RAPID COMMUNICATION

MK615 inhibits pancreatic cancer cell growth by dual inhibition of Aurora A and B kinases

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Abstract

AIM: To investigate the anti-neoplastic effect of MK615, an anti-neoplastic compound isolated from Japanese apricot, against human pancreatic cancer cells *in vitro*.

METHODS: Three human pancreatic cancer cell lines PANC-1, PK-1, and PK45H were cultured with MK615 at concentrations of 600, 300, 150, and 0 μg/mL. Growth inhibition was evaluated by cell proliferation assay, and killing activity was determined by lactate dehydrogenase (LDH) assay. Expression of Aurora A and B kinases was detected by real-time polymerase chain reaction (PCR) and Western blotting. Cell cycle stages were evaluated by flow cytometry.

RESULTS: The growth inhibitory rates of MK615 at 150, 300, and 600 μg/mL were 2.3% ± 0.9%, 8.9% ± 3.2% and 67.1% ± 8.1% on PANC1 cells, 1.3% ± 0.3%, 8.7% ± 4.1% and 45.7 ± 7.6% on PK1 cells, and 1.2 ± 0.8%, 9.1% ± 2.1% and 52.1% ± 5.5% on PK45H cells, respectively (P <0.05). The percentage cytotoxicities of MK615 at 0, 150, 300, and 600 μg/mL were 19.6% ± 1.3%, 26.7% ± 1.8%, 25.5% ± 0.9% and 26.4% ± 0.9% in PANC1 cells, 19.7% ± 1.3%, 24.7% ± 0.8%, 25.9% ± 0.9% and 29.9% ± 1.1% in PK1 cells, and 28.0% ± 0.9%, 31.2% ± 0.9%, 30.4% ± 1.1% and 35.3 ± 1.0% in PK45H cells, respectively ($P < 0.05$). Real-time PCR and Western blotting showed that MK615 dually inhibited the expression of Aurora A and B kinases. Cell cycle analysis revealed that MK615 increased the population of cells in G2/M phase.

CONCLUSION: MK615 exerts an anti-neoplastic effect on human pancreatic cancer cells *in vitro* by dual inhibition of Aurora A and B kinases.

INTRODUCTION

MK615, an extract from the Japanese apricot, *Prunus mume* Siebold et Zuccarini (*ume* in Japanese), contains several triterpenoids and has been shown to exert an anti-neoplastic effect against human cancers. Previous studies have revealed that MK615 has anti-neoplastic effects against gastric cancer[1], breast cancer[2], hepatocellular carcinoma[3], and colon cancer[4]. The mechanisms responsible for the anti-neoplastic effect of MK615 include induction of apoptosis[1,2] and autophagy[4], and suppression of Aurora A kinase[3] in cancer cells. However, the entire mechanisms of the anti-neoplastic effects of MK615 have not been elucidated.

In the present study, we cultured human pancreatic cancer cell lines in the absence and presence of MK615 for the first time, and found that MK615 had an anti-neoplastic effect that was exerted by dual inhibition of Aurora A and B kinases.

MATERIALS AND METHODS

Reagents

MK615 was provided by Japan Apricot Co., Ltd. (Gunma, Japan). MK615 is derived from Japanese apricot fruit[1].

Cell culture

Three human pancreatic cancer cell lines PANC-1 (PANC1), PK-1 (PK1) and PK-45H (PK45H) were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 mL/L

heat-inactivated fetal calf serum (FCS) and incubated at 37℃ in a humidified atmosphere consisting of 50 mL/L CO_2 in air.

