



# Sensitization of 5-Fluorouracil-Resistant SNUC5 Colon Cancer Cells to Apoptosis by $\alpha$ -Mangostin

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## Abstract

5-fluorouracil (5-FU) is a chemotherapeutic agent commonly used for treatment of solid tumors, including colorectal cancer. However, chemoresistance against 5-fluorouracil (5-FU) often limits its success for chemotherapy and, therefore, finding out appropriate adjuvant(s) that might overcome chemoresistance against 5-FU bears a significant importance. In the present study, we have found that  $\alpha$ -mangostin can sensitize 5-FU-resistant SNUC5/5-FUR colon cancer cells to apoptosis. Exposure of  $\alpha$ -mangostin induced significant DNA damages and increased the intracellular 8-hydroxyguanosine (8-OH-G) and 4-hydroxynonenal (4-HNE) levels in SNUC5 and SNUC5/5-FUR cells. Western blot analysis illustrated that  $\alpha$ -mangostin-induced apoptosis was mediated by the activation of the extrinsic and intrinsic pathways in SNUC5/5-FUR cells. In particular, we observed that Fas receptor (FasR) level was lower in SNUC5/5-FUR cells, compared with SNUC5 cells and that silencing FasR attenuated  $\alpha$ -mangostin-mediated apoptosis in SNUC5/5-FUR cells. Together, our study illustrates that  $\alpha$ -mangostin might be an efficient apoptosis sensitizer that can overcome chemoresistance against 5-FU by activating apoptosis pathway.

**Key Words:** 5-fluorouracil (5-FU),  $\alpha$ -mangostin, Oxidative damages, Apoptosis

## INTRODUCTION

The global incidence of colorectal cancer continues to increase largely due to the growth and aging of population, combined with an adoption of cancer-causing behaviors such as smoking and physical inactivity. In 2012, it was reported that 103,170 new cases of colon cancer patients and 51,690 mortalities occurred in the United States (Siegel *et al.*, 2012). 5-fluorouracil (5-FU) is a chemotherapeutic agent that has been widely used for treatment of colorectal cancer patients. 5-FU is a uracil analog with a fluorine atom existing at C-5 position (Wohlhueter *et al.*, 1980). The anti-tumorigenic mechanisms of 5-FU are associated with the inhibition of thymidylate synthase and incorporation of 5-FU into RNA and DNA (Pinedo and Peters, 1988). However, 5-FU is also cytotoxic to normal cells and exhibits many undesirable side-effects. In addition, the ultimate response rate of colorectal patients against 5-FU is very poor, mostly due to the acquisition of 5-FU chemoresistance (Jones *et al.*, 1995).

Due to an increased concern regarding to chemoresistance against 5-FU, finding out new chemotherapeutic adjuvants

that can decrease chemoresistance against 5-FU has aroused a significant interest in the last decades. 5-FU is commonly administered to colorectal cancer patients in combination with other types of chemotherapeutic agents in the clinical setting (Longley *et al.*, 2003). In addition, colorectal cancer patients often adopt natural antioxidants or dietary supplements during the 5-FU chemotherapy based on the belief that they would exhibit beneficial effects. In fact, a combination of selected natural compounds were shown to increase the anti-tumorigenic effects of 5-FU by increasing its sensitivity in cancer cells (Sinha and Honey, 2012). In the present study, we have evaluated the cytotoxic effects of our in-house natural chemicals on the growth of 5-FU-resistant colorectal cancer cells, SNUC5/5-FUR cells and found that  $\alpha$ -mangostin can induce a significant apoptotic cell death.

