Original article

The naturally occurring xanthone α-mangostin induces ROS-mediated cytotoxicity in non-small scale lung cancer cells

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ABSTRACT

Small cell lung cancer (NSCLC) accounts for 85% of total deaths globally, and recent studies indicate the increasing risks of NSCLC in China and South Asian countries. Hence, development of new therapeutics against NSCLC has been a major concern. α-Mangostin, a naturally occurring xanthone, found abundantly in pericarps of mangosteen fruit is well known for its medicinal importance. The anticancer properties of α-mangostin against several types of cancer are also well documented. But the mechanism of action of α-mangostin against lung cancer is not well understood and requires further investigation. Therefore in the present study, we explored the therapeutic potential of α-mangostin against A549 cells. Treatment of A549 cells with α-mangostin resulted in a dose-dependent loss of cell viability, while the non-malignant cells such as hPBMC and WI-38 remained unaffected. Further we observed that the ROS plays an important role in α-mangostin -induced apoptosis in A549 cells, and administration of N-acetyl cysteine significantly abrogates α-mangostin -mediated cytotoxicity in lung cancer cells. Overall, α-mangostin induces ROS-mediated cytotoxicity in NSCLC cells.

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1. Introduction

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. Not all tumors are cancerous; benign tumors do not spread to other parts of the body. Possible signs and symptoms include a lump, abnormal bleeding, prolonged cough, unexplained weight loss and a change in bowel movements. While these symptoms may indicate cancer, they may have other causes. Over 100 types of cancers affect humans. Lung cancer is responsible for most number of cancer- related deaths worldwide, and among the cancer affected patients, non-small cell lung cancer (NSCLC) accounts for 85% of the total death percentage, while small cell lung cancer (SCLC) is responsible for only 15% of mortality (Jemal et al., 2005; Smith et al., 2009; Yang et al., 2015). Mainly the poor prognosis,

accelerated metastasis and high rate of tumor recurrence are responsible for reduced survival rate and high lethality of NSCLC (Chan et al., 2002). Although the frequency of lung cancer is not very significant in the developed countries, recent epidemiological studies indicate that incidence of NSCLC had significantly increased in the Asian countries including China (Zhou, 2014; Fan et al., 2015). According to National Central Cancer Registry (NCCR) in 2010, the estimated lung cancer incidence in China was 46.08 per 100,000 populations, and over 600,000 patients were newly diagnosed. Hence lung cancer has become the major concern in China, leading to a high mortality rate of 37.00 per 100,000 populations. Although the platinum-based chemotherapy, e.g. cisplatin, is used for the treatment of advanced NSCLC patients (Gridelli and Sacco, 2016), but the acquisition of chemoresistance has significantly resulted in the poor survival rate of the patients (Jiang et al., 2016). Hence development of novel therapeutic agents against NSCLC is an urgent need for the improved treatment of the disease.

Mangosteen Linn (Garcinia mangostana) is a type of fruit mainly a grows in the Asian counties such as Thailand, Philippines, Sri Lanka, Malaysia, India and Myanmar, which have a significant medical importance, due to its usage in the treatment of trauma, abdominal pain, skin infection, dysentery and also wound-

healing properties (Peres et al., 2000) α-mangostin is a naturally occurring xanthone, found abundantly in pericarps of mangosteen fruits, which has diverse biological importance such as anti-inflammatory, antifungal, anti-tumor, antiparasitic, antioxidant, cardio-protective, and antibacterial properties to name a few (Jindarat, 2014). The anticancer properties of α-mangostin against various carcinomas are well documented (Kumazaki et al., 2015; Verma et al., 2016; Xia et al., 2016a, 2016b). But there are not much reports on the effect of α-mangostin on lung cancer cells. Hence in the present study we have investigated the effect of α-mangostin on NSCLC cells A549 and also on the noncancerous cells such as lung fibroblasts WI-38 cells and human peripheral blood mononuclear cells (PBMC).