Cell proliferation assay

Cells were plated at 5×10^3/well in 96-well plates in DMEM containing 100 mL/L FCS and cultured with or without MK615 at concentrations of 600, 300, or 150 μg/mL. For the negative control wells, cells were cultured with 10 mL/L DMSO alone. After 48 h, 3-(4, 5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTT; 5 g/L) was added to each well and the cells were incubated for 4 h. Then according to the manufacturer's recommendation, 100 μL of solubilization solution/stop mix was added and the plates were left to stand for 60 min. The absorbances at 570 nm (A_{570}) and 630 nm (A_{630}) were then measured with an enzyme-linked immunoassay (ELISA) reader. The actual counts were calculated by subtracting A_{570} from A_{630}. Each assay was performed in triplicate and the average absorbance was calculated. The growth inhibition rate was calculated using the ratio of absorbance at each drug concentration relative to the absorbance without drug.

LDH assay

Lactate dehydrogenase (LDH) assay was performed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). Briefly, cells were plated at 5×10^3/well in 96-well plates in DMEM containing 100 mL/L FCS. Cells were cultured with or without MK615 at concentrations of 600, 300, or 150 μg/mL. For the negative control wells, cells were cultured with 10 mL/L DMSO alone. The medium was removed and 100 μL of CytoTox-ONE™ Reagent was added. The cells were then incubated at 22℃ for 10 min and 50 μL of stop solution was added. The plates were shaken for 10 s and the absorbances at 560 nm (A_{560}) and 590 nm (A_{590}) were measured with an ELISA reader. To obtain the maximum LDH release, 10 μL of lysis solution (10 ×) was added to the positive control wells 45 min prior to harvest. After 48 h, 50 μL of supernatant was transferred into a fresh 96-well plate and incubated with 50 μL of substrate mix for 30 min at room temperature. Fifty microliters of stop solution was added, and the absorbance at 490 nm (A_{490}) was measured with an ELISA reader. Each assay was performed in triplicate and the average absorbance was calculated. The percentage cytotoxicity was calculated using the formula: Percentage cytotoxicity = 100 × (Experimental - Culture medium background)/ (Maximum LDH release - Culture medium and lysis solution background).

Real-time PCR

The total RNA of each cell line was isolated using a Total RNA Isolation kit (MACHEREY-NAGEL, Düren, Germany). Reverse transcription reactions were performed using a Rever Tra Ace α-First Strand cDNA Synthesis kit (TOYOBO, Osaka, Japan). Briefly, 1 μg of total RNA, oligo dT-primer, and dNTPs were incubated at 65℃ for

5 min, followed by addition of 10 μL of cDNA synthesis mixture and further incubation at 50℃ for 50 min. The reaction was terminated by adding 1 μL of RNaseH and incubating the mixture at 37℃ for 20 min.

Real-time PCR was performed with an ABI Prism 7700 Sequence Detector (Applied Biosystems, Warrington, UK). The PCR reaction was carried out in a final volume of 2 μL of cDNA, 12.5 μL of 2 × SYBR Green (Applied Biosystems), 0.5 μL of 25 nmol/L sense and antisense primers, and H_2O up to 25 μL. The PCR conditions consisted of 40 amplification cycles at 95℃ for 30 s and 60℃ for 30 s. The sequences of the primers were as follows: GAPDH, 5'-CCACCCAGAAGACTGTGGAT-3' (sense) and 5'-TTCAGCTCAGGGATGACCTT-3' (anti-sense); Aurora A, 5'-TTGGAATATGCACCACTTGGA-3' (sense) and 5'-ACTGACCACCCAAATCTGC-3' (anti-sense); and Aurora B, 5'-GGGAGAGCTGAAGATTGCTG-3' (sense) and 5'-GGCGATAGGTCTCGTTGTGT-3' (anti-sense). The level of expression was calculated using the formula: Relative expression (t) = (Copy number of target molecule/ Copy number of GAPDH) × 1000. Samples were assayed in triplicate. Means and standard deviations were calculated from the data obtained. The *t* value was calculated from the mean of three different assays.

