Effects of Mangostin on the Activity of Protein Kinase A and Expression of Estrogen Receptor in Human Breast Cancer Cell Line

Marselina Irasonia Tan and B. Noor Emma Sophianie

***Abstract*—** Mangostin is a xanthone derivative compound, contained in the fruit hull of mangosteen (*Garcinia mangostana* Linn) and has the potential as an anticancer agent. This compound has aromatic groups and some groups similar to estrogen. Previous research showed that mangostin inhibited cAMP phosphodiesterase activity, which is correlated with PKA (protein kinase A). The aim of this study was to observe the effects of mangostin on PKA activity and the expression of estrogen receptor (ER) in human breast cancer cell line MCF-7. MCF-7 cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS (fetal bovine serum) and incubated at 36.5°C, with a 90% humidity level and a 5% CO_2 level. MTT assay showed that mangostin had a cytotoxic effect on MCF-7 with the IC_{50} of 4.03 µg/mL. For the experiments, MCF-7 cells were cultured in SFDM (serum free defined medium) and divided into 5 groups: a control group; $M_{0.4}$: cells treated with mangostin 0.4 µg/mL; $M_{0.8}$: cells treated with mangostin 0.8 µg/mL; $M_{1.6}$: cells treated with mangostin 1.6 µg/mL; and $X_{0.8}$: cells treated with xanthone 0.8 µg/mL. After 48 hours, total protein from each group was isolated. The level of active PKA from each group was measured using ELISA method. The effect of mangostin on the presence of ER-protein was examined using Western blotting hybridization. Our results showed that mangostin induced MCF-7 cell death, presumably apoptosis. The ELISA result showed that mangostin increased PKA activity. The average active PKA level in the control, $M_{0.4}$, $M_{0.8}$, $M_{1.6}$ group and $X_{0.8}$ was 130.18 ng, 161.02ng, 159.79 ng, 185.31 ng, and 169.91 ng respectively. On the other side, mangostin downregulated the expression of estrogen receptor, which probably affected the proliferation activity of cells. In conclusion, it was shown that mangostin increased PKA activity level but decreased the expression of ER in human breast cancer cell line MCF-7, which furthermore reduced cell proliferation activity.

***Keywords*—**Mangostin, anticancer, protein kinase A, estrogen receptor.

I. Introduction

ALMOST one third of cancer patients in the USA suffer from breast cancer. In year 2000, it was recorded that 180,000 women suffered breast cancer and 40,000 patients died because of this disease[1]. Until now cancer has been treated through surgery, radiotherapy, and chemotherapy, which all produce side effects. Therefore, researches on finding an effective therapy are still conducted. Among these therapies, one approach is the use of natural products with anticancer activity.

Many tropical plants such as mangosteen (*Garcinia mangostana* Linn.) have any potential as therapeutic agents. The hull of mangosteen[2] contains secondary metabolites such as oxygenated and prenilated xanthone[3]. Among these, mangostin-α is a bioactive compound found in the mangosteen hull. This compound had antibacterial activity, especially towards *Staphylococcus aureus, Shigella dysenteriae, Shigella flexneri, Escherichia coli, Vibrio cholerae*, and *Helicobacter pyroli*[2]. Furthermore it had also the potential as anti-fungi, anti-inflammation, anti-oxidant, anti-tumor, anti-histamine, anti-malaria, and anti-proliferation for breast cancer cells SKBR3, and also induced apoptosis of human leukemia cell lines[2,3,4]. However, until now, the mechanism of anticancer activity of mangostin-α is still obscure.

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Other xanthone derivatives contained in the hull of mangosteen are mangostanol, α-mangostin, and β-mangostin (Fig 1.). These substances have an inhibitory effect on cAMP phosphodiesterase[5,6], and therefore might also have an effect on the activity of Protein Kinase A (PKA). PKA is one of important components in signal transduction and proliferation activity. PKA can also influence the expression of estrogen receptor[7]. Until now the effect of mangostin on PKA activity and estrogen receptor in breast cancer cell line MCF7 is still unclear. In this study, we observed the effect of mangostin on the PKA activity and estrogen receptor in breast cancer cell, MCF7. Our previous study using MTT assay showed that mangostin has a cytotoxic effect with IC_{50} of 4.03 µg/mL (~ 4 µg/mL).