2. Materials and methods

2.1. Cell culture and maintenance

Human lung fibroblast cells WI-38 and non-small cell lung cancer cells A549 were cultured in DMEM complete medium supplemented with 10% FBS. Human Peripheral Blood mononuclear cells (hPBMC) were harvested from the blood samples of the healthy donors. hPBMC were isolated by density gradient centrifugation technique, using Ficoll-Hypaque (1:1) (Histopaque 1077, Sigma Aldrich-USA), following the published protocol (Bhattacharya et al., 2013).

2.2. MTT assay

Cultured WI-38, A549 and hPBMC were seeded at a density of 1×10^4 cells/ml, grown to ~80% confluency and treated with α-mangostin (0–50 μM) for 24 h. After treatment, cellular viability was monitored by MTT assay.

2.3. Determination of apoptosis by FACS

Induction of apoptosis in A-MANGOSTIN -treated A549 cells was monitored by AnnexinV/PI double staining, using BD Fluorescence Activated Cell Sorter (ARIA II). Cultured A549 cells (1×10^4 cells/ml) were treated with varying concentrations of α-mangostin (0–10 μM) for 24 h. Induction of apoptosis was determined by flow cytometry using Annexin V-FITC apoptosis kit (Cayman Chemicals) following the protocol supplied by the manufacturers.

2.4. Determination of migration by Boyden chamber assay

Cultured A549 cells (1×10^4 cells/ml) were treated with varying concentrations of α-mangostin (0–10 μM) for 24 h and approximately 1×10^3 cells from each treatment groups were placed in the upper portion of the Boyden chamber, containing serum free medium. Cells, which invaded to the lower surface of the membrane, containing FBS supplemented DMEM, were then stained with crystal violet. The crystal violet complex thus formed, was dissolved in 10% acetic acid, and the absorbance was measured at 600 nm. The absorbance is directly proportional to the extent of migration.

2.5. Determination of ROS by DCF-DA staining

Cultured A549 cells (1×10^4 cells/ml) treated with varying concentrations of α-mangostin (0–10 μM) for 24 h, incubated with 25 μM 2′,7′- dichlorodihydrofluorescin diacetate (DCFH-DA) for 30 min at 37 °C and the images were taken by a confocal microscope (Olympus FluoView-1000). Fluorescence intensities

were calculated from approximately 1000 cells taken from different fields.

2.6. Estimation of antioxidant enzymes

For these assays A549 cells (untreated and treated with α-mangostin) were collected, resuspended in 200 μl of assay buffers, sonicated and centrifuged at 15,000 rpm for 20 min at 4 °C. The supernatant was collected and total protein was estimated by Bradford method. Catalase activity was measured according to protocol reported by Seizer& Beer in 1952 (Beers and Sizer, 1952). Briefly, 20 μl of the sample was added to 100 μl of assay buffer [50 mM potassium phosphate buffer (pH-7)] in a quartz cuvette to which 180 μl of 30 mM H_2O_2 was added. Decomposition of H_2O_2 was monitored spectrophotometrically at 240 nm for 4 min at room temperature. For each samples the catalase activity was measured by calculating μmol of H_2O_2 consumed per minute per milligram of protein.

For determination of cellular glutathione peroxidase (GPx) activity, 10 μl of sample was added to 170 μl of the assay buffer [50 mM potassium phosphate buffer with 1 mM EDTA and 2 mM sodium azide, pH-7], 30 μl of GSH (10 mM), 30 μl of glutathione reductase (2.4 U/ml) and 30 μl of 1.5 mM NADPH (dissolved in 0.1% sodium bicarbonate) in a quartz cuvette. The whole mixture was incubated at 37 °C for 10 min and the reaction is started by adding 30 μl of 2 mM H_2O_2. The consumption of NADPH was monitored spectrophotometrically at 340 nm at 37 °C for 10 min and the specific activity was calculated from nanomoles of NADPH consumed per minute per mg of protein (Das et al., 2012).

