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ORIGINAL ARTICLE

Nobiletin flavone inhibits the growth and metastasis of human pancreatic cancer cells via induction of autophagy, G0/G1 cell cycle arrest and inhibition of NF-kB signalling pathway

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Summary

Purpose: *The anticancer effects of nobiletin have not been fully explored against the human pancreatic cancer cells. Therefore this study was undertaken to evaluate the anticancer effects of nobiletin against the MIAPaCa-2 human pancreatic cancer cells along with evaluating its effects on autophagy, cell cycle phase distribution, cell migration and invasion and NF-kB signalling pathway.*

Methods: *Cell proliferation was evaluated by CCK-8 assay while cell cycle analysis was carried out by flow cytometry. Effects on cell migration and invasion were evaluated by wound healing assay and transwell assays respectively. Transmission electron microscopy (TEM) and western blot were used to study the effects on autophagy and NF-kB signalling pathway.*

Results: *The results revealed that nobiletin restrained the proliferation rate of the MIAPaCa-2 human pancreatic cancer cells and showed an IC_{50} of 6.12 μM. However, nobiletin exhibited very high IC_{50} against the normal ms-1 pancreatic cells. TEM showed that nobiletin triggered autophagy in the MIAPaCa-2 cancer cells which was accompanied by enhancement in the expression of LC3B II and LC3-I, and decrease in the expression of p62. Cell cycle analysis showed that nobiletin caused accretion of the MIAPaCa-2 cells in the G0/G1 phase of the cell cycle activating G0/G1 cell cycle arrest. The G0/G1 arrest of MIAPaCa-2 cells was also concomitant with depletion of cyclin D1 and CDK4 expression. Nobiletin suppressed the migration of the MIAPaCa-2 cancer cells reminiscent of the anti-metastatic potential of nobiletin. Finally, nobiletin also blocked the NF-kB signalling pathway in a concentration-dependent manner.*

Conclusions: *Taken together, nobiletin may prove valuable as a promising drug candidate for pancreatic cancer treatment provided further studies are carried out on it, particularly toxicological studies.*

Key words: *pancreatic cancer, nobiletin, autophagy, cell migration, cell cycle arrest.*

Introduction

Pancreatic carcinoma (PC) is reported among one of the major causes of cancer mortality-related worldwide. In US and China ranks 4th and 7th respectively in terms of deaths associated with cancer [1,2]. In US alone about 2-3 million people die due to PC annually [2]. There is a poor prognosis of PC in patients due to the poor progress made in its early detection [3,4]. Although the primary cause of PC remains unclear in recent years, studies have revealed its association with diabetes mellitus, insulin resistance and obesity [5,6]. In this regard there is an immediate need to find new drugs for treatment of this deadly disease. In the past, natural products have proved to be an important source

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of anticancer drugs, particularly plants, because of their vast diversity and it is assumed that they can support with sources of further more drugs to fight a lethal disease like cancer [7]. Pharmacological studies revealed that flavonoids show different biological activities, like anticancer activity [8]. Some flavonoids were reported to halt cancer cell proliferation, hence they may be regarded as crucial for systemic therapy development of PC [9]. Nobiletin is a flavonoid obtained from Citrus depressa Hayata belonging to Rutaceae, which is a common fruit in Okinawa, Japan. Nobiletin is well known to show antiinflammatory properties and in addition it has been revealed to suppress the proliferation of human skin, prostate, colon and breast carcinoma cell lines [10,11]. Amid 42 flavonoids, nobiletin revealed major antiproliferative effect against six different human carcinoma cell lines [12]. In the recent past, nobiletin revealed its ability to suppress cyclooxygenase-2 protein expression and prostaglandin E2 production (COX-2 and PGE2 respectively) *in vitro* [13]. Nobiletin also revealed its apoptotic effect on gastric carcinoma cell line (TMK-1) and inhibition of cell cycle progression [14]. In the current study, we assessed the effect of nobiletin on the growth and metastasis of human pancreatic cancer cells via induction of autophagy, G0/G1 cell cycle arrest and inhibition of NF-kB signalling pathway.

Methods

Cell viability determination

MIAPaCa-2 human pancreatic cancer cell line and ms-1 cells (normal pancreatic cells) were obtained from Thermo Fischer Scientific, China. Cell viability of MIAPaCa-2 and ms-1 cells was performed by using CCK-8 assay. In brief, 96-well plates were used to seed the transfected MIAPaCa-2 and ms-1 cells. The cells were then treated with different concentrations of nobiletin for 24 h at 37°C. Later the cell culture was treated with 10 µL of CCK-8 solution for 2 h and was incubated at 37°C (95% O_2 and 5% CO_2). For the determination of cell viability microplate reader was used to get the optical density (OD) at 450 nm wavelength.