Mangostin induced apoptosis through the inhibition of Ca^{2+}ATPase activity[8]. Additionally, mangostin and other xanthone derivatives induced the release of cytochrome c from the mitochondria in PC12 cells, which promoted apoptosis. In leukemia cell lines HL60, K562, NB4 and U937, and breast cancer cell SKBR3, mangostin induced apoptosis by increasing caspase 3[2,3].

The aim of this research study was to evaluate the effects of mangostin on the activity of PKA and the expression of

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estrogen receptor in breast cancer cell line.

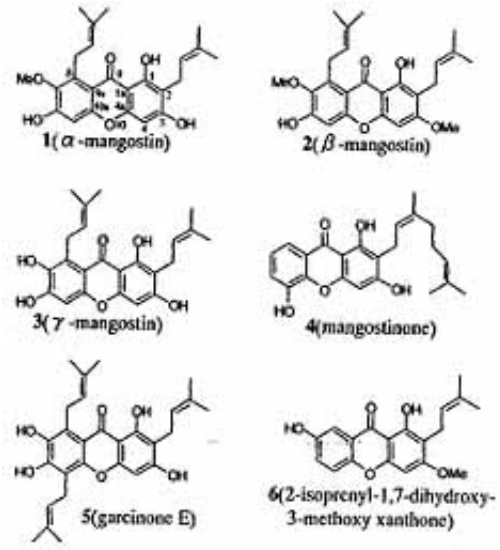

Fig 1. Bioactive compounds in mangosteen *Garcinia mangostana* Linn.)[3]

II. METHODOLOGY

A. Materials

Breast cancer cell line MCF-7 was used in this study. Cells were cultured in DMEM medium. Mangostin was purchased from Indofine Chemical Company, whereas xanthone was purchased from SIGMA.

B. Methods

1) Cell culture

Breast cancer cells were cultured in DMEM with 10% FBS in 25 cm^2culture flasks and incubated at 36.5^oC, with 90% humidity level and 5% CO_2 level. In the preliminary study, MTT Assay was performed to find out the 50% inhibition concentration (IC_{50}) of mangostin and this concentration is used for this study.

2) Cell treatment with mangostin

MCF-7 cells were cultured in 6-well multiwell plates. On the first day, 500,000 cells were cultured with a culture medium of 2% FBS. After that, cells were cultured with SFDM (containing putrescine, hydrocortisone, transferin, insulin and sodium selenite) for 48 hours. Cells were treated with mangostin with the concentrations of 0.4 µg/mL ($M_{0.4}$), 0.8 µg/mL ($M_{0.8}$), 1.6 µg/mL ($M_{1.6}$) respectively. The effect of mangostin was compared to the effect of xanthone (as the positive control) with the concentration of 0.8 µg/mL ($X_{0.8}$). As control (K), cells were treated with 0.02% ethanol, the solvent of mangostin. Medium was exchanged each day. Cell morphology was observed at 0, 24 and 48 hours incubation time using phase contrast microscope. After 48 hours cultivation, total cell protein was isolated and the concentration was measured using Bradford method by spectrophotometer at 570 nm.

3) PKA activity

Active Protein Kinase A was measured using *PKA Kinase Activity Assay Kit* (Stressgen Bioreagent) based on ELISA method. Activity of the enzyme was determined according to the protocol of the kit. Detection of active PKA was conducted using a microplate reader at 450 nm.

4) Electrophoresis and Western blot analysis

To detect estrogen receptor, total protein from each group was electrophoresized using SDS PAGE 10% and then the proteins in the gel were transfered to a PVDF membrane. Estrogen receptor was observed using Western blot analysis. To detect the Estrogen receptor, *rabbit monoclonal antibody Estrogen Receptor (SP1)* from Neo Marker was used. Detection of estrogen receptor was performed based on ECL system using *Western Blot Kit* (Zymed Lab Inc). Furthermore, the PVDF membrane was exposed to a luminescence film. Protein band density was analyzed using Scion image.

