Anticancer Activity of Phloretin Against Human Gastric Cancer Cell Lines Involves Apoptosis, Cell Cycle Arrest, and Inhibition of Cell Invasion and JNK Signalling Pathway

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Background: Gastric cancer is one of the most commonly diagnosed cancers and causes significant mortality worldwide. In this study, the antiproliferative and anticancer effects of Phloretin were evaluated against gastric cancer cell lines.

Material/Methods: MTT assay was used to assess the proliferation rate of gastric cancer cells. DAPI and annexin V/PI were used for detection of apoptotic cell death. Cell invasion was investigated by Transwell assays and the expression of the proteins was estimated by immunoblotting.

Results: The results revealed that Phloretin exerts anticancer effects on all the gastric cancer cell lines used in this study. However, the anticancer effects were more significant (p<0.05) on the AGS cell line. Further, the effect of Phloretin on the viability of normal GES-1 cells was minimal. Apoptosis assays showed that Phloretin triggers apoptotic cell death in AGS gastric cancer cells. Phloretin could also cause the arrest of the AGS gastric cancer cells in the G2/M phase of the cell cycle and suppress their ability to migrate. Western blotting analysis revealed that Phloretin significantly decreased the expression of p-JNK and p-38. However, comparatively lower effects were observed on the expression of JNK and P38.

Conclusions: We showed that Phloretin is an important molecule for the treatment of gastric cancer.

MeSH Keywords: Apoptosis • Cell Cycle Checkpoints • Cell Migration Assays

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Background

Gastric cancer (GC) is one of the most common types of cancers and more than 50% of all cancers detected in East Asian countries are gastric cancers. Despite a decline in the frequency of GC, it is still reported to be one of the most commonly diagnosed cancers worldwide [1,2]. The treatment for GC generally involves chemotherapy, but the clinical outcomes are still very poor and the adverse effects of the anticancer agents used for treatment of GC affect the overall health of GC patients [3]. Therefore, research directed at identifying the new drug candidates for GC are urgently needed. The present study was undertaken to evaluate the anticancer effects of Phloretin on human gastric cancer cell lines. Phloretin is a plant-derived natural product found to show a number of bioactivities such as anticancer, antimicrobial, and antioxidant activities [4]. For instance, Phloretin has been reported to trigger apoptotic cell death of oesophageal cancer cells [5]. Furthermore, it has been found that Phloretin can enhance the anticancer potential of cisplatin on lung cancer cells [6]. Similarly, Phloretin has been shown to induce apoptotic cell death in human leukemia cells [7]. Phloretin, being a natural flavonoid (dihydrochalcone), has little cytotoxicity on normal cells [8]. In this study, we observed that Phloretin reduced the cell viability of gastric cancer cells. Although Phloretin exerted its antiproliferative effects on all the cell lines, the lowest IC_{50} of 8 µM was observed against the AGS gastric cancer cell line. Interestingly, Phloretin showed comparatively lower cytotoxicity on the normal gastric GES-1 cells, with an IC_{50} of 120 µM. Given these results, the underlying mechanism for the anticancer effects of clearly shown by DAPI staining. Furthermore, the percentage of apoptotic AGS cells increased with increased dosage of Phloretin. Examination of the effect of Phloretin on invasion of the AGS gastric cancer cells revealed that Phloretin inhibits the migration of AGS cells. Additionally, the effect of Phloretin was also assessed on the protein expression of JNK signalling and it was observed that Phloretin modulates the JNK signalling pathway in gastric cancer cells.

Cell viability and colony formation assay

The viability of gastric cancer cells was determined by MTT assay. In brief, the cultured gastric cancer cells were seeded at the density of 1.5×10^4 in 96-well microtiter plates. This was followed by the addition of 20 µl MTT solution (2.5 mg/ml) in all the wells, then the absorbance at 570 nm was assessed using an ELISA plate reader. To assess the impact of Phloretin on the colony formation potential of Phloretin, the AGS cells were collected at exponential phase of growth and the cells were then counted using a hemocytometer. The plating of the cells was carried out at 200 cells/well. The plates were then kept at 37°C for 48 h to permit the cells to adhere. This was followed by the addition of various concentrations (0, 4, 8, and 16 µM) of Phloretin. Following treatment with Phloretin, the cells plates were again incubated for 6 days. After incubating the cells for about 6 days, they were subjected to washing with PBS and fixation with methanol. The AGS cells were then stained with crystal violet followed by microscopy.

Detection of apoptosis

The gastric cancer AGS cells were seeded in 6-well plates (2×10^5 cells per well). The cells were then DAPI-stained for 20 min at room temperature to detect the apoptosis by fluorescence microscopy, as previously reported [9]. To determine the percentage of apoptotic cells, an FITC-Annexin V/PI Apoptosis detection kit was employed as per the instructions of the manufacturer (Beijing Biosea Biotechnology, China).