## MATERIALS AND METHODS

### Reagents, antibodies and cell culture

Purified  $\alpha$ -mangostin was directly provided from Dr. Young-

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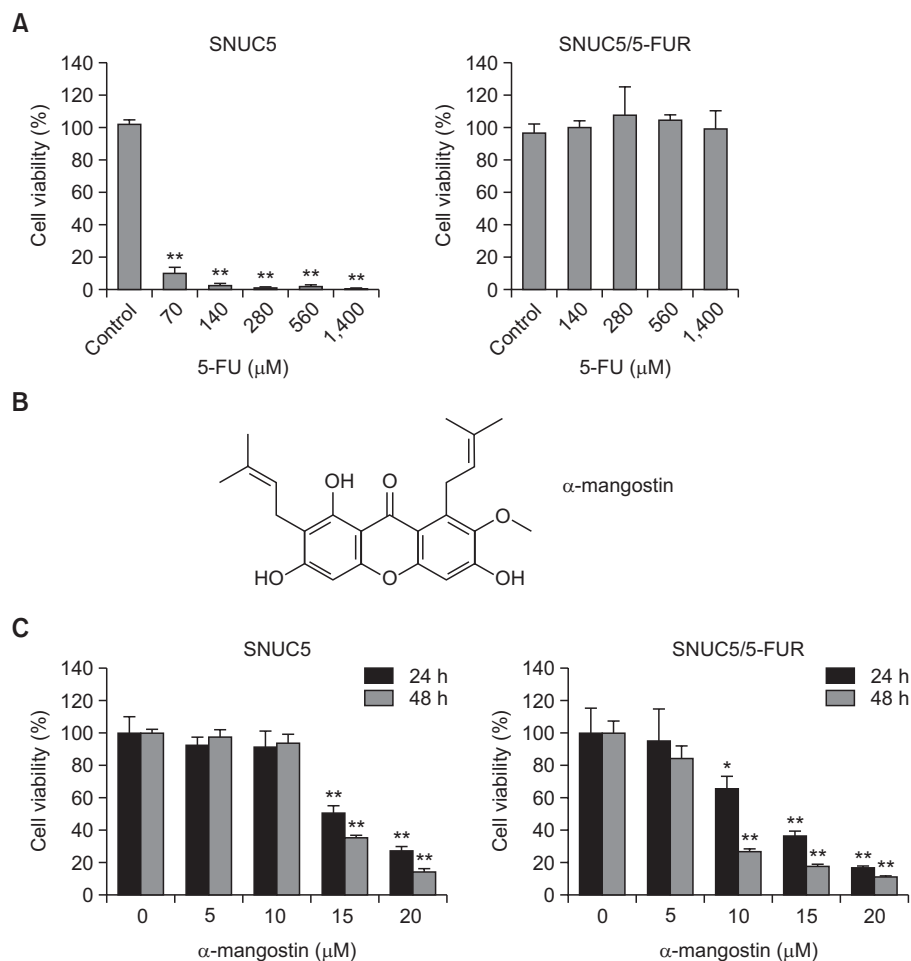
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**Fig. 1.** The induction of cell death in SNUC5 and SNUC5/5-FUR cells by  $\alpha$ -mangostin. (A) The cell viability of SNUC5 and SNUC5/5-FUR cells against 5-FU was examined by MTT assay. (B) Chemical structure of  $\alpha$ -mangostin. (C) MTT assay results illustrate a concentration-dependent cell death of SNUC5 and SNUC5/5-FUR cells by  $\alpha$ -mangostin. Asterisks indicate a statistical significance with \* $p$ <0.05 and \*\* $p$ <0.01, compared with the control group.

