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ORIGINAL ARTICLE

Inhibition of cancer cell growth in gemcitabine-resistant pancreatic carcinoma by mangiferin involves induction of autophagy, endogenous ROS production, cell cycle disruption, mitochondrial mediated apoptosis and suppression of cancer cell migration and invasion

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Summary

Purpose: *Pancreatic cancer causes considerable mortality across the globe and its treatment options are limited. Besides, the development of drug resistance among the pancreatic cancer cells makes it even more difficult to manage. In this study the anticancer effects of mangiferin were examined against the Mia-PaCa2 gemcitabine-resistant pancreatic cancer cells.*

Methods: *Cell proliferation of pancreatic cancer cell line Mia-PaCa2 and normal cell line HTERT-PINE was examined by MTT assay, while apoptosis was detected by fluorescent microscopy and western blot. The effects on cell cycle, reactive oxygen species (ROS) generation and mitochondrial membrane potential (MMP) were evaluated by flow cytometry. The fact that mangiferin induced autophagy was demonstrated by fluorescent microscopy in combination with western blot.*

Results: *Mangiferin inhibited the growth of the Mia-PaCa2 cells and exhibited an IC_{50} of 10 μM. Of note, the toxic effects of mangiferin were less on the normal cells. Mangiferin induced apoptosis in the Mia-PaCa2 cells which was associated with enhancement of Bax/Bcl-2 ratio. Further investigations revealed that mangiferin triggered autophagy in the Mia-PaCa2 cells as evidenced from the elevated expressions of the LC3 II and Beclin-1. The antiproliferative effects of mangiferin were also accompanied by generation of endogenous ROS and cell cycle arrest. Investigation of the effects of mangiferin on the invasion and migration of the Mia-PaCa2 cells showed this molecule suppressed the migration and invasion potential of the Mia-PaCa2 cells.*

Conclusions: *Mangiferin could be utilised for the development of systemic therapy for pancreatic cancer provided further in depth experiments are carried out along with its toxicological studies.*

Key words: *pancreatic cancer, mangiferin, apoptosis, autophagy, cell migration*

Introduction

Naturally occurring antioxidants have shown significant potential to combat cancer by scavenging reactive oxygen species (ROS) [1]. Mangiferin, a pharmacologically important C-glucosylated xanthone, is generally obtained from different parts of *Mangifera indica* [2]. Mangiferin reduces DNA damage and thereby halts the development and progression of cancer [3]. A number of studies have indicated the anticancer properties of mangiferin. It has been shown to prompt apoptotic cell death of myeloma cells [4]. It has also been shown to inhibit the proliferation of breast cancer cells

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via modulating the expression of matrix metalloproteinases (MMP) [5]. Mangiferin has also been shown to increase the oxaliplatin-induced apoptosis in several cancer cell types [6]. In yet another study mangiferin was found to cause cell cycle arrest of lung cancer cells [7]. However, the anticancer effects of this molecule have not been examined against human pancreatic cancer cells lines. Ranked as the 7th major cause of cancer-related deaths in China, pancreatic cancer takes 2.5 million lives annually across the world [8]. Owing to poorest prognosis, late diagnosis, emergence of drug resistance and the adverse effects of chemotherapeutic drugs, pancreatic cancer is often difficult to manage [9]. In this study the anticancer effects of mangiferin were examined against the gemcitabine-resistant human Mia-PaCa 2 pancreatic cancer cells. Its effects on cell autophagy, endogenous ROS production, cell cycle, apoptosis and cell migration and invasion were also investigated.

Methods

Cell viability assay

The viability of the cancer cells was measured by MTT assay. In brief, as the confluence of the Mia-PaCa2 and HTRET-HPNE cells reached around 70%, they were seeded in 96-well plates with DMEM medium and treated with 0-100 µM of mangiferin. After a 24-h incubation, the cells were incubated with MTT for another 4 h. After this, the medium was removed and the colored formazan product was solubilized by 200 µl of dimethyl sulfoxide. The viability of the Mia-PaCa2 and the HTRET-HPNE normal cells was then determined by taking absorbance at 570 nm using spectrophotometer.