Cell cycle analysis

To investigate the distribution of the AGS gastric cancer cells in different phases of the cell cycle, approximately 1×10^6 cells in each well in 6-well plates were kept at 37°C overnight to allow the cells to adhere. This was followed by treatment with various doses of Phloretin (0, 10, 20, and 40 µM), followed by incubation at 37°C. Finally, the distribution of the AGS cells in various cell cycle phases was determined by flow cytometry.

Cell invasion assay

The cell invasion ability of gastric cancer AGS cells was evaluated by Transwell assay. Briefly, the cells were seeded at 2×10^5 cells/ml density and incubated for 24 h at 37°C. Thereafter, 200-ml cell suspensions were added into the upper chamber and the complete medium was added into the bottom wells. After 24-h culturing, the cells in the upper chambers were removed and cells that migrated through the chambers were subjected to fixation with methyl alcohol followed by staining with crystal violet. Finally, the number of cells that migrated was determined by counting the cells under an inverted microscope (Magnification 200×, 10 different fields).
Western blotting

The gastric cancer AGS cells were harvested and lysed with lysis buffer. The protein extracts were incubated at 99°C for 15 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 blocked using 5% skimmed milk powder. Membrane incubation with primary antibodies (p-JNK, JNK, p-P38, P38) was performed for 24 h at 4°C. The membranes were incubated with horseradish peroxidase-linked secondary biotinylated secondary antibodies at 1:1000 dilution for 2 h. Washing of the membranes with PBS was followed by visualization of the immunoreactive bands using the ECL-PLUS kit according to the guidelines of the manufacturer. The immune complexes development was carried out using an ECL detection kit according to the manual. The bands were analyzed using the GelGDoc2000 imaging system.

Statistical analysis

The experiments were repeated 3 times and are presented as mean ±SD. The t test (for comparisons between 2 samples) and one-way ANOVA followed by Tukey’s test (For comparisons between more than 2 samples) with GraphPad 6 were used for statistical analysis (P<0.05).

Results

Anticancer effects of Phloretin on AGS cells

To confirm the growth inhibitory effects of Phloretin (Figure 1A) on the gastric cancer cell lines, the gastric cancer cells were subjected to treatment with Phloretin at varied doses. We observed that Phloretin displayed significant anticancer effects against all the gastric cancer cell lines. However, the highest antiproliferative effects were observed against the AGS cell line, with an observed IC50 of 8 µM (Table 1). Nonetheless, Phloretin showed lower growth inhibitory effects on normal human GES-1 cells (IC50 of 80 µM). The effect of Phloretin on the growth of AGS cells exhibited a concentration-dependent pattern (Figure 1B). Further, evaluation of the effect of Phloretin on the colony-formation potential revealed that Phloretin suppressed the colony-forming potential of the AGS gastric cancer cells in a dose-dependent manner (Figure 1C).
Phloretin triggers apoptosis in AGS cells

To determine the cause of the anticancer effects, Phloretin treated AGS cells were subjected to DAPI staining. It was observed that Phloretin caused apoptosis in AGS cells in a dose-dependent manner (Figure 2). The analysis of the apoptotic cell population was performed by flow cytometry as depicted in Figure 3. The apoptotic AGS cells increased from 1.25% in control to 46.3% at 40 µM doses of Phloretin. To assess whether the apoptosis was due to activation of the mitochondrial apoptotic pathway, the protein expression of Bax and Bcl-2 was examined. The results revealed that the protein expression of Bax was increased in a dose-dependent pattern and the expression of Bcl-2 decreased (Figure 4).

Phloretin triggers cell cycle arrest

To examine the impact of Phloretin on the distribution of AGS in different phases of the cell cycle, cells were treated with 0, 4, 8, and 16 µM of Phloretin for 24 h. We found that the percentage of cells at G2 phase increased in a dose-dependent manner causing cell cycle arrest (Figure 5). At 4 µM, there was slight increase in the G2-phase cells and at 16 µM there was a marked increase in G2-phase cells.

Phloretin inhibits cell invasion of AGS gastric cancer cells.

The impact of Phloretin on cell invasion of gastric cancer AGS cells was also investigated (Figure 6). The results of cell invasion assay at 20 µM Phloretin for 24 h indicated that Phloretin reduced the invasiveness of the AGS cells.

Phloretin inhibits the JNK/P38 signalling pathway

The effect of Phloretin was also evaluated on the JNK/P38 signalling pathway. The results showed that Phloretin significantly decreased the expression of p-JNK and p-P38. However, comparatively smaller effects were observed on the expression of JNK and P38 (Figure 7).