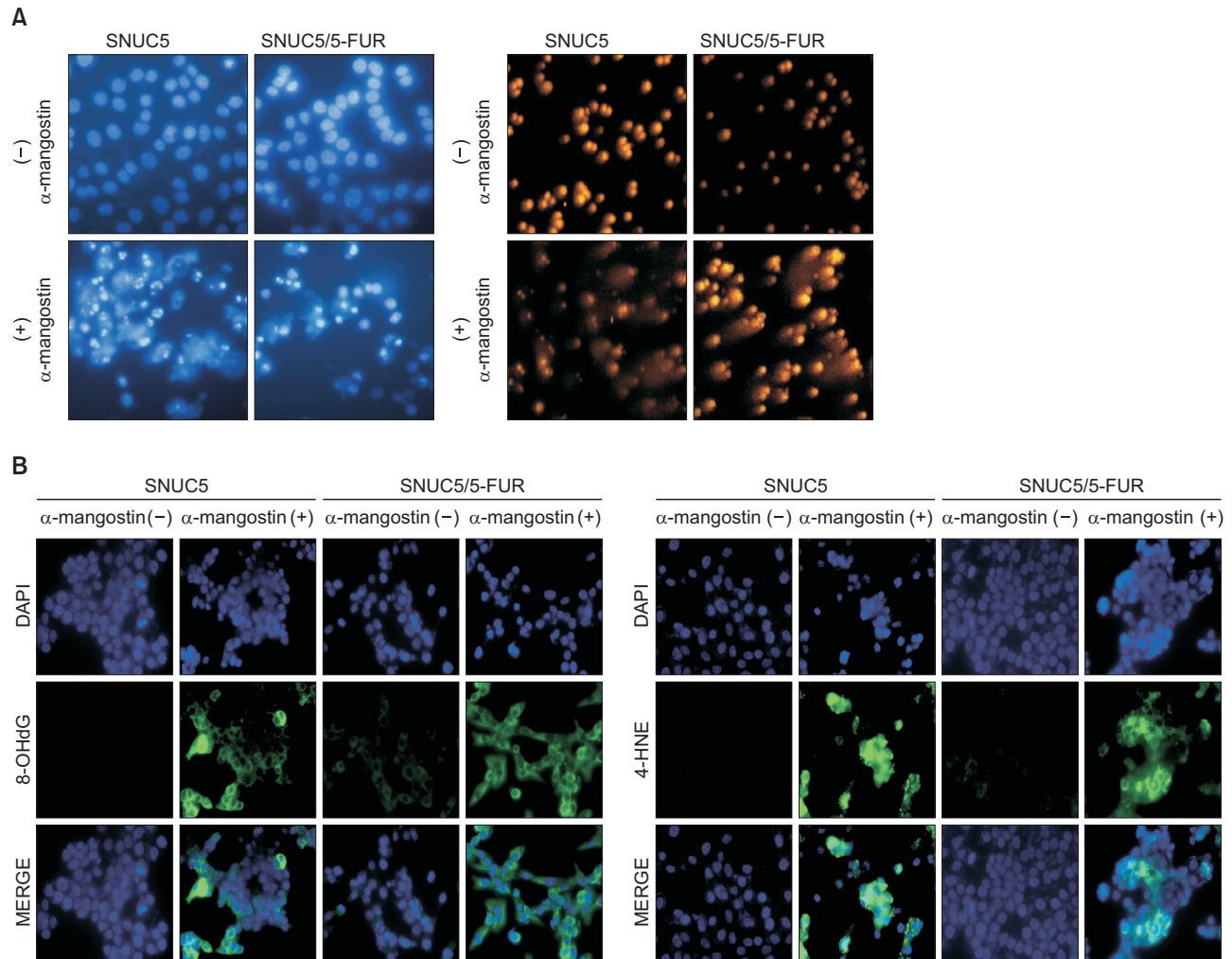
Won Chin (College of Pharmacy, Dongguk University, Goyang, Republic of Korea) and the integrity of  $\alpha$ -mangostin was confirmed by NMR spectroscopy. Pure  $\alpha$ -mangostin was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM as a stock solution. 5-FU, puromycin, MTT and lentiviral shRNAs plasmid against Fas receptor (FasR) were purchased from Sigma (St. Louis, MO, USA). Lentiviral helper vectors (pMD2.G and psPAX.2) were acquired from Addgene (Cambridge, MA, USA). RPMI-1640 media, fetal bovine serum (FBS), penicillin/streptomycin, and phosphate-buffered saline (PBS) were purchased from Welgene (Daegu, Republic of Korea). All apoptosis-related antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). SNUC5 and 293T cells were acquired from the Korean Cell Line Bank (Seoul, Republic of Korea). SNUC5/5-FUR cells were acquired from the Research Center for Resistant Cells of Chonun University (Gwangju, Republic of Korea). SNUC5 cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere of RPMI-1640 medium, containing 10% FBS and penicillin/streptomycin (100 unit/ml). SNUC5/5-FUR cells were grown in the same media of SNUC5 cells, supplemented with 140  $\mu$ M 5-FU in order to provide a constant chemoresistance.