Acridine orange and ethidium bromide staining

The Mia-PaCa2 cells were grown in 6well plates (0.6×10^6 cells/well) and incubated for 12 h. The Mia-PaCa2 cells were subjected to mangiferin treatment for 24 h at 37°C. As the cells sloughed off, 10 µl cell culture were put onto glass slides and stained with a solution of acridine orange (AO) and ethidium bromide (EB). The slides were then cover slipped and examined with a fluorescent microscope.

Determination of ROS and MMP

The ROS and MMP levels were estimated by culturing of the Mia-PaCa2 cells for 24 at 37°C and subsequently treated with varied doses of mangiferin for 24 h. Next, the medium was discarded and the cells were treated 5 µM DCH-DA for estimation of ROS or rhodamine 123 (Rh123) for estimation of MMP by flow cytometry.

GFP-LC3 transfection for the detection of autophagy

For the detection of autophagy, the Mia-PaCa2 cells were grown to 70% confluence and transfected with GFP-LC3 plasmids using Lipofectamine 2000 (Invitrogen, California, USA) as per the manufacturer's instructions. The transfected cells were then treated with varied concentrations of mangiferin (0, 5, 10 and 20 µM) for 24 h and subsequently monitored by fluorescent microscopy.

Cell cycle, migration and invasion analysis

Following the incubation of the Mia-PaCa2 pancreatic cancer cells with varied concentrations of mangiferin (0, 5, 10 and 20 µM) for 24 h, the cells were washed with phosphate buffered saline (PBS). Afterwards, the Mia-PaCa2 cells were stained with propidium iodide (PI) and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer. The cell migration and invasion assays were performed as described previously [10].

Western blotting

The Mia-PaCa2 cells were harvested and washed with ice-cold PBS. The pellet was then suspended in a lysis buffer at 4°C and then shifted to 95°C. Thereafter, the protein content of each cell extract was checked by Bradford assay. About 40 µg of protein was loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then treated with tris-buffered saline (TBS)

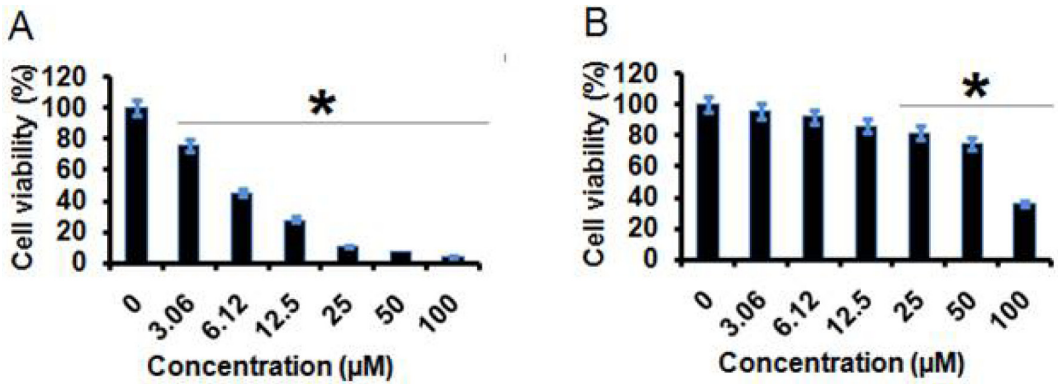

Figure 1. MTT assay showing the effects of mangiferin on the viability of **A:** Mia-PaCa2 and **B:** normal HTRET-HPNE cells. The values represent the mean of three experiments ± SD (*p<0.05).

and exposed to primary antibody at 4°C. Thereafter, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent.

Statistics

The experiments were performed in triplicate and data are shown as mean±SD. Statistical analysis was done using Student's *t*-test and one-way ANOVA. P value <0.05 was considered as statistically significant difference.