Transmission electron microscopy

The MIAPaCa-2 cells were treated with 6.25 µM of nobiletin. These cells were later treated with 4% glutaraldehyde in 0.05M sodium cacodylate buffer for fixation. After post fixation was done with 1.5% OsO_4 these cells were dehydrated with alcohol. Then, the cells were organised for implanting in Epon 812 and further observation was carried out by using Zeiss CEM 902 electron microscope.

Cell cycle analysis

The MIAPaCa-2 pancreatic carcinoma cells were cultured in DMEM medium at a cell density of 1×10^5 cells/ml. The cells were treated with different concentrations of nobiletin molecule (0, 6.12, 12.5 and 25 µM). Firstly, the cells were harvested and then fixation was done by using 70% ethanol and then treated with 20 µg/ml of RNase A. Later washing and staining was done with phosphate buffered saline (PBS) and annexin V/propidium iodide (PI) solution of 20 µl (20 µg/mL) respectively. Distribution of MIAPaCa-2 pancreatic carcinoma cells to different phases of cell cycle was examined by FACS Calibur flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA, USA).

Cell invasion assay

The impact of nobiletin over the invasion capacity of MIAPaCa-2 cells was examined by the transwell chamber assay with 6-mm pore sized Matrigel coated with polyvinylpyrrolidone-free polycarbonate filter. In the upper chamber about 160 ml of cell culture was placed and only Dulbecco's modified Eagle's medium (DMEM) was placed in the bottom chamber. There was a time gap of 1 day and during that time these cells were incubated and transferred of the upper chamber and the fixation of invaded cells was done with methanol. Later, staining of the cell culture was done for 40 min with 1.60% crystal violet dye. Inverted microscope was used to photograph and analyze the number of invaded cells at 200× magnification.

Wound healing assay

Wound healing assay was used to evaluate cell migration [15]. 24-well plates were used to seed MIAPaCa-2 cells. At 98% confluence a scratch wound was made manually by a 100 µL sterile pipette tip in each well and photographed instantly (0 h). The dose of nobiletin was fixed at 6.12 or 0.1% DMSO was added to the cells, later incubated at 37°C and after 48 h the scratch area was photographed. Image J software was used to measure the distance between two adjacent cells (National Institutes of Health, Bethesda, MD).

Western blot analysis

Western blotting technique was used to determine the protein expression. The nobiletin (0, 6.12, 12.5 and 25 µM) treated MIAPaCa-2 cells were harvested by centrifugation. Lysis buffer containing the protease inhibitor was used to lyse MIAPaCa-2 cells. Forty µg from each lysate were separated using SDS-PAGE and finally transferred electrophoretically on PVDF membrane (polyvinylidene difluoride). Blocking of the membrane was done with fat free milk at room temperature for 1 h. After blocking, the membranes were exposed at 4°C to antibodies overnight. Consequently, incubation of membranes with secondary antibodies was done and Odyssey Infrared Imaging System (LI-COR, USA) was used to detect signals. For normalization, Actin was used.

Statistics

All the experimental data obtained from individual triplicate experiments was subjected to Duncan's multiple range tests for comparisons and analyzed through one-way ANOVA. Considering $p<0.05$ as statistically significant, all the data was expressed as mean±SD.

Results

Nobiletin induces selective cytotoxicity in MIAPaCa-2 human pancreatic cancer cells

The CCK-8 assay was used to determine the effects of nobiletin (Figure 1A) on the growth of the MIAPaCa-2 human pancreatic cancer cells and ms-1 normal cells. Nobiletin caused a substantial reduction in the proliferation rate of the MIAPaCa-2 cells. The effects of nobiletin on the proliferation rate of the MIAPaCa-2 cells were concentration-dependent and IC_{50} of 6.12 μM was reported for this molecule against the pancreatic cancer cells (Figure 1B). Interestingly, the cytotoxic effects of nobiletin on the normal ms-1 cells were less pronounced and an IC_{50} of 75.5 μM was reported for nobiletin against these normal cells (Figure 1B).

