induction by levopimaric acid in cisplatin-resistant A549 human lung cancer cells

Levopimaric acid facilitated in bringing about autophagy, its detection and extent being evaluated by TEM. Levopimaric acid-treated cells showed vacuole formation, and autophagosome formation (Figure 4) which is suggestive of autophagy. Furthermore, to confirm autophagy induction by levopimaric acid, western blotting analysis was performed and the expression of autophagy-related proteins were obtained and indicated that this molecule decreased LC3 II, increased LC3 I and p62 protein expressions (Figure 5), giving more support of the fact that levopimaric acid results in autophagy induction.

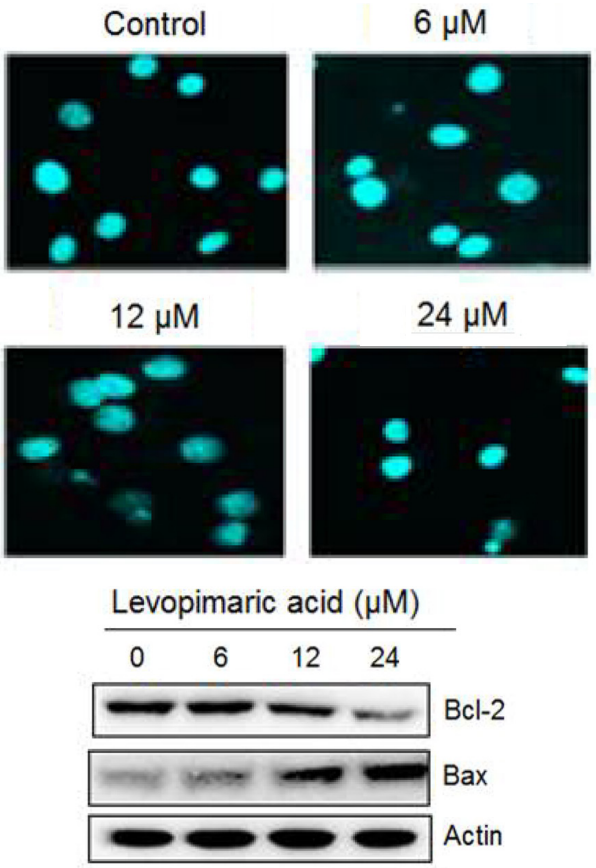

Figure 7. Effect of levopimaric acid on the cellular morphology as well as apoptosis-related proteins (Bax and Bcl-2). Cellular morphology was evaluated by DAPI staining using fluorescence microscopy, while effects on Bax and Bcl-2 were examined by western blot method. The Figure shows that levopimaric acid induces apoptosis in A549 cells by increasing Bax/Bcl-2 ratio. The experiments were performed in triplicate.

Levopimaric acid raised ROS and declined MMP in A549 human lung cancer cells

The influence of levopimaric acid on ROS and MMP in A549 human lung cancer cell line was assessed by flow cytometry. These cells were subjected to treatment with different concentrations of the molecule, i.e. 0, 6, 12 and 24 µM. Exposure to target molecule initiated a substantial escalation in ROS percentage, nearly touching 210 (Figure 6A), which indicated that the target molecule resulted in escalation of intracellular ROS concentrations in a dose-dependent manner. The results also revealed that MMP percentage in levopimaric acid-treated cells reduced considerably from 100% to just above 20% with increasing levopimaric acid concentrations (0, 6, 12 and 24 µM) (Figure 6B).

Levopimaric acid induces apoptosis in A549 lung cancer cells

DAPI staining indicated that A549 lung cancer cells have shrunken, condensed, and fragmented nuclei after exposure to levopimaric acid for 24 h (Figure 7A), which clearly indicates that the test molecule causes apoptosis induction. Untreated cells showed low fluorescence, smooth, flattened and normal nuclei. To confirm autophagy induction in cisplatin-resistant A549 lung cancer cells, western blotting analysis was performed. The results revealed that when the cells were treated with varying doses of levopimaric acid, the expression of apoptosis-associated proteins was altered, resulting in down-regulation of BCL-2 and up-regulation of BAX protein, clearly indicating onset of apoptosis (Figure 7B).

