

# **Research Article**

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# MK615, A Compound Extract from the Japanese Apricot "*Prunus mume*" Inhibits *In vitro* Cell Growth and Interleukin-8 Expression in Non-small Cell Lung Cancer Cells

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## Abstract

The Japanese apricot "*Prunus mume*," which is also known as the Ume fruit in Japan, is a centuries-old traditional Japanese medicine, and it is a commonly consumed food. MK615, a compound extract from Ume fruits, has been shown to have anti-tumor and anti-inflammatory effects. In this study, we assessed the effects of MK615 on the *in vitro* growth of nine non-small cell lung cancer (NSCLC) cell lines and the HBEC4 immortalized bronchial epithelial cell line. While MK615 inhibited the *in vitro* cell growth of the majority of the NSCLC cell lines, the growth-inhibitory effects varied among the cell lines, and some cell lines exhibited MK615 resistance. In the H1299 and H157 NSCLC cell lines that are highly sensitive to MK615, the induction of autophagy was observed after MK615 treatment. In addition, cell-cycle analysis showed that MK615 increased the proportion of cells in the G0-G1 phase in H1299 and H157 cells. In H1792 cells that overexpress IL-8, MK615 down-regulated IL-8 expression at the mRNA and protein levels in a dose-dependent manner. These results suggest that MK615 has multiple anti-tumor activities including the inhibition of cell proliferation, autophagy induction, G0-G1 cell cycle arrest and the down-regulation of IL-8 expression in NSCLC cells.

#### Introduction

Lung cancer is the leading cause of cancer related deaths throughout the world [1]. Lung cancer is divided into two major histological subtypes: small cell lung cancer and non-small cell lung cancer (NSCLC), and the latter represents 80-85% of all lung cancers [2]. The majority of patients with NSCLC have locally advanced or metastatic disease at initial diagnosis, and systemic chemotherapy for such patients remains marginally effective. Moreover, chemotherapeutic agents occasionally cause serious adverse effects. Thus, there is an urgent need to develop more effective and less toxic anti-tumor agents for NSCLC therapy.

The Japanese apricot "*Prunus mume*" (Figure 1A), which is also known as the Ume fruit in Japan, is a centuries-old traditional Japanese medicine, and it is a commonly consumed food. Ume fruits contains many natural chemical substances including citric acid, malic acid and triterpenoids [3]. MK615 (Figure 1B), a compound extract from Ume fruits, has been shown to have anti-tumor effects against various human cancers including malignant melanoma [4] and colon [5],

breast [6], hepatocellular [7,8], esophageal [9] and pancreatic cancers [10]. However, the MK615 anti-tumor effects on lung cancer remain to be elucidated. Furthermore, a previous study demonstrated that MK615 inhibits the release of inflammatory cytokines, interleukin-6 and tumor necrosis factor-alpha by mitogen-activated protein kinase (MAPK) and NF-kB p65 activation in lipopolysaccharide (LPS)-induced macrophage-like cells [11]. This finding suggests that MK615 potentially inhibits tumor-related cytokine production. Here we describe the inhibitory effects of MK615 on *in vitro* cell growth and interleukin-8 (IL-8) expression in NSCLC cells.

#### Materials and Methods

#### **MK615** preparation

MK615 (Figure 1B) represents the extracted components from Ume fruits [5-8]. The MK615 preparation procedure was previously described [6]. Briefly, Ume fruits were squeezed in a press, and the juice was heated and concentrated. The condensed extract was dissolved in water and neutralized by NaOH. The MK615 solution was then sterilized in an autoclave.



Figure 1: Pictures of (A) the Japanese apricot "*Prunusmume*," which is also known as the Ume fruit in Japan, and (B) MK615, a compound extract from Ume fruits.