III. RESULT AND DISCUSSION

A. Effects of mangostin on breast cancer cell MCF-7

MCF-7 cell morphology was observed on the 0, 24, and 48 hours incubation time of mangostin/xanthone treatment. MCF-7 cells treated for 48 hours with either mangostin or xanthone had similar epitheloid cell morphologies with the control group (Figure 2). The higher the concentration of mangostin used, the greater the number of cells that died. We supposed that mangostin and xanthone could diffuse into the cancer cells due to their hidrophobic characteristic, which furthermore cause cell death, probably apoptosis. Mangostin caused apoptosis in leukemia cells, HL-60 and breast cancer cells, SKBR-3[2,3]. We suggested that cell death in MCF-4 was stimulated by the inhibition of Ca^{2+}ATPase activity in the endoplasmic reticulum. Mangostin inhibited Ca^{2+}ATPase, and consequently blocked the release of calcium ions from the endoplasmic reticulum to the cytoplasm[8].

B. Effect of mangostin on the activity of protein kinase A

Protein kinase A activity in MCF-7 cells was measured using ELISA (Fig 3). The data were analyzed statistically using one way variance analysis (ANOVA). Statistical analysis showed that there was no significant difference in Protein Kinase A activity among groups (Fig 3), however, activated PKA tended to increase in cells treated with mangostin and xanthone (Fig 3 and 4). The higher the concentration of mangostin, the greater the number of protein kinase A activated.

In the control group, active PKA was 130.18 ng. Cells treated with mangostin 0.4 µg/mL ($M_{0.4}$) and 0.8 µg/mL ($M_{0.8}$) exhibited similar active PKA levels, i.e., 161.02 ng and 159.79 ng respectively. Active PKA increased by 23.69% in cells treated with mangostin 0.4 µg/mL ($M_{0.4}$), and by 22.75% in cells treated with mangostin 0.8 µg/mL ($M_{0.8}$). This showed that mangostin with concentrations of 0.4 µg/mL and 0.8

µg/mL had a similar effect on PKA activity in MCF-7. Cells treated with mangostin 1.6 µg/mL ($M_{1.6}$) increased their active PKA by 42.35% (185.31 ng). The increase of active PKA in cells treated with xanthone ($X_{0.8}$) was 30.51% (169.91ng) (Fig 4).

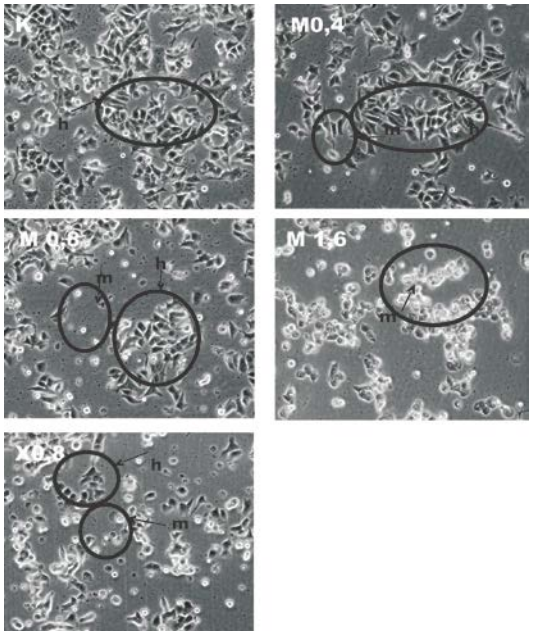

Fig 2. Breast cancer cell MCF-7 after mangostin treatment (magnification 100X). K: control group; $M_{0.4}$: MCF-7 treated with mangostin 0.4 µg/mL; $M_{0.8}$: MCF-7 treated with mangostin 0.8 µg/mL; $M_{1.6}$: MCF-7 treated with mangostin 1.6 µg/mL; $X_{0.8}$: MCF-7 treated with xanthone 0.8 µg/m. L.h: living cells, m: dead cells.

A boxplot diagram for active PKA (Fig 3) showed that in groups of cells treated with mangostin 0.8 µg/mL ($M_{0.8}$) and 1.6 µg/mL ($M_{1.6}$), certain groups of cells exhibited a very high concentration of active PKA. This might have resulted from a variety of cell cycle conditions in the treated cells, which might then have an effect on cell response to mangostin or xanthone.

Increase of active PKA in cells treated with mangostin or xanthone probably resulted from an increase of cAMP concentration in the cells. This could occur through two pathways, i.e., the increase of adenilil cyclase activity as an enzyme for cAMP synthesis or through the inhibition of cAMP phosphodiesterase as an enzyme for cAMP hydrolysis[9].