Western blotting

Anti-Aurora A and Aurora B, nuclear factor-κB (NF-κB), and phosphorylated NFκB at serine residue 536 [pNF-κB (ser536)] antibodies were purchased from Cell Signaling Technology (Beverly, MA). Cells (5-10×10^6) were cultured with MK615 (300 μg/mL) for 48 h, washed twice with cold PBS, lysed with 200 μL of 5 g/L SDS, and centrifuged at $10\,000 \times g$. The supernatants were adjusted to contain equal amounts of protein by dilution, using a BCA Protein Assay kit (PIERCE, Rockford, IL). Samples (20 μg protein) were run on 125 g/L SDS-PAGE with 10% gel and electroblotted onto PVDF membranes. The blots were blocked for 1 h with 50 g/L nonfat milk powder and 1 mL/L Tween 20 in Tris-NaCl, followed by overnight incubation with primary antibody (dilution 1:1000) at 4℃. After extensive washing, the blots were incubated with the secondary horseradish-peroxidase-conjugated antibody (dilution 1:2000) for 2 h at room temperature. Immunoreactive bands were visualized by using an enhanced chemiluminescence detection system (Amersham Life Sciences, Arlington Heights, IL).

Cell cycle analysis

Cells were cultured with 300 μg/mL MK615 for 48 h, then collected by trypsinization. According to the manufacturer's instructions, cell cycle analysis was performed with a Cycle TEST PLUS DNA Reagent kit (Becton-Dickinson, Mountain View, CA) on a FACScaliber (Becton-Dickinson). Results were analyzed with CellFIT software.

Statistical analysis

For the MTT assay, LDH assay, and real-time PCR, Student's *t* test (two-sided) was used to compare the data obtained from MK615-treated and MK615-untreated groups. A *P* value less than 0.05 was considered statistically significant.

Table 1 Cell cycle analysis showing MK615 treatment increased the cell population in G2-M phase in all three pancreatic cancer cell lines.				
	MK615	**G0-G1**	**S**	**G2-M**
PANC1	(-)	68.1	24.2	7.8
	(+)	71.6	15.4	13.0
PK1	(-)	55.9	42.2	1.8
	(+)	68.8	26.0	5.2
PK45H	(-)	56.0	21.1	22.9
	(+)	59.0	11.7	29.3

Figure 2 Lysis of pancreatic cells by MK615. MK615 lysed PANC1, PK1, and PK45H cells at concentrations of 150, 300, and 600 μg/mL. The percentage cytotoxicities at 150, 300, and 600 μg/mL MK615 were significantly higher than those at 0 μg/mL MK615 ($^aP < 0.05$). Cells were cultured for 48 h. The assay was carried out in triplicate.
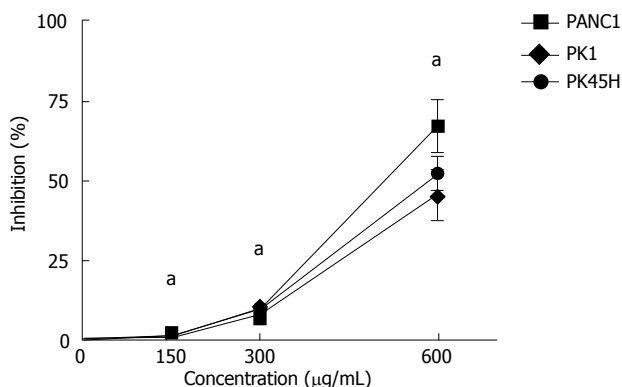

Figure 1 Growth inhibition of pancreatic cancer cells by MK615. MK615 significantly inhibited the growth of PANC1, PK1, and PK45H cells in a dose-dependent manner ($^aP < 0.05$). Cells were cultured for 48 h. The assay was carried out in triplicate.

RESULTS

MK615 inhibited growth of the three pancreatic cancer cell lines (Figure 1). The growth inhibitory rates of MK615 at 150, 300 and 600 μg/mL were 2.3% ± 0.9%, 8.9% ± 3.2% and 67.1% ± 8.1% on PANC1 cells, 1.3% ± 0.3%, 8.7% ± 4.1% and 45.7% ± 7.6% on PK1 cells, and 1.2% ± 0.8%, 9.1% ± 2.1% and 52.1% ± 5.5% on PK45H cells, respectively. The growth inhibition induced by MK615 at all concentrations was significantly higher than that at 0 μg/mL ($P < 0.05$).