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#### Cell lines and culture conditions

Nine NSCLC cell lines (i.e., NCI-H157, NCI-H441, NCI-H460, NCI-H661, NCI-H838, NCI-H1299, NCI-H1395, NCI-H1792 and HCC827) [12,13] and the HBEC4 immortalized human bronchial epithelial cell line [14] were kindly provided by Drs. John D. Minna and Adi F. Gazdar of the University of Texas Southwestern Medical Center at Dallas. The cancer cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum. The HBEC4 cells were cultured in Keratinocyte-SFM medium (Invitrogen, Carlsbad, CA) containing 50 µg/ml bovine pituitary extract (Invitrogen) and 5 ng/ml EGF (Invitrogen).

#### Cell growth assay

Twenty-four hours after plating the cells (H1299:  $1\times10^4$  cells; H1395:  $2\times10^5$  cells; and all other cell lines:  $1\times10^5$  cells) on 6-well plates, the cells were treated with 1,5, and 10  $\mu$ l/ml MK615. After 48 and 96 h, the trypan blue-negative viable cells were counted.

## Immunofluorescence staining

Autophagy was evaluated by immunostaining of microtubuleassociated protein 1 light chain 3 (LC3), which is an autophagosome marker [15,16]. The cells were treated with or without 10  $\mu$ l/ml MK615 and harvested after 6 h. The cells were then mounted on glass slides using a Cytospin (Shandon, Pittsburgh, PA; model Cytospin 2). The mounted cell specimens were fixed with 4% paraformaldehyde for 10 min at room temperature. The specimens were incubated with an anti-LC3 antibody (Medical & Biological Laboratories Co., Nagoya, Japan) at 4°C overnight. The specimens were subsequently incubated with a FITC-conjugated antibody (MP Biomedicals-Cappel, Irvine, CA) for 1 h at room temperature. The specimens were then immediately observed for the intracellular localization of FITC using a fluorescence microscope (Olympus, Tokyo, Japan; Model AX80). Non-treated cells (i.e., those incubated without MK615) were used as control specimens.

## Western blotting

After treatment with or without 10 µl/ml MK615 for 4 and 8 h, the cells were washed with phosphate-buffered saline, and lysed with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) containing 2% ß-mercaptoethanol. Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using AnykD Criterion TGX precast gel (Bio-Rad Laboratories), and electro transferred by semi-dry blotting (Bio-Rad Laboratories) onto a PVDF membrane. After the membrane was blocked with 5% skim milk in TBS buffer containing 0.1% Tween-20, it was incubated with an anti-LC3 antibody (Medical & Biological Laboratories) and an anti-ß-actin antibody (Medical & Biological Laboratories) diluted with Can Get Signal Immunoreaction Enhancer Solution (TOYOBO, Osaka, Japan). The membrane was washed with TBS buffer containing 0.1% Tween-20 and then incubated with an IgG-HRP conjugated antibody (Medical & Biological Laboratories). Following the membrane was washed again, developed using 0.56 mM 3, 3'-Diaminobenzidine and 0.006% H<sub>2</sub>O<sub>2</sub> in 50 mM Tris-HCl, pH7.6.

## Cell cycle analysis

Twenty-four hours after MK615 treatment, the cells were harvested and fixed with ethanol. The cells were then treated with 200  $\mu$ g/ml RNase A, stained with 10  $\mu$ g/ml propidium iodide, and analyzed on a FACS Caliber instrument using Cell Quest software (Nippon Becton Dickinson, Tokyo, Japan).

#### Quantitative real-time RT-PCR

*IL-8* mRNA expression was evaluated using quantitative real-time RT-PCR [17]. Briefly, 24 h after  $1 \times 10^5$  cells were plated in each well of a 6-well plate, the culture medium was replaced with 2 ml of growth medium containing MK615 at different concentrations. After 24 h, the cells were harvested, and total RNA was extracted using an RN easy mini kit (Qiagen, Chatworth, CA), and cDNA was synthesized from 2 µg of total RNA using the Super Script II First-Strand Synthesis kit and an oligo (dT) primer system (Invitrogen). An *IL-8* TaqMan probe (assay ID: Hs00174103\_m1) was purchased from Applied Biosystems (Tokyo, Japan). For quantitative analysis, the *TBP* gene was used as an internal reference to normalize the input cDNA. PCR was performed using a Gene Amp 7700 Sequence Detection System and its software (Applied Biosystems). The comparative Ct method was used to compute the relative expression values.