We suggest that the increase of active PKA in MCF-7 was the cause of inhibition of cAMP phosphodiesterase. Xanthone derivatives such as mangostanol, α-mangostin, and β-mangostin derived from mangosteen (*Garcinia mangostana* Linn.) had an inhibitory effect on cAMP phosphodiesterase[5]. The mechanism by which they inhibit cAMP phosphodiesterase, is still unclear.

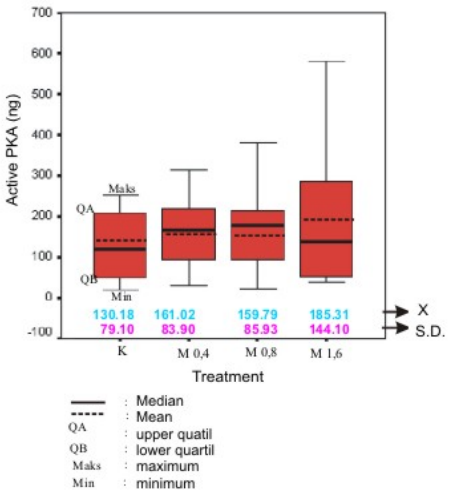

Fig 3.Boxplot diagram of active PKA in control and treatment groups. K: control group. $M_{0,4}$: MCF-7 cells treated with mangostin 0.4 µg/mL; $M_{0,8}$: MCF-7 cells treated with mangostin 0.8 µg/mL; $M_{1,6}$: MCF-7 cells treated with mangostin 1.6 µg/mL.

The inhibitory effect of mangostin on cAMP phosphodiesterase could increase cAMP concentration in MCF-7. A high concentration of cAMP induced the activity of PKA. Similarly, in our study we observed that higher mangostin concentration resulted in higher inhibition of cAMP phosphodiesterase activity in MCF-7; consequently, this could increase PKA activity[6].

The increase in PKA activity could influence several factors in cells, such as CREB (*cAMP response element binding*) activation[10], inhibition of BAD activity (one of proapoptosis protein)[11], inhibition of MAPK pathway[9,12], and estrogen receptor transactivation[13]. Increase of CREB activation and inhibition of BAD activity could increase cell proliferation, whereas inhibition of MAPK pathway and RE transactivation could reduce proliferation activity. Our microscopy observation results (Fig 2) suggest that the increase of PKA induced cell death.

Inhibition of MAPK pathway by high concentrations of PKA could reduce proliferation activity of MCF-7 cells. We suggested that PKA might phosphorylate Raf which bind to Ras and trigger inhibition of MEK1/2 and ERK1/2 phosphorylation (Fig 5). Inhibition of MAPK pathway could reduce proliferation activity. Inhibition effects of proliferation due to high PKA could be related to the declining transactivation activity of estrogen receptor, which is important as transcription factor for genes which have ERE (*Estrogen binding element*) and play a role in cell proliferation (Fig 5). The decrease of this gene expression was affected by PKA activity, which could phosphorylate serine 236 in CII zinc finger of DNA binding domain of ER. Phosphorylation in this position could inhibit RE monomer binding to ERE[13]. Phosphate molecules will insert into the ER monomer and disturb electrostatic force between the monomers, so that ER dimer cannot be formed. Moreover, phosphate molecules inhibit the interaction of water molecules which link the

surface of both of the ER monomers (Fig 5). Water molecules can bridge contact between serine 236 in DBD molecules and methionine of another DBD.

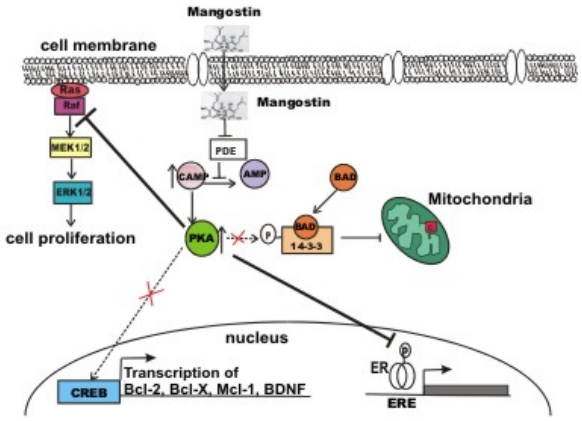

Fig 5. Hypothesis on the effect of the increase of protein kinase activity due to mangostin in MCF-7. High PKA activity inhibit MAPK pathway, specifically to MEK1/2 and ERK1/2. Due to PKA phosphorylation, ER transactivation on genes which have ERE was inhibited.