### Cell viability assay

Cell viability was determined by MTT assay. SNUC5 and SNUC5/5-FUR cells were seeded at a density of  $3 \times 10^4$  cells in 96-well culture plates with 100  $\mu$ l RPMI 1640 media, 10% FBS and 1x penicillin/streptomycin (100 unit/ml). After an exposure to  $\alpha$ -mangostin, cells were washed with 1x PBS three times and 50  $\mu$ l MTT stock solution (2 mg/mg) was added into the each well, followed by an incubation for 4 h. After discarding the media, the resulting formazan crystals were dissolved in 150  $\mu$ l DMSO and the absorbance at 540 nm was measured by spectrophotometer. The percentage of cell viability was statistically analyzed by Student t-test.

### Hoechst 33342 staining and single cell gel electrophoresis (alkaline comet assay)

SNUC5 and SNUC5/5-FUR cells were seeded in 6 well plates at 70% confluence and exposed to 20  $\mu$ M  $\alpha$ -mangostin for 48 h. After fixation, the nuclear morphology of cells was evaluated by the cell-permeable Hoechst 33342 dye. The degree of oxidative cellular DNA damage, e.g. DNA fragmentation, was determined by the alkaline comet assay as described by Singh (2000).



**Fig. 2.** The induction of oxidative DNA and lipid damages in SNUC5/5-FUR cells occurs by  $\alpha$ -mangostin treatment. (A) Hoechst 33342 staining (Left Panel) and the alkaline comet assay (Right Panel) were conducted to visualize the genomic DNA integrity of SNUC5 and SNUC5/5-FUR cells after 20  $\mu$ M  $\alpha$ -mangostin for 24 h. (B) Immunofluorescence using antibodies against 8-OH-G and 4-HNE was conducted to visualize the oxidative damages in SNUC5 and SNUC5/5-FUR cells after 20  $\mu$ M  $\alpha$ -mangostin for 24 h.

**Immunofluorescence against 8-hydroxyguanosine (8-OH-G) and 4-hydroxynonal (4-HNE)**

In order to measure the changes of 8-hydroxyguanosine (8-OH-G) and 4-hydroxynonal (4-HNE) levels, SNUC5 and SNUC5/5-FUR cells grown on a slice glass were permeabilized by 4% paraformaldehyde. After washing with cold 1x PBS, cells were incubated with 1% bovine serum albumin (BSA) for 30 min and hybridized with primary antibodies against 8-OH-G (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 4-HNE (Abcam, Cambridge, MA, USA) overnight at 4°C. After washing with 1x PBS three times, the slides were probed with FITC-conjugated rabbit secondary antibody and the fluorescent images were obtained with a C2 confocal microscope (Nikon Korea, Seoul, Republic of Korea).