Cytotoxicity of MK615 against pancreatic cancer cell lines was evaluated by LDH assay (Figure 2). The percent specific lysis by MK615 at 0, 150, 300, and 600 μg/mL was 19.6% ± 1.3%, 26.7% ± 1.8%, 25.5% ± 0.9% and 26.4% ± 0.9% in PANC1 cells, 19.7% ± 1.3%, 24.7% ± 0.8%, 25.9% ± 0.9% and 29.9% ± 1.1% in PK1 cells, and 28.0% ± 0.9%, 31.2% ± 0.9%, 30.4% ± 1.1% and 35.3% ± 1.0% in PK45H cells, respectively. The percent specific lysis induced by MK615 at all concentrations was significantly higher than that at 0 μg/mL ($P < 0.05$).

Moreover, we examined whether MK615 was able to inhibit Aurora kinases in the pancreatic cancer cell lines. Aurora kinases mRNA expression is shown in Figure 3. The inhibition rate of Aurora A and Aurora B mRNA was 38.4% ± 2.9% ($P = 0.02$) and 40.8% ± 3.2% ($P = 0.03$) in PANC1 cells, 7.6% ± 1.1% ($P = 0.05$) and 43.4% ± 4.5% ($P = 0.03$) in PK1 cells, and 8.7% ± 2.5% ($P = 0.05$) and 10.5% ± 2.1% ($P = 0.05$) in PK45H cells, respectively, indicating a significant inhibition of mRNA expression of both Aurora kinases by MK615.
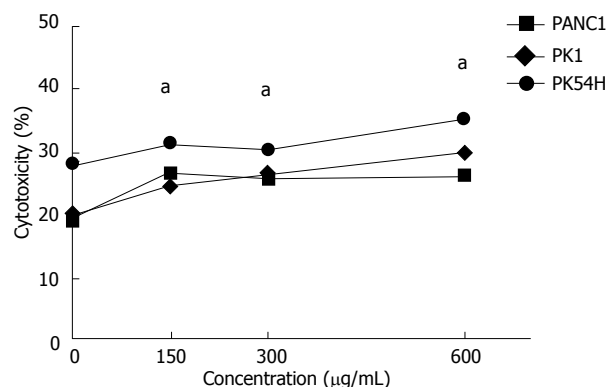
Figure 4 shows the protein expression of Aurora kinases. After 48 h of incubation with 300 μg/mL MK615, expressions of both Aurora A and B proteins were decreased in PANK1 and PK45H cells. Because endogenous Aurora A expression was low in PK1 cells, no significant decrease in its expression was observed. Consequently, MK615 decreased NF-κB expression in PANK1 and PK45H cells, but no evident decrease in NF-κB expression in PK1 cells was observed. The decrease in NF-κB expression was more evident with the use of anti-phosphorylated NF-κB antibody [pNF-κB (ser536)].

In addition, we analyzed the effect of MK615 on the cell cycle. MK615 increased the proportion of cells in G2-M phase (Table 1) from 7.8% to 13.0% in PANC1 cells, from 1.8% to 5.2% in PK1 cells, and from 22.9% to 29.3% in PK45H cells.

DISCUSSION

MK615 is isolated from Japanese apricot and contains many defined and undefined substances, including several triterpenoids[1], some of which exert an anti-neoplastic effect. In the present study, MK615 inhibited the proliferation of all three pancreatic cancer cell lines (Figure 1), and LDH assay showed that the anti-proliferative effect of MK615 on these cells was due not only to cell anti-proliferative effect, but also to direct cell killing effect (Figure 2).