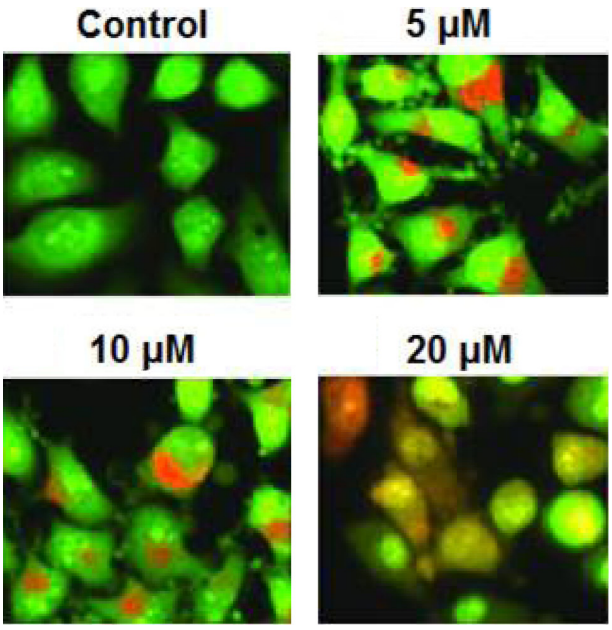

Figure 2. AO/EB staining showing nuclear morphology of the mangiferin-treated Mia-PaCa2 cells. The Figure depicts that with increasing dose the cell morphology shows characteristics of apoptosis. Red fluorescence indicates apoptotic cells, while green fluorescence indicates normal cells. Note that apoptotic cells increased significantly with increasing drug dose. The experiments were performed in triplicate.

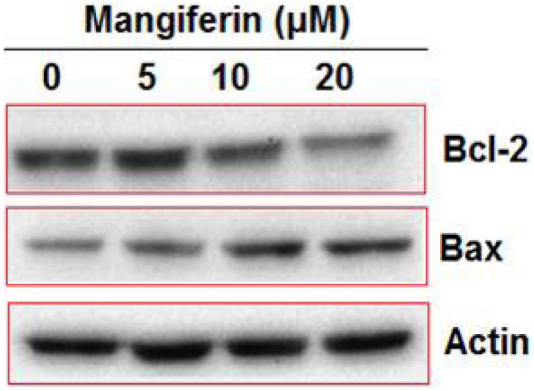

Figure 3. Effect of mangiferin on the expression of Bax and Bcl-2 in Mia-PaCa2 cells as depicted by western blot analysis. The Figure shows that mangiferin treatment led to decrease of Bcl-2 and increase of Bax dose-dependently. The experiments were performed in triplicate.

Results

Mangiferin inhibits the proliferation of Mia-PaCa2 gemcitabine-resistant pancreatic cancer cells

The effects of mangiferin on the viability of Mia-PaCa2 gemcitabine-resistant pancreatic cancer cells and the HTRET-HPNE normal cells were assessed by MTT assay at concentrations ranging from 0 to 320 μM. It was observed that mangiferin suppressed the growth of the Mia-PaCa2 cells in a dose-dependent manner (Figure 1A). The IC_{50} of mangiferin against the Mia-PaCa2 cells was 10 μM. Nonetheless, the effects of mangiferin on the