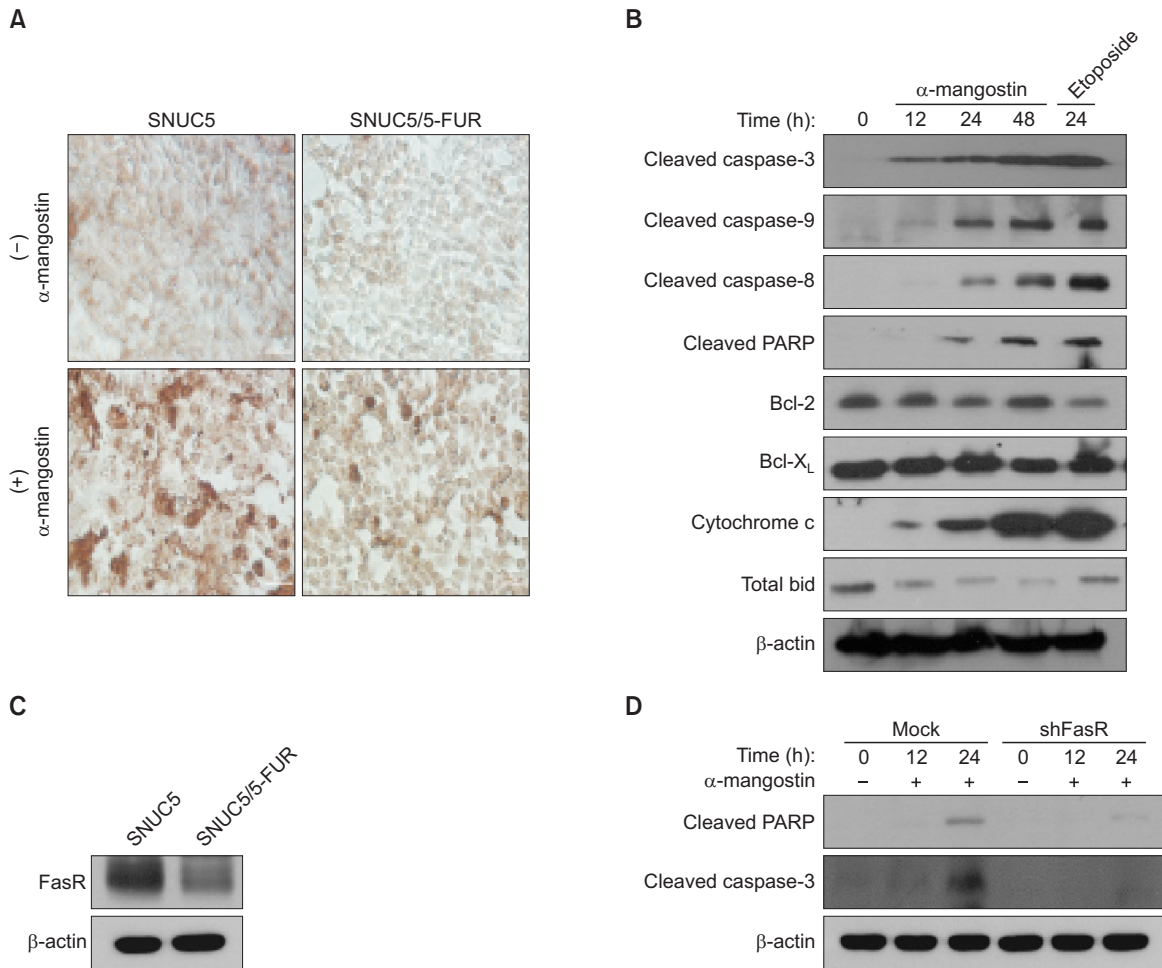
**TUNEL assay**

After an exposure of  $\alpha$ -mangostin to SNUC5 and SNUC5/5-FUR cells ( $7.5 \times 10^4$  cells on a cover glass), TUNEL assay was conducted using DeadEND™ fluorometric TUNEL system kit (Promega, Madison, WI, USA) as recommended by the manu-

facturer.

**Western blot analysis**

After appropriate treatment, cells were collected and washed three times with 1x phosphate-buffered saline (PBS). After centrifugation at 3,000 rpm for 5 min, cells were resuspended with 200  $\mu$ l RIPA buffer [50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate (DOC), 0.1% sodium dodecylsulfate (SDS), 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF)] and kept on ice for 30 min. Cells were centrifugation at 12,000 rpm for 10 min and the supernatant was collected for the measurement of protein concentration by BCA Protein Assay Kit (Thermo Fisher Scientific Inc, Waltham, MA, USA). Cell lysates were resolved and transferred onto PVDF membranes (BioRad, Hercules, CA, USA). Membranes were incubated in blocking buffer [5% skim milk/1x PBST (1x PBS with 0.1% Tween-20 solution)] for 1 h and hybridized with the appropriate primary antibodies in 1x PBS solution, containing 3% skim-milk overnight at 4°C. After washing three times with 1x



**Fig. 3.** Apoptotic induction of SNUC5/5-FUR cells by  $\alpha$ -mangostin. (A) TUNEL assay illustrates the induction of apoptosis in SNUC5 and SNUC5/5-FUR cells after 20  $\mu$ M  $\alpha$ -mangostin treatment for 24 h. (B) Western blot analysis illustrates that 20  $\mu$ M  $\alpha$ -mangostin activates the extrinsic and intrinsic apoptosis pathways in SNUC5/5-FUR cells. Etoposide (10  $\mu$ M) was included as a positive control. (C) Western blot assay results show that the expression of FasR was lower in SNUC5/5-FUR cells compared with that of SNUC5. (D) Lentiviral silencing of FasR attenuates  $\alpha$ -mangostin-induced apoptosis in SNUC5/5-FUR cells.