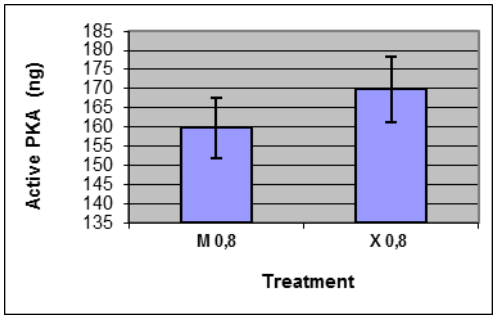

Fig 6. Active PKA in cells treated with mangostin 0.8 µg/mL (M 0,8) and xanthone 0.8 µg/mL (X0,8)

Statistical analysis on active PKA showed that there was a significant difference between cells treated with xanthone 0.8 µg/mL and mangostin 0.8 µg/mL (Fig 6). Active PKA in cells treated with xanthone 0.8 µg/mL was higher than in cells treated with mangostin 0.8 µg/mL. This difference might be related to the side chain differences (R group) in mangostin. Mangostin is a xanthone derivative, which has an R Group hydroxyl at positions C1, C3 and C6; methoxy group at position C7 and 3-methyl-2 butenyl at C2 and C8. These R groups of xanthones determine the effect of mangostin or xanthone derivatives on PKA. The result could be due to the difference in R groups between mangostin and xanthone. R groups in xanthones could influence the increase of PKA effectively. Until now it is still unclear how these R groups regulate the effect of mangostin on PKA. Several R groups have been suggested to affect the anticancer activity of mangostin. Xanthone derivatives with a hydroxyl group on C1 and C3 and a methyl group on C2 had a significant effect on MCF cell growth[14].

C. Effect of mangostin on the expression of estrogen receptor

The effect of mangostin on the expression of ER was observed using western blot analysis. Fig 7 shows that the density of ER protein in the control group was similar to cells treated with mangostin 0.4 µg/mL. ER protein band in cells treated with mangostin 0.8 µg/mL were very thin, whereas bands were not expressed in cells treated with mangostin 1.6 µg/mL and xanthone 0.8 µg/mL. Based on this result, we concluded that mangostin reduced the expression of ER protein

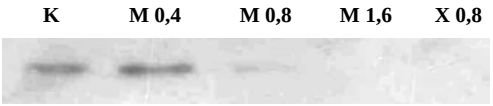

Fig 7. Autoradiogram of western blot analysis on the expression of ER in MCF-7 cells treated with mangostin with concentration of 0.4 µg/mL (M0,4); 0.8 µg/mL (M0,8); 1.6 µg/mL (M1,6) and with xanthone with concentration of 0.8 µg/mL (X0,8). K is control group

Mangostin with concentration of 0.4 µg/mL appeared not to affect the expression of ER compared to control. In cells treated with mangostin 0.8 µg/mL, the expression of ER was clearly lower compared to cells treated with 0.4 µg/mL, although both treatments showed no significant differences in active PKA. This showed that downregulation of ER in MCF-7 treated with 0.8µg/mL was not correlated with the increase in PKA activity. This could be a direct effect of mangostin on the expression of ER. Reduced ER gene expression on cells treated with mangostin 0.8 µg/mL might be related to the characteristic of mangostin. Mangostin could interact antagonistically to estrogen. Antagonistic substances to estrogen could activate proteosom 26S which then could degrade the ER protein complex[7].

In cells treated with mangostin 1.6 µg/mL and xanthone 0.8 µg/mL, reduction in ER gene expression was probably related to the activation of proteosome 26S. Additionally it might also be inhibited by the high activity of PKA. PKA could inhibit MAPK pathway, which furthermore affected the inhibition of ERBF-1 (*Estrogen receptor B factor-1*) as a transcription factor for the ER gene[15].

From this research study, we conclude that mangostin reduced proliferation activity of MCF-7 cells and induced cell death through the inhibition of MAPK pathway and the expression of ER. Decrease in proliferation of cell activity resulted from high activity of PKA in MCF-7 treated with mangostin.

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