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ORIGINAL ARTICLE

Inhibition of kidney cancer cell growth by Mulberroside-A is mediated via mitochondrial mediated apoptosis, inhibition of cell migration and invasion and targeting EGFR signalling pathway

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Summary

Purpose: *Kidney cancer is a lethal type of malignancy with high mortality. The chemotherapeutic agents used for the treatment of kidney cancer have several adverse effects, therefore, there is need to explore new molecules for the treatment of this disease. In the current study, the anticancer activity of plant-derived stilbenoid Mulberroside-A (MA) was evaluated against the A498 kidney cancer cell line and the underlying mechanism was explored.*

Methods: *The A498 cell viability was determined by WST-1 assay. DAPI and AO/EB staining were employed for the detection of apoptosis. Matrigel and wound heal assay were used for cell invasion and migration study, respectively. Protein expression was checked by immunoblotting.*

Results: *The results showed that MA could inhibit the growth of the A498 cells dose-dependently. The IC_{50} of 20 μM was observed for MA against the kidney cancer A498 cells. The anticancer effects of MA against these cells were due to apoptotic cell death. Apoptosis was associated with alteration of the Bax/bcl-2 ratio. In addition, MA could also suppress the migration and invasion of the kidney cancer A498 cells by targeting EGFR signalling pathway.*

Conclusion: *In conclusion, MA may prove beneficial in the treatment of kidney cancer.*

Key words: *apoptosis, cell invasion, cell migration, mulberroside-A, kidney cancer*

Introduction

From the very past, plants have been employed in the treatment of human diseases [1]. Modern medicines have improved the overall health, but still around 70% of the world's population is dependent on plants for their primary health care needs [2]. The plants and plant derived formulations have been shown to exhibit minimal toxicity compared to their synthetic counterparts [3]. A large percentage of currently used drugs come from either plants or microbes and these natural sources continue to provide mankind with more and more drugs [4]. *Morus alba* is an important medicinal plant. The seeds and other parts of this plant have been reported to exhibit a wide array of bioactivities, including also anticancer activities. *Morus alba* is a rich source of molecular scaffololds with anticancer activity [5,6]. MA is generally isolated from *Morus alba* and it is generally formed by

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glycosylation of oxyresveratrol. It has been reported to be of immense pharmacological potential and has been reported to exhibit a wide array of bioactivities [7]. However, the anticancer effects of MA are largely unknown. The current study was therefore performed to examine the anticancer effects of MA against the kidney cancer cells. Kidney cancer in adults generally arises from the renal parenchyma or renal pelvis. The pelvis cancers include around 10% of all the diagnosed kidney cancer cases and the rest 90% of kidney carcinomas are adenocarcinomas originating from the renal parenchyma [8]. Kidney cancer is responsible for significant mortality and its incidence is still increasing. Although there are treatments available for this type of cancer, however there are frequent relapses and these treatments have a number of side effects. Herein, we report that MA inhibits the growth of kidney cancer cells. The anticancer effects of MA are attributed to its ability to induce apoptotic cell death as indicated by the DAPI and AO/EB staining. Besides, MA could also suppress the migration and invasion of kidney cancer cells by targeting EGFR.

Methods

Cell lines and culture conditions

Kidney cancer A498 cell line was obtained from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China, and was cultured in RPMI 1640 complete medium containing 10%u fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), at 37°C in a humidified incubator.

Assessment of cell viability

The cell viability of the kidney cancer cells was assessed by WST-1 colorimetric assay. Briefly, the A498 kidney cancer cells were seeded in 96-well plates at the density of 2×10^5 cells/well and treated with different concentrations of MA. The cells were then incubated with WST-1 at 37°C for 4 hrs. The absorbance at 450 nm was then taken by a microplate reader to determine the viability of kidney cancer cells.

Detection of apoptosis

The nuclear morphology of the A498 cancer cells was assessed by fluorescence microscopy after subjecting the cells to DAPI staining. Ten fields with 100 cells/field were randomly selected for estimation of the cells with condensed nuclei. AO/EB double staining was used for the determination of apoptotic kidney cancer cells as described previously [9].