PBST solution, the membrane was hybridized with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature and washed 3 times with 1x PBST solution for 30 min. Membrane was finally visualized by using enhanced chemiluminescence (ECL) detection system. The total actin expression in the Western blot denotes an equal loading of samples.

#### Generation of stable cells by lentiviral transduction

Generation of stable knock-down SNUC5/5-FUR cells was conducted by lentiviral transduction. Briefly, 293T packaging cells were transfected with 3  $\mu$ g FasR shRNA construct together with 3  $\mu$ g pMD2.G and 3  $\mu$ g psPAX.2 lentiviral helper vectors, using JetPEI reagent (Polyplus-Transfection, NY, USA). After 72 h, viral supernatant was collected and filtered. SNUC5/5-FUR cells were then transduced with viral supernatant containing 10  $\mu$ g/ml polybrene for 12 h at 37°C and selected with 3  $\mu$ g/ml puromycin for 48 h.

## RESULTS

### Treatment of $\alpha$ -mangostin sensitizes SNUC5/5-FUR cells to 5-FU-mediated cell death

In the present study, we have attempted to find out novel natural compound(s) that might exhibit significant inhibitory effects on the growth of 5-FU resistant colon cancer cells. To this end, we have exposed SNUC5/5-FUR cells to individual chemicals from our in-house natural compound library and assessed the resulting cell viability by MTT assay. SNUC5/5-FUR cells were previously established from 5-FU susceptible SNUC5 colorectal cancer cells after a continuous exposure to 5-FU for six months (Kang *et al.*, 2014). The sensitivity of SNUC5 and SNUC5/5-FUR cells against 5-FU were examined and confirmed in our hands (Fig. 1A). Our results show that  $\alpha$ -mangostin (Fig. 1B) exhibited the most potent growth-inhibitory effects among natural chemicals (data not shown). Supporting this observation, an exposure of  $\alpha$ -mangostin suppressed the growth of both SNUC5 and SNUC5/5-FUR cells in a time- and concentration-dependent manner (Fig. 1B).



### Treatment of $\alpha$ -mangostin provides direct oxidative damages on SNUC5 and SNUC5/5-FU cells

We next suspected that the growth-inhibitory mechanism of  $\alpha$ -mangostin could be ascribed to the disruption of genomic integrity. Consistent with this idea,  $\alpha$ -mangostin caused significant changes in the nuclear morphology of SNUC5 and SNUC5/5-FUR cells, as visualized by Hoechst 33342 staining (Fig. 2A, left panel). The alkaline comet assay also showed that  $\alpha$ -mangostin induced substantial genomic DNA strand breakages in SNUC5 and SNUC5/5-FUR cells (Fig. 2A, right panel).

It is generally accepted that disruption of cellular integrity is largely attributable to the generation of intracellular reactive oxygen species (ROS), resulting in direct oxidative damages on intracellular macromolecules. To examine this hypothesis, we have exposed  $\alpha$ -mangostin to SNUC5 and SNUC5/5-FUR cells, and measured the resulting 8-OH-G and 4-HNE levels by immunofluorescence. As a result, we observed that  $\alpha$ -mangostin significantly increased the formation of 8-hydroxyguanosine (8-OH-G) and 4-hydroxynonenal (4-HNE) in SNUC5 and SNUC5/5-FUR cells (Fig. 2B). This result illustrates that  $\alpha$ -mangostin exhibits inhibitory effects on the growth of SNUC5 and SNUC5/5-FUR cells through the generation of intracellular oxidative damages.