Autophagy inducing effects of nobiletin on the MIAPaCa-2 human pancreatic cancer cells

Next, TEM analysis of the nobiletin-treated MIAPaCa-2 cells was performed. It was witnessed that nobiletin caused extensive development of autophagic vesicles or autophagosomes in the MIAPaCa-2 cells which are the trademarks of autophagy (Figure 2). Furthermore, nobiletin also triggered increase in the protein levels of LC3-I and LC3-II, indicative of autophagy. Nonetheless, the expression levels of p62 were found to decrease in a dose-dependent fashion (Figure 3).

Nobiletin led to induction of G0/G1 cell cycle arrest in MIAPaCa-2 cells

The MIAPaCa-2 human pancreatic cancer cells were treated with different concentrations of nobiletin and the distribution of pancreatic cancer cells at each phase of the cell cycle was examined by flow cytometry. The results disclosed that the G0/G1 phase cells increased remarkably upon nobiletin treatment. The percentage of G0/G1 phase cells were found 50.1, 62.2, 73.1 and 82.0% at 0, 6.12, 12.5 and 25 μM concentrations of nobiletin respectively, indicative of G0/G1 arrest of the MIAPaCa-2 cells (Figure 4). Western blot analysis was also performed to scrutinize the effects of nobiletin on cell cycle associated proteins. The results ex-

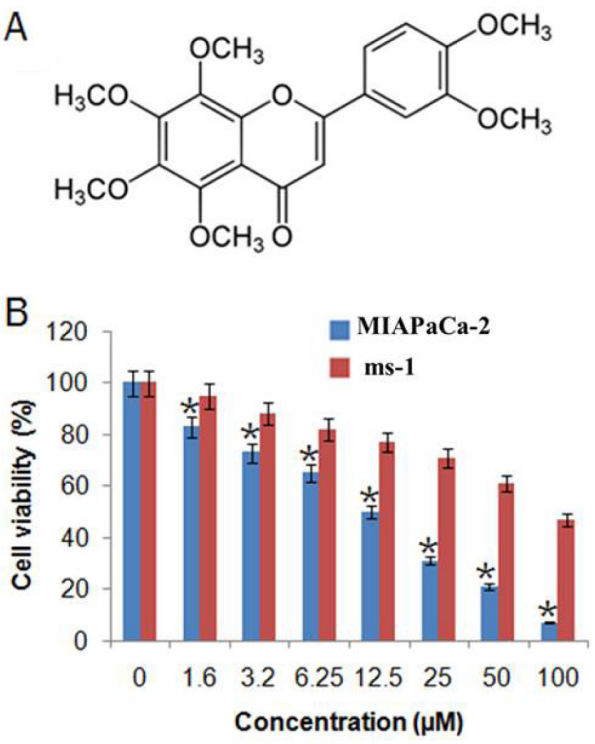

Figure 1. A: Chemical structure of nobiletin; **B:** CCK-8 assay showing dose-dependent and selective cytotoxicity induced by nobiletin in MIAPaCa-2 human pancreatic cancer cells exerting lesser cytotoxicity to the normal pancreatic cells. The data is the mean ± SD of three individual experiments. *p<0.01.

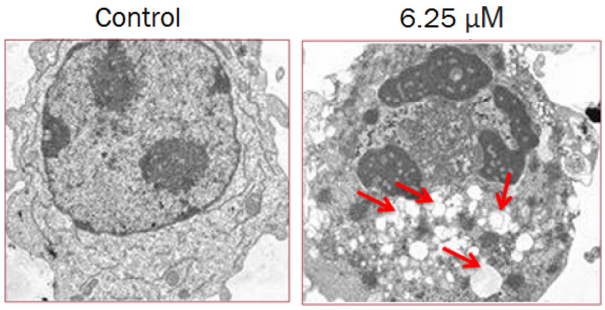

Figure 2. Transmission electron microscopy (TEM) analysis showing initiation of autophagic vacuoles or autophagosomes in MIAPaCa-2 human pancreatic cancer cells on treatment with 6.25 μM dose of nobiletin. The experiments were performed in triplicate. Arrows indicate the presence of autophagosomes which shows that nobiletin treatment induces autophagy in the pancreatic cells.