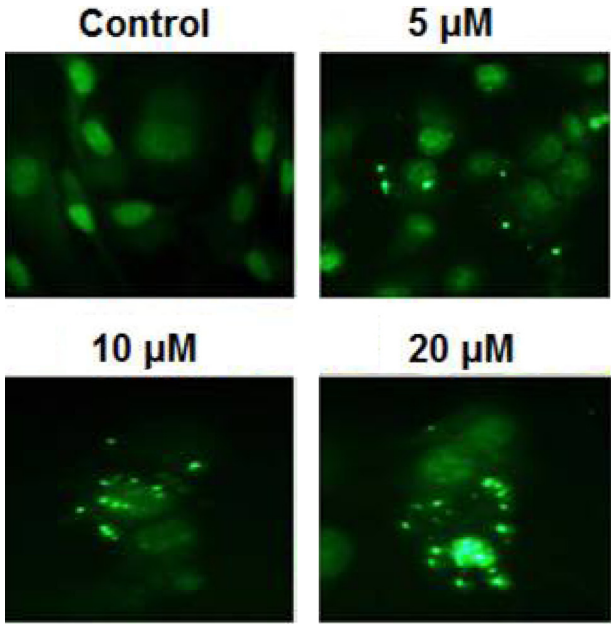

Figure 4. Effect of mangiferin on the expression of LC3 expression as depicted by fluorescence microscopy of the mangiferin-treated Mia-PaCa2 cells. The Figure indicates that mangiferin treatment led to induction of autophagy in a dose-dependent manner. The experiments were performed in triplicate.

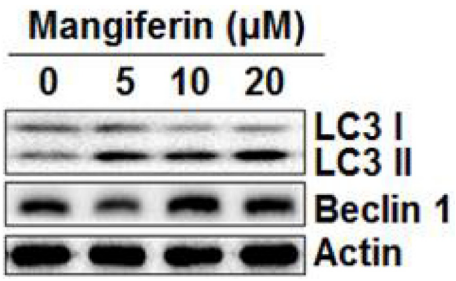

Figure 5. Effect of mangiferin on the expression of autophagy proteins in Mia-PaCa2 cells as depicted by western blot analysis. The Figure indicates that mangiferin treatment led to induction of apoptosis in a dose-dependent manner as revealed by the increase in expression of LC3-I, LC3-II and Beclin-1. The experiments were performed in triplicate.

viability of the HTRET-HPNE cells were less pronounced. The IC_{50} of mangiferin against the normal HTRET-HPNE cells was 75 μM (Figure 1B).

Mangiferin induces autophagy in Mia-PaCa2 cells

The Mia-PACa2 cells were transfected with GFP-LC3 vectors and treated with different concentrations of mangiferin and it was found that mangiferin. This showed increase in the expression of the LC3 expression as indicated by fluorescence microscopy, suggesting that mangiferin induced autophagy in the Mia-PaCa2 cells (Figure 2). In addition, mangiferin also enhanced the expression of LC3 II and Beclin-1, confirming the mangiferin-induced autophagy (Figure 3).

Mangiferin induces apoptotic cell death of Mia-PaCa2 cells

To decipher the mechanism behind the antiproliferative effects of mangiferin-treated Mia-PACa2 cells were stained with AO/EB and DAPI. The AO/EB staining showed that the orange colored cells increased in number as the concentration of mangiferin was increased (Figure 4). The effects of mangiferin were examined on the Bax and Bcl-2 expression of Mia-PaCa2 cells which are considered as important biomarker proteins of apoptosis. The results of the western blot analysis showed that Bax expression increased dose-dependently and that of Bcl-2 decreased, indicative of apoptosis (Figure 5).

Mangiferin causes increase of ROS in Mia-PACa2 cells

The effects of mangiferin were examined by fluorescence microscopy on the Mia-PaCa2 cells at different concentrations (0, 5, 10, and 20 μM) and the results showed that this molecule caused significant increase in the ROS levels of the Mia-PaCa2 cells (Figure 6) and that these effects were found to be concentration-dependent.

Mangiferin decreases the MMP levels of Mia-PaCa2 cells

Since mangiferin caused production of ROS, its effects were examined on MMP levels by fluorescence microscopy on the Mia-PACa2 cells at different concentrations (0, 5, 10, and 20 μM). The results showed that mangiferin causes substantial decrease in the MMP levels of the Mia-PaCa2 cells and, like with that of ROS, these effects were found to be concentration-dependent (Figure 7).