### Silencing Fas receptor (FasR) attenuates the induction of apoptosis by $\alpha$ -mangostin in SNUC5/5-FUR cells

It is known that oxidative stresses in cells can lead to apoptosis if cellular damages are huge and unable to be properly repaired (McIlwain *et al.*, 2015). By conducting the TUNEL assay, we observed that  $\alpha$ -mangostin indeed caused apoptosis in SNUC5 and SNUC5/5-FUR cells (Fig. 3A). Previous studies have demonstrated that apoptosis can occur by the extrinsic and/or intrinsic pathway (Elmore, 2007). To identify which pathway(s)  $\alpha$ -mangostin employed to induce apoptosis in SNUC5/5-FUR cells, we conducted Western blot assay. Our results show that  $\alpha$ -mangostin induced the activation of Caspase-3, -8, -9 and PARP, together with a release of cytochrome c. In addition, we observed that a cleavage of total Bid occurred while the Bcl-2 and Bcl-X<sub>L</sub> levels were not affected. These results illustrate that  $\alpha$ -mangostin is an efficient inducer of apoptosis in SNUC5/5-FUR cells through the activation of extrinsic and intrinsic pathways (Fig. 3B). In subsequent experiments, we observed that Fas receptor (FasR) expression was much lower in SNUC5/5-FUR cells, compared with that of SNUC5 cells (Fig. 3C). Based on this observation, we next silenced the FasR expression by lentiviral transduction in SNUC5/5-FUR cells and exposed  $\alpha$ -mangostin. As a result, we observed that  $\alpha$ -mangostin failed to induce the activation of Caspase-3 and PARP in SNUC5/5-FUR cells when the FasR expression was silenced (Fig. 3D). These results illustrate that FasR activation is critical in  $\alpha$ -mangostin-induced apoptosis in SNUC5/5-FUR cells.

## DISCUSSION

Due to their roles in imparting damages on intracellular macromolecules, reactive oxygen species (ROS) are often regarded as major culprits for various pathogenesis, including cancer (Schieber and Chandel, 2014). Superoxide anion (O<sub>2</sub><sup>-</sup>), hydroxyl peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxyl radicals (OH<sup>•</sup>) are well-

known examples of ROS and they are formed as a result of aerobic metabolism. It is known that many chemotherapeutic agents, including 5-FU also exhibit cytotoxic effects on the growth of tumors through the generation of intracellular oxidative stress (Hwang *et al.*, 2007). Concurrent with this idea, we observed that  $\alpha$ -mangostin exhibited significant cytotoxic effects on the growth of SNUC5/5-FUR cells (Fig. 1C) through the generation of oxidative damages (Fig. 2B). This implies that oxidative stress is responsible for the growth inhibition of SNUC5/5-FUR cells by  $\alpha$ -mangostin.

We have observed that  $\alpha$ -mangostin induced a significant apoptosis in SNUC5/5-FUR cells (Fig. 3A). Our results showed that  $\alpha$ -mangostin induced the activation of Caspase-8 and Caspase-9 in SNUC5/5-FUR cells (Fig. 3B), both of which are critical executioners of the extrinsic and intrinsic pathways, respectively. Although the activation of both apoptotic pathways by  $\alpha$ -mangostin was clearly evident, it is notable that the Bcl-2 and Bcl-X<sub>L</sub> expressions were unaffected in this process (Fig. 3B), implying that protein(s) other than Bcl-2 and Bcl-X<sub>L</sub> might have contributed to the mitochondria-dependent apoptosis by  $\alpha$ -mangostin, leading to the release of cytochrome c and a subsequent Caspase-9 activation. We speculate that the cleavage of total Bid (Fig. 3B) could have possibly played a role in this event because the truncation of Bid, a member of the Bcl-2 family, can alternatively initiate a mitochondrial-dependent apoptosis (Kantari and Walczak, 2011). In addition, we also made following observations in the present study: (1) FasR expression was lower in SNUC5/5-FUR cells compared with that of SNUC5 cells (Fig. 3C) and (2) silencing the FasR expression in SNUC5/5-FUR cells blocked the induction of apoptosis by  $\alpha$ -mangostin (Fig. 3D). Therefore, future studies aimed at elucidating the molecular mechanisms how  $\alpha$ -mangostin-mediated FasR activation contributes to the total Bid cleavage and subsequent apoptotic events will enable us to better understand the in-depth cytotoxic mechanisms of  $\alpha$ -mangostin.

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