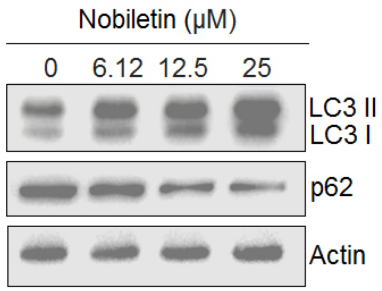

Figure 3. Effect of nobiletin on the expression of various autophagy-related protein expressions including LC3-II and LC3-I and p-62. Nobiletin triggered increase in the protein levels of LC3-I and LC3-II indicative of autophagy. Nonetheless, the expression level of p62 was found to decrease in a dose-dependent manner. The experiments were performed in triplicate.

hibited that nobiletin inhibited the expression of cyclin D1 and CDK4 (Figure 5). Both these assays prove that nobiletin caused G0/G1 cell cycle arrest in MIAPaCa-2 cells.

Suppression of cell migration and invasion induced by nobiletin flavone in MIAPaCa-2 human pancreatic cancer cells

Further, we carried out experiments in order to check the effects of nobiletin on cancer cell migration and invasion using *in vitro* wound healing assay and transwell assay respectively.

The results showed that nobiletin led to substantial decline in the migration of the MIAPaCa-2 cancer cells at a dose of 6.12 μM which is the IC_{50} value of the molecule (Figure 6). Furthermore, transwell assay showed that the invasion of the MIAPaCa-2 cells was also reduced in a dose-dependent manner as apparent from the wound width (Figure 7).

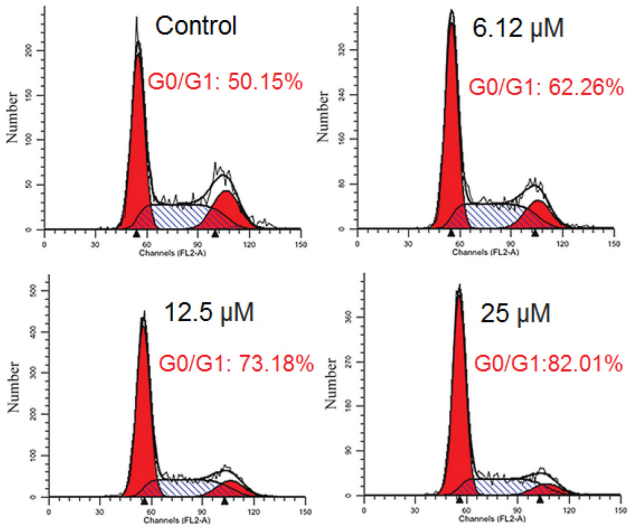

Figure 4. Effect of nobiletin on the cell cycle phase distribution of MIAPaCa-2 human pancreatic cancer cells. The results showed that it led to G0/G1 cell cycle arrest in a dose-dependent manner. The experiments were performed in triplicate.

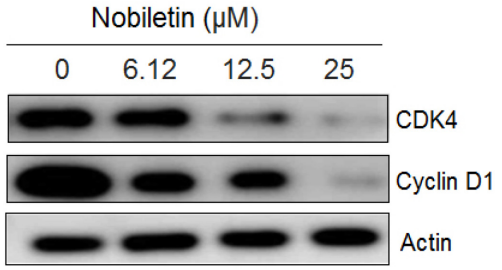

Figure 5. Effect of nobiletin on the expression levels of cyclin-D1 and CDK-4 proteins as revealed by western blot method in MIAPaCa-2 human pancreatic cancer cells. The experiments were performed in triplicate.

Nobiletin blocks the NF-kB signalling pathway

The effects of nobiletin were also examined on the NF-kB signalling pathway at 0, 6.12, 12.5 and 25 μM using western blot assay. This signalling pathway has been reported to play a crucial role in

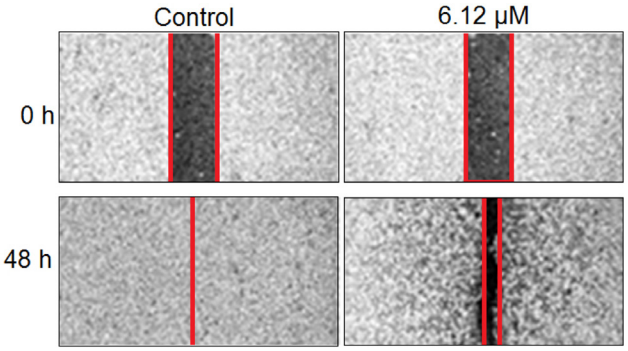

Figure 6. Treatment of MIAPaCa-2 pancreatic cancer cells resulted in the inhibition of cell migration as predicted by wound healing assay. The experiments were performed in triplicate.