Mangiferin triggers the G2/M phase arrest of the Mia-PaCa2 cells

The effects of mangiferin were also examined on the distribution of the Mia-PaCa2 cells at 0, 5, 10 and 20 μM concentrations by flow cytometry and the results revealed that that mangiferin triggered G2/M arrest of the cell cycle. The percentage of the G2/M phase pancreatic cancer cells was around

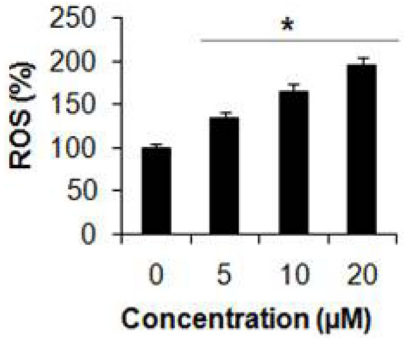

Figure 6. Effect of mangiferin on ROS levels of mangiferin-treated Mia-PaCa2 cells as measured by flow cytometry. The Figure shows that mangiferin treatment led to increase of ROS production. The values represent the mean of three experiments±SD (*p<0.05).

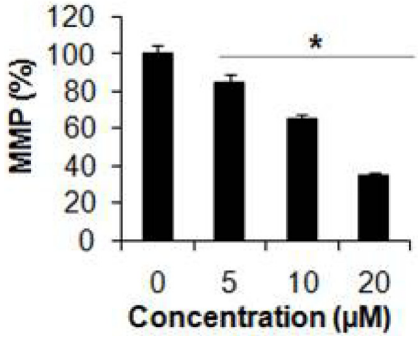

Figure 7. Effect of mangiferin on MMP levels of mangiferin-treated Mia-PaCa2 cells as measured by flow cytometry. The Figure shows that mangiferin treatment led to decrease of MMP. The values represent the mean±SD of three experiments (*p<0.05).

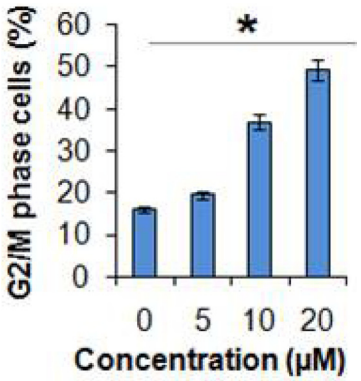

Figure 8. Cell cycle phase distribution of Mia-PaCa2 cells upon treatment with varied concentrations of mangiferin as assessed by flow cytometry. The Figure shows that mangiferin treatment led to increase of G2/M phase cells. The values represent the mean±SD of three experiments (*p<0.05).

40% at 20 μM while it was 16% in the control cells (Figure 8). These effects of mangiferin were concentration dependent

Mangiferin inhibits cell migration and invasion of Mia-PaCa2 cancer cells

Next, the effect of mangiferin on the migration and invasion of the Mia-PaCa2 cancer cells was investigated by transwell assay. The results showed

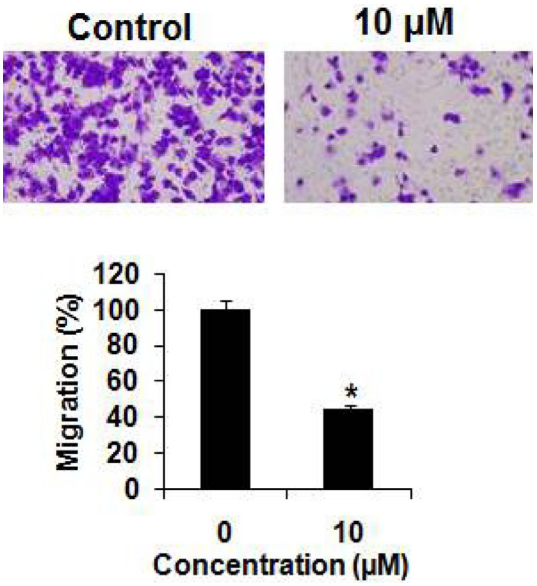

Figure 9. Inhibition of migration of the Mia-PaCa2 cells by mangiferin at indicated concentrations as depicted by transwell assay. The Figure shows that mangiferin treatment led to inhibition of cell migration at 10μM. The values represent the mean of three experiments ± SD (*p<0.05).