In a previous study from our laboratory, MK615 was shown to be capable of inhibiting Aurora kinase A in human hepatocellular carcinoma[3]. This prompted us to investigate the effects of MK615 on Aurora B kinases. Aurora kinases are key mediators of cell division by controlling chromatid segregation[6-8]. There are three isoforms of Aurora kinases in mammals, Aurora A, Aurora B, and Aurora C. Structurally, all three share a similar domain organization, consisting of the N- and C-terminal domains, and a central region containing the catalytic domain, whose sequence is conserved among all three Aurora kinases[9]. Despite their sequence similarities, however, the functions and cellular localization of Aurora kinases differ. Aurora A is localized at the centrosome and forms the mitotic spindle apparatus
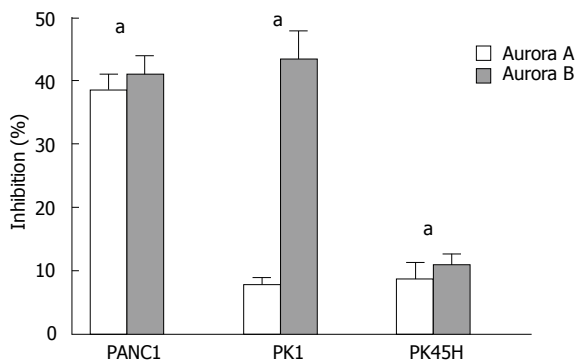
Figure 3 Dual inhibition of mRNA expression of Aurora A and Aurora B kinases by MK615. MK615 significantly inhibited the transcription of Aurora A and Aurora B mRNA. The percentage inhibition in PANC1, PK1, and PK45H cells was statistically significant ($^aP < 0.05$). RNA was extracted from the cells cultured with MK615 at a concentration of 300 μg/mL for 48 h.
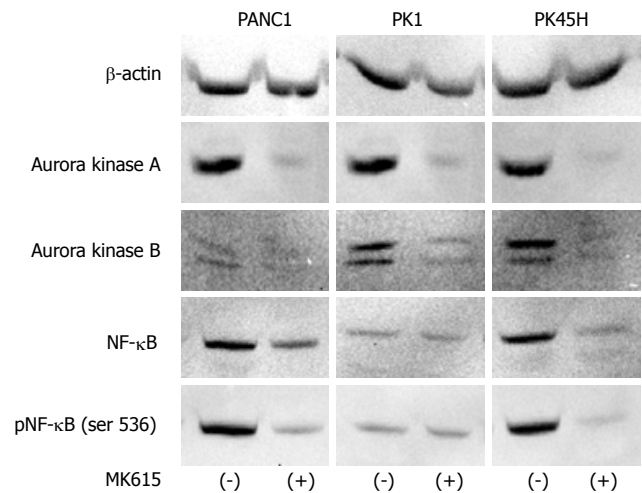

Figure 4 Inhibition of protein expression of Aurora A and Aurora B kinases, and NF-κB by MK615. MK615 dually inhibited the expression of Aurora A and Aurora B kinases in PANC1 and PK45H cells. However, no inhibition was evident in PK1 cells. NF-κB inhibition was more evident when anti-phosphorylated NF-κB antibody was used. Protein was extracted from the cells cultured with MK615 at a concentration of 300 μg/mL for 48 h.

that plays a crucial role in segregating chromosomes into daughter cells. Aurora B is localized in inner-centromeric chromatin during metaphase-anaphase transition, and relocalizes to microtubules in the spindle midzone during telophase, being required for accurate cytokinesis[10,11]. Unlike Aurora A and Aurora B kinases, the function of Aurora C still remains unclear. Over-expression of Aurora A and Aurora B is frequently observed in various human cancers[12-15].