#### Enzyme-linked immunosorbent assay

Twenty-four hours after  $1 \times 10^5$  cells were plated in each well of a 6-well plate, the culture medium was replaced with 2 ml of growth medium containing MK615 at different concentrations. Forty-eight hours after MK615 treatment, the IL-8 protein concentration in the culture medium of H1792 cells was determined using an enzymelinked immunosorbent assay (ELISA) with a human IL-8 duo set kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

# Statistical analysis

Statistical analysis were performed using GraphPad Prism, version 5.0 for Mac OS X (GraphPad Software, San Diego, CA). A *P*<0.05 was considered statistically significant.

## Results

# The effect of MK615 on cell growth varies among NSCLC cell lines

We first evaluated the effect of MK615 on the *in vitro* cell growth of nine NSCLC cell lines and the HBEC4 immortalized bronchial epithelial cell line, which served as a non-cancerous control (Figure 2A). MK615 significantly inhibited the growth of the H661, H838, H1299, H157, H1395 and HCC827 cells at  $\geq 5 \mu$ l/ml and that of H1792 cells at  $\geq 1 \mu$ l/ml, whereas <10  $\mu$ l/ml MK615 did not affect HBEC4 cell growth. In contrast, some cell lines exhibited MK615 resistance; 10  $\mu$ l/ml MK615 significantly but weakly inhibited H441 cell growth, and a significant growth-inhibitory effect was not observed in H460 cells even at a 10  $\mu$ l/ml.

The proportions of viable cells after 48 h of MK615 treatment are summarized in Figure 2B and Table 1. The proportion of viable cells was significantly lower for H661, H838, H1299 and H157 cells treated with  $\geq 5 \ \mu/ml$  MK615, and the value was significantly higher for H441 and H460 cells treated with 10  $\ \mu/ml$  compared to the HBEC4 non-cancerous control. These results indicate that the majority of NSCLC cell lines are sensitive to MK615 and that the growth-inhibitory effect varies among cell lines.

# The MK615 cytotoxic effects involve autophagy induction and G0/G1 cell-cycle arrest in NSCLC cells

A previous study demonstrated that autophagy is induced by MK615 in colon cancer cells [5]. Therefore, to evaluate the mechanisms of MK615-mediated cell death in NSCLC cells, we next examined





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	Day 2					Day 4						
Cell line	5 µl/ml		10 µl/ml			5 µl/ml			10 µl/ml			
HBEC4	103.2	±	10.1	44.5	±	8.5	99.6	±	15.2	29.9	±	5.3
H661	38.2	±	9.2	0.0	±	0.0	23.3	±	3.8	0.0	±	0.0
H838	51.8	±	4.4	16.0	±	1.0	62.1	±	1.5	12.1	±	3.0
H1299	56.2	±	9.2	0.0	±	0.0	40.2	±	1.8	0.0	±	0.0
H157	54.5	±	6.2	23.1	±	6.6	47.9	±	11.5	14.2	±	5.3
H1395	77.3	±	4.9	40.8	±	7.9	74.9	±	5.1	29.6	±	4.6
H1792	82.7	±	4.2	48.5	±	11.6	73.8	±	4.7	36.3	±	7.1
HCC827	83.8	±	8.4	63.8	±	11.6	66.3	±	11.3	48.1	±	5.6
H441	93.8	±	11.7	67.8	±	11.4	77.4	±	10.9	56.9	±	12.9
H460	84.8	±	11.8	80.0	±	10.1	93.0	±	17.0	79.8	±	19.6

Table 1: The percentage of viable cells after MK615 treatment in nine NSCLC cell lines and the HBEC4 immortalized bronchial epithelial cell line.

whether autophagy is induced by the MK615 treatment using the H1299 and H157 cell lines because these lines are highly sensitive to the cytotoxic effect of MK615. To test this idea, we performed immunostaining of LC3, which is a yeast Apg8 mammalian homologue,



Figure 3: (A) Autophagosome formation by MK615 treatment (10 µl/ml) in the H1299 and H157 cell lines for 6 h. An increase of LC3 expression was clearly observed after 6 h treatment in both cell lines, as compared to the non-treatment control. (B) Western blot analysis of LC3 expression in MK615-treated (10 µl/ml) and untreated H1299 cells for 4 and 8 h. The ratios of the LC3-II/LC3-I levels were increased at 4 and 8 hMK615 post-treatment, as compared to the non-treatment control. **B**-Actin expression levels were used as a loading control.