Discussion

Plants are natural chemical factories which have the capacity to produce a wide array of secondary metabolites. These metabolites have diverse structures and play vital functions in plants [10]. Since plants are always exposed to extreme environmental conditions, they have evolved to produce different types of secondary metabolites [11]. These metabolites have been shown to be beneficial in the treatment of human diseases [12]. Phloretin, a dihydrochalcone, has been reported to possess a number of bioactivities [4]. In this study, we evaluated the anticancer activity of Phloretin on human gastric cancer cell lines. Phloretin was found to exhibit antiproliferative effects on all the gastric cancer cell lines used. However,
more profound anticancer effects were observed on the AGS gastric cancer cell line with IC_{50} of 8 µM. Unlike gastric cancer cell lines, the anticancer effects of Phloretin on the normal GES-1 cells were very small. Previous studies have also reported the anticancer effects of Phloretin on several types of cancers, including but are not limited to, lung, colorectal, and breast cancers [6,13,14]. Moreover, it has been reported that flavonoids such as Phloretin are safe for human consumption [15]. They are consumed in significant amounts by humans in their normal diet [16]. Flavonoids have been reported to induce apoptosis in cancer cells [17]. Apoptosis is an imperative mechanism by which harmful and unwanted cells are eliminated from the body [18]. In this study, we observed that Phloretin exerts its anticancer effects on the AGS cancer cells by induction of apoptosis. The results of DAPI and annexin V/PI staining confirmed that Phloretin exerts its anticancer effects by triggering apoptosis in cancer cells. These observations are also supported by previous studies wherein Phloretin was observed to trigger apoptotic cell death in cancer cells. For example, Phloretin induces apoptosis in breast cancer cells by via the JNK signalling pathway [19]. The Bax/Bcl-2 ratio is also an important determinant of apoptosis. Higher expression of Bax causes the discharge of Cyt c, ultimately favoring apoptosis [20]. In this study, we also observed that Phloretin causes upregulation of Bax, which is also associated with decreased level of the anti-apoptotic protein Bcl-2. Apart from apoptosis, the arrest of cancer cells in different phases of the cell cycle is an important mechanism by which proliferation of cancer cells is prevented by chemotherapeutic agents [20]. In this study, we observed that Phloretin induces G2/M cell cycle arrest in
Figure 4. Effect of Phloretin on the expression of Bax and Bcl-2, as depicted by immunoblotting analysis. The experiments were repeated 3 times and data are expressed as mean ±SD (p<0.05).

Figure 5. Phloretin triggered G2/M cell cycle arrest in AGS cancer cells as determined by flow cytometry. Experiments were repeated 3 times.
Phloretin suppressed the invasion of AGS gastric cancer cells, as determined by Transwell assays. The experiments were repeated 3 times and data are expressed as mean ±SD (p<0.05).

Figure 6. Phloretin suppressed the invasion of AGS gastric cancer cells, as determined by Transwell assays. The experiments were repeated 3 times and data are expressed as mean ±SD (p<0.05).

gastric AGS cancer cells and the percentage of the AGS cells in G2 phase of the cell cycle increased with increased concentration of Phloretin. These results are also complimented by previous studies wherein Phloretin was reported to trigger cell cycle arrest in glioblastoma cells [21]. Cell invasion capacity is an important factor in metastasis of cancer cells [22] and in this study we observed that Phloretin could inhibit the invasion of the human gastric cancer cell lines. This suggests that Phloretin may be an important anti-metastatic agent in vivo. Since previous studies have shown that Phloretin exerts anti-cancer activity via the JNK signalling pathway [18], we examined the effects of Phloretin on the expression of some important proteins of this pathway. Phloretin significantly decreased the expression of p-JNK and p-38. However, comparatively smaller effects were observed on the expression of JNK and P38. Previous investigations have revealed that the JNK pathway is activated in several types of cancers and it is believed that this pathway can be utilized as a target for anticancer agents.

Figure 7. The effect of Phloretin on the JNK/P38 pathway, as depicted by Western blotting. The experiments were repeated 3 times.

Our results show that Phloretin inhibits the proliferation of human gastric cancer cells, with minimal effects on normal cells. Further, the anticancer effects of Phloretin were found to be due the induction of apoptosis and cell cycle arrest. The results of this study suggest that Phloretin may be useful in the treatment of gastric cancer. However, further studies, such as in vivo evaluation, of Phloretin are urgently required.

Conclusions

Our results show that Phloretin inhibits the proliferation of human gastric cancer cells, with minimal effects on normal cells. Further, the anticancer effects of Phloretin were found to be due the induction of apoptosis and cell cycle arrest. The results of this study suggest that Phloretin may be useful in the treatment of gastric cancer. However, further studies, such as in vivo evaluation, of Phloretin are urgently required.

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