Our results demonstrated that MK615 inhibited the growth of, and showed cytotoxicity against, all three pancreatic cancer cell lines. The former effect was dose-dependent, while the latter was not. Although the mechanism responsible for the anti-neoplastic effect of MK615 has not been fully elucidated, the results of real-time PCR (Figure 3) and Western blotting (Figure 4) indicated that MK615 inhibited the expression of Aurora A and B kinases. There are several inhibitors of Aurora kinases, including the anti-Aurora B inhibitor, hesperadin[16], anti-Aurora A and B, ZM447439[17], and anti-Aurora A, B, and C, VX-680[18]. As shown in the present study, MK615 dually inhibited both Aurora A and B kinases. Inhibition of Aurora kinases mRNA expression varied in three pancreatic cancer cell lines. In PANC1 and PK45H cells, Aurora A and Aurora B mRNA expression was inhibited to the same degree, whereas Aurora B mRNA was inhibited more efficiently by MK615 than Aurora A mRNA in PK1 cells. Consequently, MK615 efficiently inhibited Aurora A and Aurora B in PANC1 and PK45H cells at the protein level (Figure 4), whereas such inhibition was not evident in PK1 cells. Because the protein expression of Aurora A in PK1 cells was weak, the deficient inhibition of Aurora kinases may be due to the low expression of endogenous Aurora A in these cells. Although MK615 suppresses unknown molecules located upstream from Aurora A and B kinases, administration of MK615 at least down-regulates the expression of Aurora A and B kinases in a dual manner.

NF-κB plays a central role in cell proliferation[19-21] and is consistently activated in some human cancers[22-24]. Aurora A binds the E2 ubiquitin ligase UBE2N, and UBE2N phosphorylates IκBα. Activated IκB translocates NF-κB from the cytoplasm to the nucleus, resulting in activation of the NF-κB complex[25]. MK615 inhibits the expression of Aurora A, resulting in inhibition of NF-κB activation[26-28].

A previous study from our laboratory showed that MK615 induced cell-cycle arrest at the end of G2 phase in hepatocellular carcinoma[3]. In the present study, MK615 treatment increased the cell population in G2-M phase in all three pancreatic cancer cell lines. MK615 inhibits Aurora A and Aurora B kinases and may induce cancer cell death at the G2-arrest check point[29,30].

In conclusion, MK615 exerts an anti-neoplastic effect on human pancreatic cancer cells *in vitro via* dual inhibition of Aurora A and B kinases. Although further investigation is needed, MK615 seems to have the property anti-Aurora kinase compound.

COMMENTS

Background
MK615 is an extract from the Japanese apricot. Previous studies have revealed that MK615 has anti-neoplastic effects against human cancers. The mechanisms of anti-neoplastic effect of MK615 include apoptosis, autophagy, and suppression of Aurora A kinase. However, the entire mechanisms of the anti-neoplastic effects of MK615 have not been elucidated.

Research frontiers
Pancreatic cancer is one of the leading causes of death worldwide, and the 5-year survival rate is 2%-20%. There has been a need for chemotherapeutic drugs that are less toxic and can inhibit or suppress pancreatic cancer, and MK615 is a promising candidate.

Innovations and breakthroughs
There are not many inhibitors of Aurora kinases. MK615 is a natural compound which strongly inhibits Aurora A and B kinases.

Applications
MK615 shows an anti-neoplastic effect on human pancreatic cancer cells *in vitro via* dual inhibition of Aurora A and B kinases. MK615 may prove to be a therapeutic approach in pancreatic cancer therapy.

Terminology

MK615 is an extract from the Japanese apricot, *Prunus mume* Siebold et Zuccarini (*ume* in Japanese). Aurora kinases are key mediators of cell division by controlling chromatid segregation. There are three isoforms of Aurora kinases in mammals: Aurora A, Aurora B, and Aurora C.

Peer review

This is an *in vitro* study of an anti-neoplastic drug MK615 made from Japanese apricot. Its mechanism of action was studied and revealed to have inhibitory effects on Aurora A and B kinases. This is a good basic research on anti-neoplastic agent MK615 for pancreatic carcinoma.

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