		H157		H1299				
	G0/G1	S	G2/M	G0/G1	S	G2/M		
Non-treatment	50.3	16.8	32.9	48.8	20.3	30.9		
5 µl/ml	64.5	14.0	21.5	52.6	16.2	31.2		
10 µl/ml	63.4	15.5	21.1	55.6	13.8	30.6		

Table 2: Cell cycle distribution in MK615-treated H157 and H1299 cells.

for visualizing autophagosome formation because it is associated with autophagosomal membranes in its membrane-bound form [15,16]. An increase in LC3 expression was clearly observed in the H1299 and H157 cell lines after 6 hours of MK615 treatment, as compared to the non-treatment control (Figure 3A). The induction of autophagy was further confirmed by Western blot analysis showing that the ratios of the levels of LC3-II, which represents the autophagosome-associating form of LC3, to the levels of LC3-I were increased in MK615-treated H1299 cells, as compared to the levels in the untreated cells (Figure 3B). These results suggest that autophagy is involved in MK615-mediated cell death in NSCLC cells.

We further examined the MK615 effect on the cell cycle in H1299 and H157 cells. After  $\geq 5 \,\mu$ l/ml MK615 treatment for 24 h, there was an increase in the proportion of cells in the G0-G1 phase and a decrease in the S phase in both cell lines (Figure 4 & Table 2), suggesting that MK615 induces G0-G1 cell-cycle arrest in NSCLC cells.

# MK615 inhibits *IL-8* mRNA expression and protein production in NSCLC cells

In H1792 cells,  $\geq 1 \mu l/ml$  MK615 modestly inhibited cell growth (Figure 2A); however, the induction of autophagy was not obvious in this cell line (data not shown), suggesting that other MK615-mediated growth inhibition mechanisms may exist. We recently found that H1792 cells, which harbor *KRAS* mutations, highly express IL-8 through ERK-MAPK pathway activation and that IL-8 attenuation resulted in the growth inhibition of this cell line [17]. In addition, a

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previous study demonstrated that MK615 inhibits the LPS-induced activation of MAPK pathways in macrophage-like cells [11]. These findings prompted us to investigate whether MK615 treatment results in the down-regulation of IL-8 expression in H1792 cells. *IL-8* mRNA expression was significantly reduced by  $\geq 0.5 \,\mu$ l/ml MK615 treatment in a dose-dependent manner (Figure 5). Consistent with this result, we observed the dose-dependent reduction of IL-8 protein levels in H1792 cells (Figure 5). These results suggest that MK615 inhibits cell growth by down-regulating IL-8 expression in some NSCLC cells.

# Discussion

This is the first report to demonstrate the inhibitory effects of MK615 on *in vitro* cell growth and IL-8 expression in NSCLC cells. MK615 contains several triterpenoids including triterpene acids (e.g., oleanolic acid and ursolic acid) and phytosterols (e.g., ß-sitosterol) [9,18,19], which are widely present in natural foods and plants [20]. Given that these triterpenoids have been shown to have anti-inflammatory [21,22] and anti-tumor activities [23-26], some triterpenoids in MK615 appear



**Figure 5:** MK615mediates the down-regulation of IL-8 expression at the mRNA and protein levels in IL-8 overexpressing H1792 cells. The columns represent the mean ± SD from three independent experiments.\*, *P*<0.0001; \*\*\*, *P*<0.001 for comparisons of the non-treatment control (0 µl/ ml) by ANOVA using Bonferroni multiple comparison.