Cell migration assay

The migration of A498 cells was determined with wound healing assay. The cells were grown till 70% confluence and a scratch was made with a scratching device. The cells were then incubated for 48 hrs again, and the wound healing capacity was evaluated by comparing the widths of the wounds.

Matrigel invasion assay

Invasion was evaluated with the help of Matrigel®-coated invasion chambers. The MA-treated and untreated A498 cells that reached the lower surface of the membrane were subjected to staining with crystal violet (CV), and images of CV-stained cells were taken. The CV complexes formed were dissolved in 10% acetic acid and the cell invasion was determined by measuring the absorbances of the resultant solutions at 600 nm in a spectrophotometer.

Western blotting analysis

The A498 kidney cancer cells were collected and treated with lysis buffer. The extracts were boiled for 10 min in the presence of loading buffer, followed by separation of cell extracts using 15% SDS-PAGE gel. The samples were then put onto polyvinylidene fluoride membranes and subjected to blocking with skim milk (5%). Membrane incubation with primary antibodies was performed overnight at 4°C and subsequently incubated with horseradish peroxidase-linked biotinylated secondary antibodies at 1:1,000 dilution for 2 hrs. The membranes were then subjected to washing with phosphate buffered saline (PBS) and the immune-reactive bands were visualized using ECL-PLUS/Kit. The development of the immune complexes was carried out using an ECL detection kit according to the manual protocol. The bands were analyzed with Ge1GDoc 2000 imaging system.

Statistics

Each test was carried out thrice and the values are presented as mean ± SD. Student's *t*-test was employed for statistical analysis using GraphPad prim 6 software. $P<0.05$ was considered statistically significant.

Results

MA inhibits the proliferation of A498 cells

The effect of MA (Figure 1) on the viability of the kidney cancer A498 cells was investigated by WST-1 assay. It was found that MA could significantly inhibit the growth of the kidney cancer A498 cells. The IC_{50} of MA against the A498 cells was 20 µM. In addition, the anticancer effects of MA on the kidney cancer A498 cells showed a dose-dependent pattern (Figure 2).

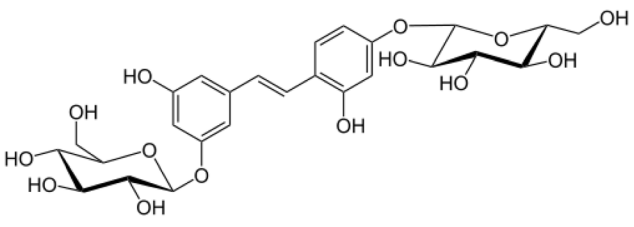

Figure 1. Chemical structure of Mulberroside-A.

MA initiates apoptotic cell death of A498 cells

Since the WST-1 assay showed that MA inhibits the viability of the kidney cancer A498 cells we sought to know about the underlying mechanism for the anticancer effects of MA on these cells so we subjected the A498 cells to DAPI and AO/EB staining. The results of DAPI staining revealed that MA caused significant alterations in the nuclear morphology which were characteristic of apoptotic cell death (Figure 3). Similarly, AO/EB staining also revealed that MA caused considerable increase in the red colour cells indicating apoptosis (Figure 4). Bax and Bcl-2 are important proteins the expression of which to a greater extent determines if the cell undergoes apoptosis or not. Herein, we observed that MA caused decline in the expression of Bcl-2 and increase in the expression of Bax, ultimately favouring apoptosis (Figure 5).