For the determination of total GSH, the cell lysates were mixed with 10% TCA (1:1 volume) and centrifuged at 150,000 RPM at 4 °C for 5 min, to eliminate the cellular proteins. This step was repeated and the resultant supernatant was checked for the residual proteins by Bradford method. Finally to the protein free supernatant, 1 mm of DTNB was added in a quartz cuvette Formation of TNB was monitored spectrophotometrically at 412 nm for 10 min. A standard curve for GSH was prepared by using known concentration of GSH (0.1–50 μM) and their respective absorbances at 412 nm, and the total GSH was calculated from the standard curve (Forman et al., 2009).

2.7. Western blot analysis

Cultured A549 cells (1×10^4 cells/ml) were treated with varying concentrations of α-mangostin (0–10 μM) for 24 h and western blots were performed for the detection of Bax and Bcl-2 proteins. The primary antibodies e.g. mouse monoclonal anti- Bcl-2 antibody (sc-7382) and rabbit polyclonal anti-Bax antibody (sc-493) were purchased from Santa Cruz (USA).

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Software. All results are expressed as the mean ± SD. Statistical significance was determined by one way Anova using Newman-Keuls Multiple Comparison Test.

3. Results

3.1. α-Mangostin induces loss of cell viability in NSCLC cells

The cytotoxic effects of α-mangostin on NSCLC cells A549 and nonmalignant WI-38 and hPBMC were determined by MTT assay. α-Mangostin -treatment resulted in a dose-dependent loss of cell viability in A549 cells after 24 h of treatment ($IC_{50} \sim 10$ μM)
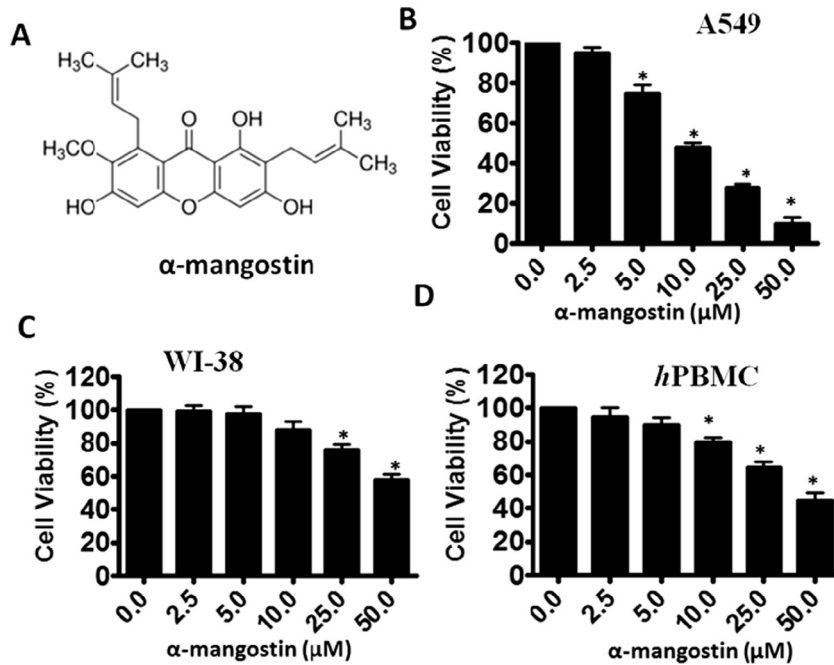
Fig. 1. Effect of xanthone α-mangostin on cell viability in NSCLC cells.

(Fig. 1). But interestingly, negligible cytotoxicity were observed in normal lung fibroblasts WI-38 and also human PBMC, with the respective IC_{50} concentrations being observed at around 50 μM α-mangostin, after 24 h of treatment.

3.2. α-Mangostin -treatment induces apoptosis in NSCLC cells via modulation of Bax/Bcl-2 ratio

α-Mangostin -treatment of A549 cells resulted in the significant induction of apoptosis in a dose-dependent fashion, as determined by annexin V/PI staining. Treatment of A549 cells with 5 μM α-mangostin, increased the apoptotic population to 18%, while in

the presence of 10 μM α-mangostin, 38% of the cells were found to be apoptotic (Fig. 2A). Moreover, we also observed that, Bax/Bcl-2 ratio was also increased in α-mangostin –treated A549 cells, confirming the involvement of apoptosis (Fig. 2B).