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to be the key substances relevant to the anti-tumor and anti-IL-8 effects in NSCLC cells.

While MK615 inhibited the in vitro cell growth of the majority of the NSCLC cell lines tested, the growth-inhibitory effects varied among the cell lines, and some cell lines exhibited MK615 resistance. Previous studies indicate that MK615 inhibits cell growth through the inhibition of the Aurora A and B kinases [8,10]. They indicate that the growth-inhibitory effect may depend on the expression level of the Aurora A and B kinases. More recently, Sakuraoka et al. [7] reported that MK615 down-regulates the expression of advanced glycation end-product (AGE)-receptor (RAGE), which in turn inhibits AGEstimulated cell growth in hepatocellular carcinoma cells, where RAGE is highly expressed [7]. These findings suggest that the MK615 growthinhibitory effects may depend on the endogenous expression levels of the Aurora A and B kinases and RAGE. Further studies are needed to verify whether the Aurora A and B kinases and RAGE contribute to MK615 sensitivity and to investigate whether there are other molecular mechanisms responsible for MK615 sensitivity in cancer cells.

In this study, we observed autophagosome formation in NSCLC cells early after MK615 treatment. Autophagy is a process of selfcannibalization, which is classified as type II programmed cell death [27]. Accumulating evidence highlight the essential role of autophagy in the regulation of cancer cell survival [28]. Autophagic cell death is morphologically characterized by the formation of cytoplasmic vacuoles, and this morphological change was observed in MK615treated breast cancer cells [6]. In addition, Mori et al. [5] reported that MK615 induces autophagy in colon cancer cells. These observations suggest that the induction of autophagy is one of the mechanisms of MK615-mediated cancer cell death. Of note, previous studies have demonstrated that several triterpenoids including ursolic acid induce autophagy in cancer cells [25,29-31]. Although the precise mechanisms of MK615-mediated autophagy induction remain unknown, some of the triterpenoids in MK615may play important roles in the autophagic cell death of cancer cells.

We found that MK615-treated cells accumulated in the G0-G1phase, and this was accompanied by a decrease of cells in the S phase in H1299 and H157 NSCLC cells, suggesting that MK615 has the ability to induce G0-G1arrest in NSCLC cells. MK615 contains oleanolic acid and ursolic acid, and both possibly induce G0-G1 cell cycle arrest. It has been shown that G0-G1 arrest is induced by oleanolic acid in osteosarcoma cells [32] and by ursolic acid in prostate cancer [25] and hepatocellular carcinoma cells [33]. In contrast, previous studies reported that MK615 induces G2-M arrest in breast [6], pancreatic [10] and esophageal cancers [10]. These inconsistent results may be due to the difference in the type of cancer cells.

Inflammation has been thought to play essential roles in tumor development and progression [34]. One of the most important cancerrelated inflammatory chemokines is the CXC chemokine interleukin-8 (IL-8), which serves as an angiogenic growth factor in several types of cancers including NSCLC [35]. We recently reported that *KRAS*mutant NSCLC cell lines, including H1792, overexpress IL-8 through ERK-MAPK pathway activation [17]. In this study, the treatment of H1792 cells with MK615 resulted in a significant reduction in the *IL-8* mRNA expression and protein production in a dose-dependent manner. Given that MK615 was shown to inhibit LPS-induced ERK phosphorylation [11], it is likely that MK615 inactivates the ERK- MAPK pathway, leading to the transcriptional down-regulation of IL-8 expression in NSCLC cells. Because IL-8 is a potent angiogenic factor, it is also likely that MK615 has the potential to suppress tumor angiogenesis in NSCLC cells.

In conclusion, we demonstrated that MK615 has multiple antitumor activities in NSCLC cells. Together with previous studies demonstrating the MK615 anti-tumor activities in various cancer types [4-10], our results strengthen the evidence indicating that MK615 is an anti-tumor agent. Further *in vivo* studies will be necessary to evaluate the therapeutic efficacy of MK615 in NSCLC.

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