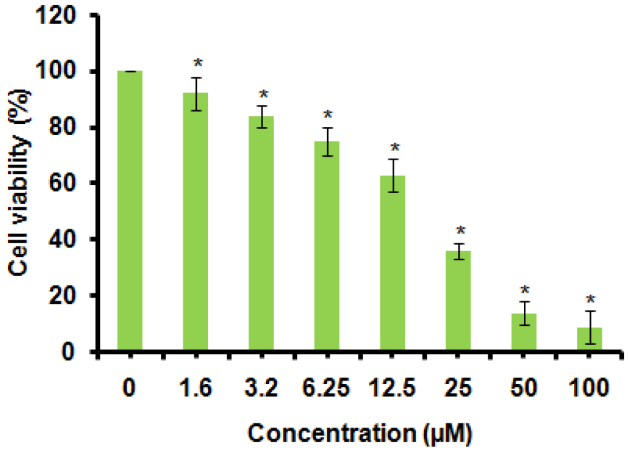

Figure 2. Effect of Mulberroside-A on the proliferation of the kidney cancer A498 cells as determined by WST-1 assay. The experiments were repeated thrice and values as shown as mean ± SD (*p <0.05).

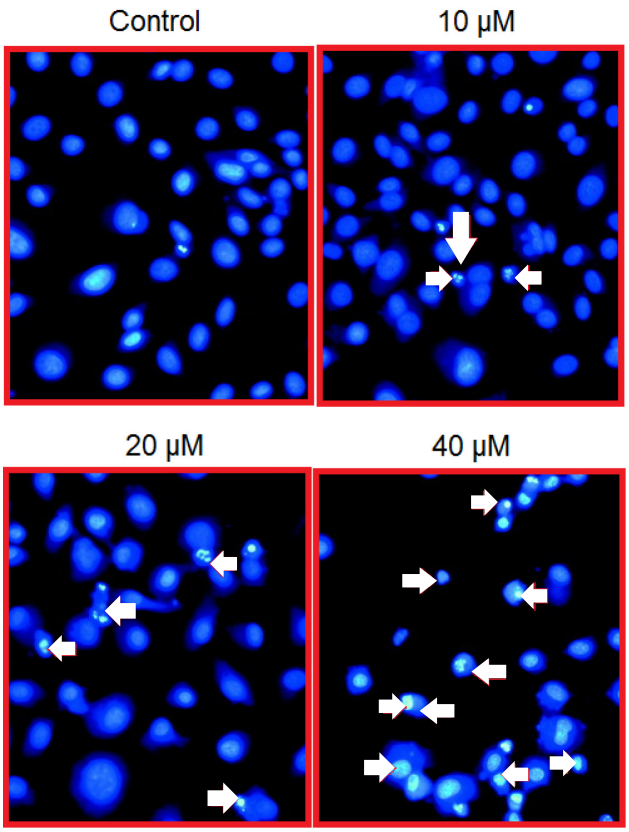

Figure 3. Mulberroside-A induces dose-dependent apoptotic cell death as indicated by DAPI staining. The experiments were repeated thrice. Arrows indicate apoptotic cells.

MA inhibits the migration of A498 cells

The effect of MA on the migration of the A498 cells was assessed by the wound healing assay. The

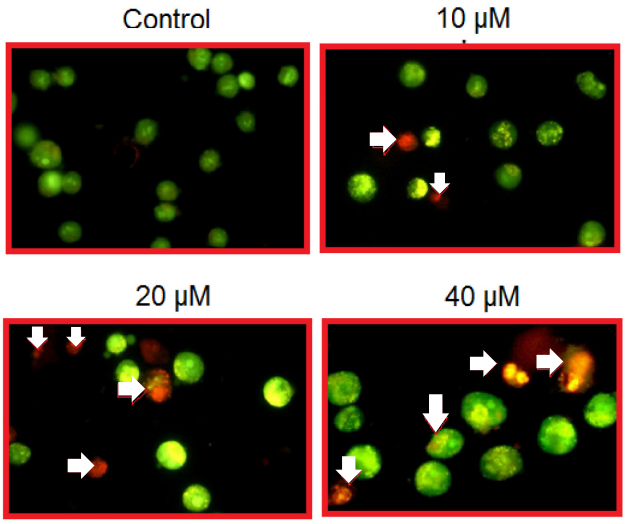

Figure 4. Mulberroside-A induces dose-dependent apoptotic cell death as indicated by AO/EB staining. The experiments were repeated thrice. Arrows indicate apoptotic cells.