3.3. α-Mangostin -treatment inhibits the migratory activity of NSCLC cells

The migratory properties of A549 cells in the absence and presence of α-mangostin was accessed by Boyden chamber assay. α-Mangostin -treatment of A549 cells resulted in the inhibition of migratory properties in a dose-dependent fashion (Fig. 2C).
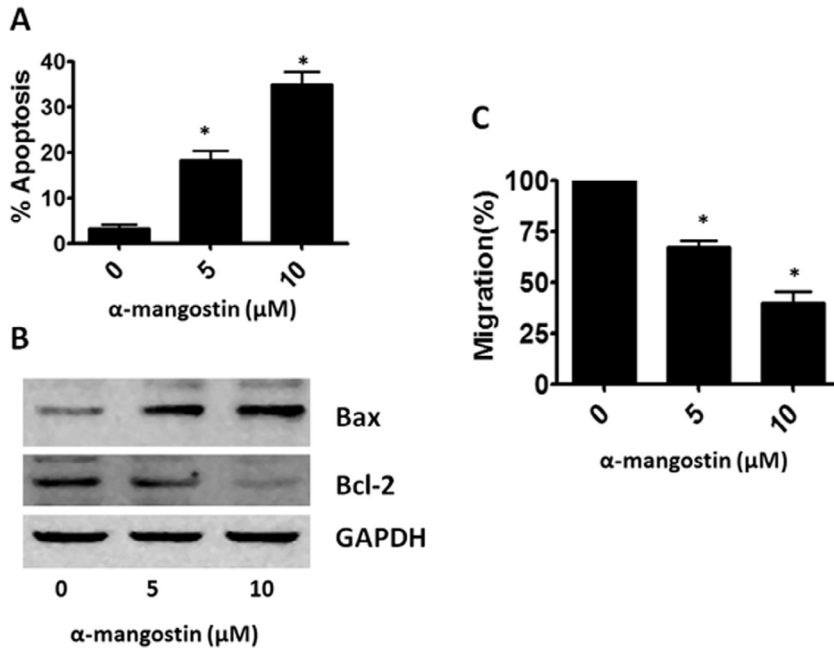

Fig. 2. Different concentration of xanthone α-mangostin treatment induces apoptosis in NSCLC cells via modulation of Bax/Bcl-2 ratio.

When treated with 5 µM α-mangostin, cellular migration was found to be inhibited by almost 33%, and in the presence of 10 µM α-mangostin, migration was inhibited by 60%.

3.4. α-Mangostin -treatment resulted in ROS-generation in NSCLC cells

α-Mangostin was known to induce ROS generation in several cancer cells, but there was no report of α-mangostin -induced ROS generation in NSCLC cells. Hence we investigated whether ROS is involved in α-mangostin -mediated cytotoxicity in A549 cells. We observed that, DCF-fluorescence, an indicator of ROS accumulation was increased in α-mangostin -treated cells in a dose-dependent fashion (Fig. 3). In the presence of 10 µM α-mangostin, ROS levels were increased by ~6-folds compared to the untreated cells (Fig. 3B).

3.5. α-Mangostin -treatment resulted in the down regulation of antioxidant machinery of NSCLC cells