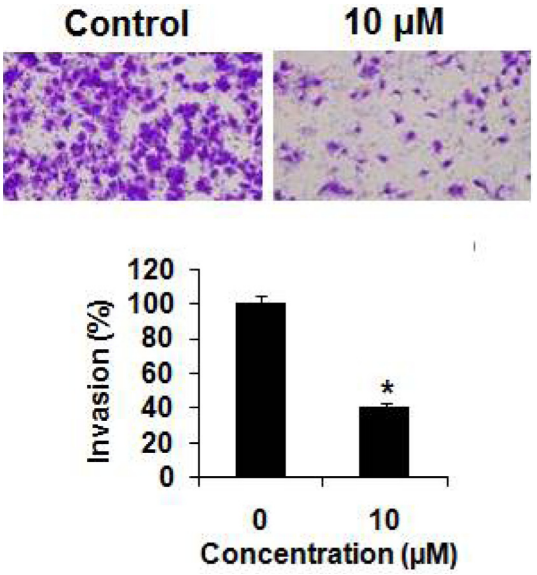

Figure 10. Inhibition of invasion of the Mia-PaCa2 cells by mangiferin at indicated concentrations as depicted by transwell assay. The values represent the mean of three experiments ± SD (*p<0.05).

that at IC_{50}, mangiferin could inhibit the migration of the Mia-PaCa2 cancer cells (Figure 9). Similar trend was observed in cell invasion (Figure 10).

Discussion

Pancreatic cancer is one of the lethal cancers across the globe and in United States pancreatic cancer is the 4th major cause of cancer-related mortality. It is believed the prognosis of pancreatic cancer is one the poorest among all cancers [11]. Herein the anticancer activities of a naturally occurring xanthone were examined against the gemcitabine-resistant pancreatic cancer cells. Plants have provided a number of pharmaceutical agents for the alleviation of human diseases [12]. Anticancer drugs such as etoposide, vinca alkaloids and others have been employed for the treatment of cancer [13]. Plants have a sophisticated mechanism to synthesize chemical entities, such as xanthones, to combat the pathogenic microbes [14]. These metabolites have also been screened by researchers to develop drugs for the treatment of human diseases [15]. In this study mangiferin was evaluated against the Mia-PaCa2 cancer cells and HTRET-HPNE normal cell line. The results showed that mangiferin could inhibit the growth of the Mia-PaCa2 cells with little toxic effects on the normal HTRET-HPNE cells. The anticancer effects of mangiferin were mainly due the induction of autophagy and apoptosis. This was associated with upregulation of autophagy LC3 II and Beclin expression and enhancement of the Bax/Bcl-2 ratio. Autophagy and apoptosis have been shown the two important processes that help in the elimination of the diseased and harmful cells from the body of an organism [16]. Previous studies have also shown that mangiferin induces apoptosis in human leukemia HL-60 cells line via NF-κB signalling pathway [17]. Several of the natural products have been reported to induce apoptosis and autophagy through production of ROS [18]. In this study we found that mangiferin causes the production of the significant amounts of ROS in the Mia-PaCa2 cells which was also associated with decline in the MMP levels. Besides apoptosis cell cycle arrest is another mechanism by which anticancer agents exert their anticancer effects and herein we observed that mangiferin triggered arrest of Mia-PaCa2 cells at the G2/M phase of the cell cycle. Previous studies have also shown that mangiferin triggers cell cycle arrest of human lung cancer cells [7]. Cell migration and subsequent invasion at distant parts of the body is the initial step for metastasis of cancers [19] and herein we observed that mangiferin caused suppression of cell migration and the invasion of

the Mia-PaCa2 cells, suggestive of the antimetastatic potential this compound.

Conclusion

The findings of the present study revealed that mangiferin inhibited the growth of the drug-resistant pancreatic cancer cells via induction of autophagy, apoptosis, cell cycle arrest and generation of ROS. Furthermore, mangiferin also inhibited the migration and invasion of the pancreatic cancer cells, suggestive of its potential as a lead molecule for pancreatic cancer systemic therapy.

Conflict of interests

The authors declare no conflict of interests.

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