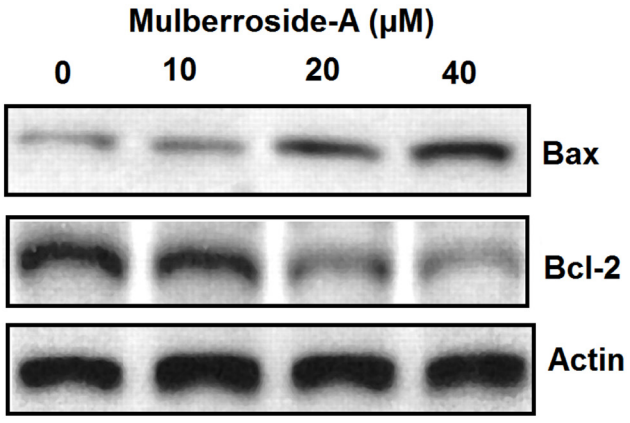

Figure 5. Effect of Mulberroside-A on the expression of Bax and Bcl-2 as depicted by western blotting. The experiments were repeated thrice. The Figure depicts that Mulberroside-A enhances the expression of Bax and decreases the expression of Bcl-2 dose-dependently.

outcomes of this experiment showed that the cells were able to migrate quickly in case of untreated A498 cells. However, the treatment of the A498 cells with MA inhibited their capacity to migrate. These results indicate that MA inhibits the migration of the A498 cells (Figure 6).

MA inhibits the invasion of A498 cells

The effects of MA on the invasion of the kidney cancer A498 cells was examined by Matrigel invasion assay. It was found that MA could significantly inhibit the invasion of A498 cells (Figure 7). The migration of the A498 cells was decreased by 40% relative to the untreated A498 cells.

MA targets EGFR signalling in A498 cells

The effects of MA were also examined on the epidermal growth factor receptor (EGFR) by western blotting. The results showed that MA caused significant decrease in the expression of p-EGFR in a dose-dependent manner. However, no apparent effects were observed on the total EGFR expression (Figure 8).

Discussion

Kidney cancer is one of the lethal malignancies and its treatments are limited, while exhibiting a number of side effects [10]. Hence there is urgent need to either screen new molecules for anticancer activity or to explore novel therapeutic targets. In this study the anticancer effects of a plant derived stilbenoid, Mulberroside-A (MA), was investigated against the kidney cancer A498 cells and an attempt was made to explore the underlying mechanism [11]. It was found that MA reduced the viability of kidney cancer A498 cells dose-dependently. Previous studies had revealed that *Morus alba* inhibits the growth of cancer cells [12]. Since MA is an important constituent of *Morus alba*, we strongly believe the anticancer activity of *Morus alba* extracts might have been at least in part due to the presence of MA. Moreover, MA is a diglucoside of oxyresveratrol which has known anticancer activity [13]. These investigations complement the results of the present study. Different molecules that inhibit the growth of cancer cells, exert their anticancer effects via a number of mech-

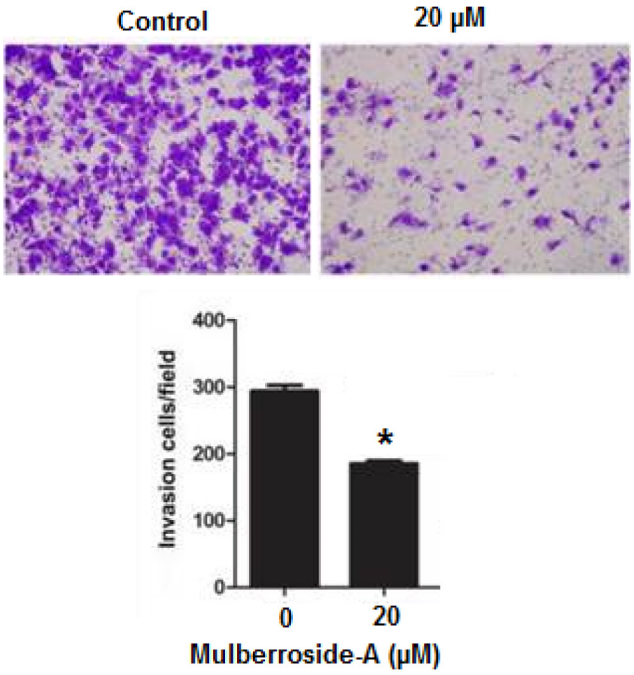

Figure 7. Effect of Mulberroside-A on the invasion of kidney cancer A498 cells. The experiments were repeated thrice and values as shown as mean ± SD (*p<0.05). The Figure reveals that Mulberroside-A inhibits the invasion of kidney cancer cells.