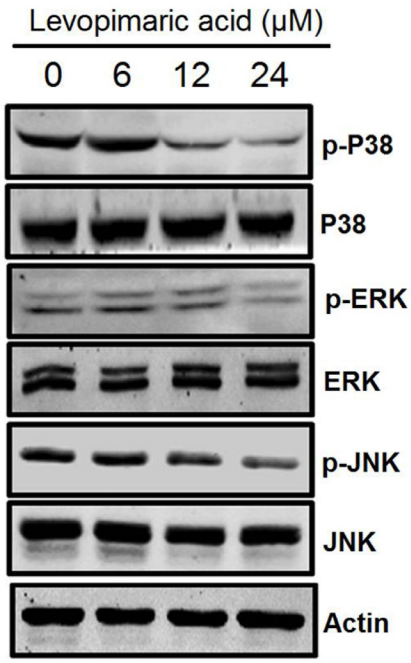

Figure 8. Effect of levopimaric acid on the ERK/MAPK/JNK signalling pathway. Levopimaric acid led to considerable reduction in p-P38, p-ERK and p-JNK protein expressions but caused no significant increase (almost constant) in P38, ERK and JNK protein expressions. The experiments were performed in triplicate.

Modulation of ERK/MAPK/JNK signalling pathway by levopimaric acid

To establish that levopimaric acid results in modulation of ERK/MAPK/JNK signalling pathway, western blotting analysis was performed. The A549 cells were treated with different concentrations (0. 6, 12, and 24 µM) of the test molecule and later were subjected to western blotting analysis and data was recorded. Data revealed significant effect of levopimaric acid on protein expression related to ERK/MAPK/JNK signalling pathway. It was observed that levopimaric acid caused no significant increase (almost constant) in P38, ERK and JNK protein expressions but resulted in considerable reduction in p-P38, p-ERK and p-JNK protein expressions (Figure 8). ERK/MAPK/JNK signalling pathway is important for cancer development and its alteration disturbs cancer growth.

Discussion

Though tobacco prevention is one of the most important strategies to fight lung carcinoma, still it is not sufficient alone to win the war. In order to increase the clinical outcome for patients, more sophisticated therapies are needed. Over the past 20 years or so, the discovery of new potential target therapies and proper application of immunotherapy in some patients with later stages of NSCLC, has been promising and substantial. Although advancements are made towards treatment of lung carcinoma, still international research is lacking publications of other types of cancer (recent bibliometric investigation). Besides high mortality rate, poor prognosis and large economic costs, full interest was not laid towards lung carcinoma up to 2013 [15]. Thus, to fight this fatal malignancy and increase survival chances, development of *in vitro* and *in vivo* novel treatment strategies are the need of the hour. Herein, studies on levopimaric acid were performed to unveil its *in vitro* anticancer effects in cisplatin-resistant human lung carcinoma cells. Levopimaric acid resulted in decreasing of cell proliferation/multiplication of human lung cancer cells selectively. No such effect was observed on normal human lung cell line, indicating its selectivity and potential of lowering the proliferation of cancer cells in a dose-dependent manner as revealed by MTS assay. Furthermore, clonogenic

assay showed that levopimaric acid resulted in suppression of colony formation in a dose-dependent manner. Next, the tested molecule was evaluated for autophagy induction through TEM and the results revealed that levopimaric acid-treated cells caused vacuole and autophagosome formation, which clearly indicate its autophagy induction potential. The validity of autophagy induction by the target molecule was assessed by western blotting analysis through autophagy-associated protein expression which clearly indicated LC3 II and P62 protein expression was decreased and LC3 I protein expression was significantly increased. Afterwards, flow cytometry was used to estimate ROS and MMP in cisplatin-resistant A549 lung cancer cells which showed an increase in ROS and decrease in MMP of these cells. Oncogenic rearrangement ROS1 has developed as well-known therapeutic target in human lung cancer, and about 1-2% of NSCLC patients are recognized with this type of rearrangement [16,17]. Although this frequency may appear low at first glance, 2,000 to 4,500 patients will be newly identified with ROS1-rearranged NSCLC every year [18,19]. Further induction of apoptosis was assessed by DAPI staining, which revealed that levopimaric acid resulted in induction of apoptosis in A549 lung cancer cells. Further support by western blotting analysis showed that the expression of apoptosis-related proteins was altered, like increase in BAX and decrease in BCL-2 protein expression in a dose-dependent manner. Finally, levopimaric acid resulted in modulation of ERK/MAPK/JNK signalling pathway. In addition, levopimaric acid caused no significant increase in P38, ERK and JNK protein expressions but led to decrease in p-P38, p-ERK and p-JNK protein expressions.

Conclusion

In conclusion, we performed different assays to unveil the *in vitro* anticancer activity of levopimaric acid. It was shown that this molecule targets dose-dependently and selectively cisplatin-resistant human lung carcinoma cells without causing too much toxicity to normal lung cells. Furthermore, these anticancer effects were mediated via autophagy, apoptosis, ROS generation, MMP collapse and modulation of ERK/MAPK/JNK signalling pathway.

Conflict of interests

The authors declare no conflict of interests.

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