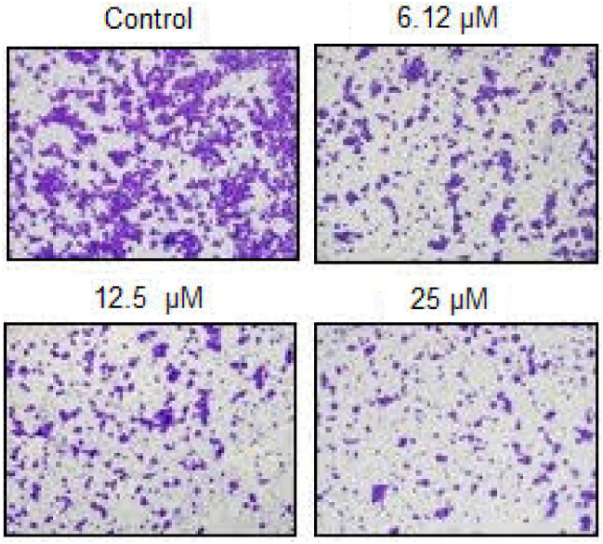

Figure 7. Effect of nobiletin on invasive potency of MIAPaCa-2 pancreatic cancer cells. Prior to the cell invasion assay cells were treated with control, 6.12, 12.5 and 25 μM of nobiletin for 24 h. There was a dose-dependent reduction in the number of invaded cells after one complete day of treatment. The experiments were performed in triplicate.

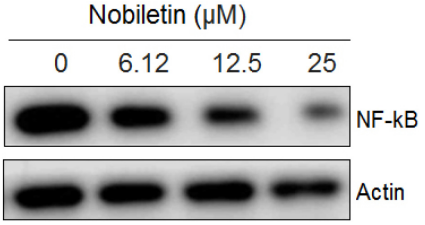

Figure 8. Nobiletin treatment led to dose-dependent inhibition of the NF-kB protein expression as shown by western blot assay. The experiments were performed in triplicate.

the carcinogenesis process and as such could be a promising therapeutic target. The results showed that nobiletin caused dose-dependent inhibition of the NF-kB protein expression (Figure 8).

Discussion

Pancreatic cancer, 4th leading cause in mortality among all cancers, is a common malignant tumor [16]. In the recent past, molecules obtained from plants have drawn astonishing attention because of their reasonably lesser toxic effects. Therefore, identifying and screening of natural products for developing competent drugs against cancer remains a continuous focus of researchers across the globe. Citrus flavonoids (C. depressa Rutaceae CDR) have revealed antiproliferative, antiinvasive and antiinflammatory effects over various human carcinoma cell lines. Though CDR contains six different flavonoids we selected nobiletin for the current study due its operative suppression of different human carcinoma cells like breast adenocarcinoma, neuroblastoma and gastric adenocarcinoma [17-19]. In the current study MIAPaCa-2 human pancreatic cancer cells were treated with nobiletin, revealling surprising effects. Selective antiproliferative effects of nobiletin were demonstrated against MIAPaCa-2 human pancreatic cancer cells with minimal effects to the normal pancreatic cells (ms-1), indicating selectivity of this molecule. In the recent past flavonoids have revealed inhibition of growth of carcinoma cells by activating autophagy and apoptosis [20]. Autophagy and apoptosis maintain tissue homeostasis thereby abolishing cancer, harmful, and unwanted cells [21]. The current study revealed that nobiletin induced autophagy in MIAPaCa-2 human pancreatic cancer cells. The expression of proteins related to autophagy was also altered. Previous studies stated that different anticancer molecules trigger autophagy in cancer cells [21]. We found in the current study that nobiletin also induced G0/G1 cell cycle arrest. NF-kB signalling pathway is closely involved in the progression and development of cancer. NF-kB pathway controls gene expression of IL6, TNFA, BCLXL, BCL2, BCLXS, VEGF and XIAP genes and hence is an important pathway in cancer development [22]. Herein, we found that nobiletin was actively involved in blocking NF-kB signalling pathway. Finally, suppression of cell migration and invasion induced by nobiletin flavone in MIAPaCa-2 human pancreatic cancer cells was also confirmed.

Conclusion

Overall, nobiletin induces significant anticancer effects on the MIAPaCa-2 human pancreatic carcinoma cells. It induced autophagy, G0/G1 cell cycle arrest and inhibition of NF-kB signalling pathway along with inhibiting both cancer cell migration and invasion in human pancreatic carcinoma cells. Hence, nobiletin may prove a potential lead molecule for the systemic treatment of pancreatic cancer, therefore it merits more investigations.

Conflict of interests

The authors declare no conflict of interests.

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