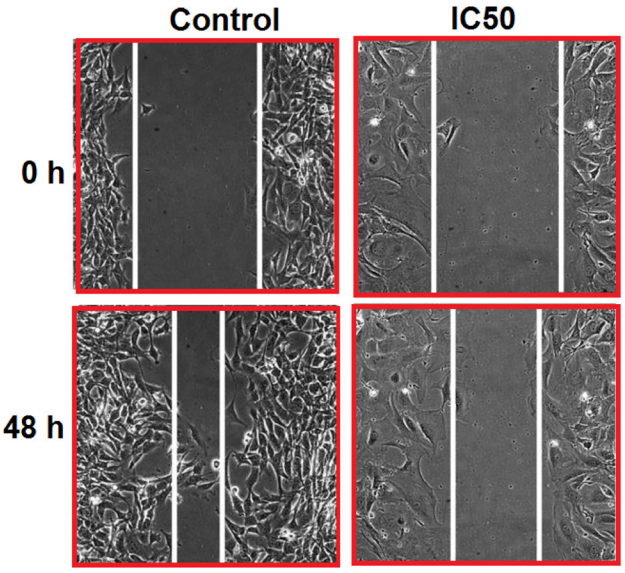

Figure 6. Effect of Mulberroside-A on the migration of kidney cancer A498 cells. The experiments were repeated thrice. The Figure shows that Mulberroside-A inhibits the migration of kidney cancer cells.

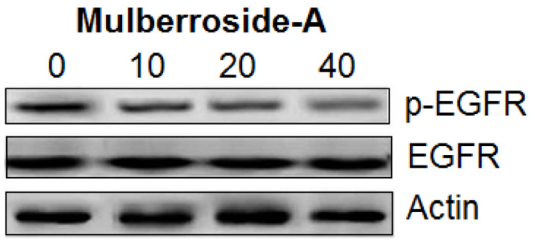

Figure 8. Effect of Mulberroside-A on the expression of EGFR as depicted by western blotting. The experiments were repeated thrice. The Figure shows that Mulberroside-A inhibits the phosphorylation of EGFR kidney cancer cells.

anisms [14]. Therefore, the next goal of the study was to assess the underlying mechanism. Apoptosis is an essential mechanism by which the harmful and lethal cells are eliminated from the body. Many of the available anticancer drugs eliminate the cancer cells by inducing apoptotic cell death [15]. In this study the DAPI and AO/EB staining revealed that MA prompts apoptosis in the A498 kidney cancer cells dose-dependently. This was also associated with alteration of the Bax and Bcl-2 expression. Cancer metastasis is an important factor that determines its prognosis. The cancers cells are capable to enter the blood stream and invade different parts of the body, creating a situation quite difficult to treat as multiple organs get involved [16]. In this study we observed that MA could significantly inhibit the proliferation and migration of cancer cells, indicating that MA could potentially inhibit the metastasis of kidney cancer *in vivo*. The epidermal growth factor receptor (EGFR) is highly expressed in cancer cells and it has been reported that inhibition of EGFR inhibits proliferation and triggers apoptosis in cancer cells [17]. In this study we observed that MA could inhibit the expression of p-EGFR, indicating the anticancer potential of this molecule.

Conclusion

Taken together, the results of the current study indicate that MA inhibits the proliferation of kidney cancer cells by triggering apoptosis. Besides it also inhibits the migration and invasion of the kidney cancer cells by targeting EGFR. Therefore, MA could prove to be an important lead molecule for the management of kidney cancer.

Conflict of interests

The authors declare no conflict of interests.

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