After confirming the involvement of ROS in α-mangostin -mediated cytotoxicity in A549 cells we further monitored the status of different antioxidant markers such as catalase, glutathione peroxidase (GPx) and reduced glutathione (GSH). Thymoquinone was reported to have the unique property of modulating both pro-oxidant as well as antioxidant machinery in cancer cells. We observed the activity levels of the antioxidant enzymes like catalase and GPx were enhanced in the presence of low α-mangostin -doses, while at IC50 or near IC50 concentrations, the activity levels of these enzymes as well as the total GSH-levels were significantly decreased (Fig. 4).
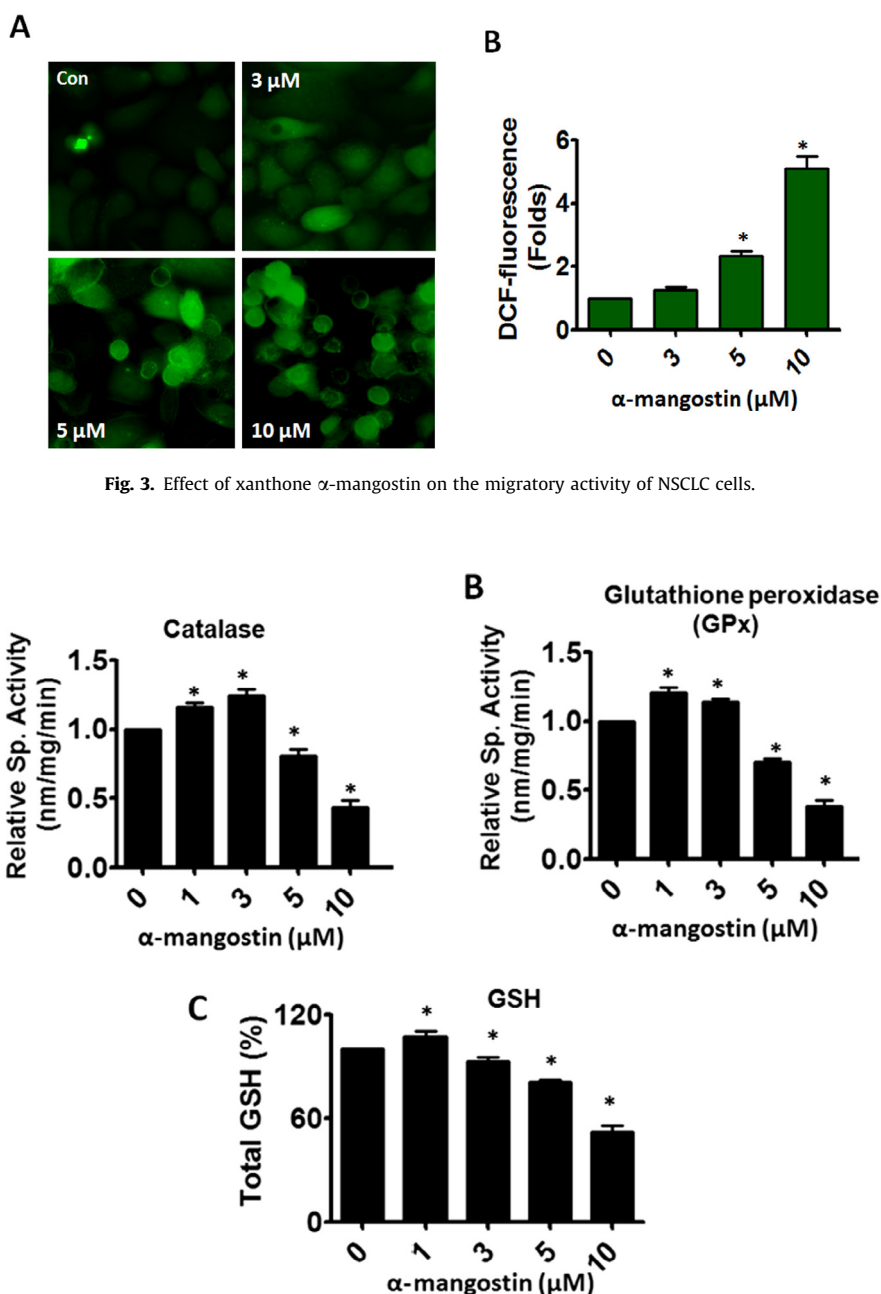

Fig. 3. Effect of xanthone α-mangostin on the migratory activity of NSCLC cells.

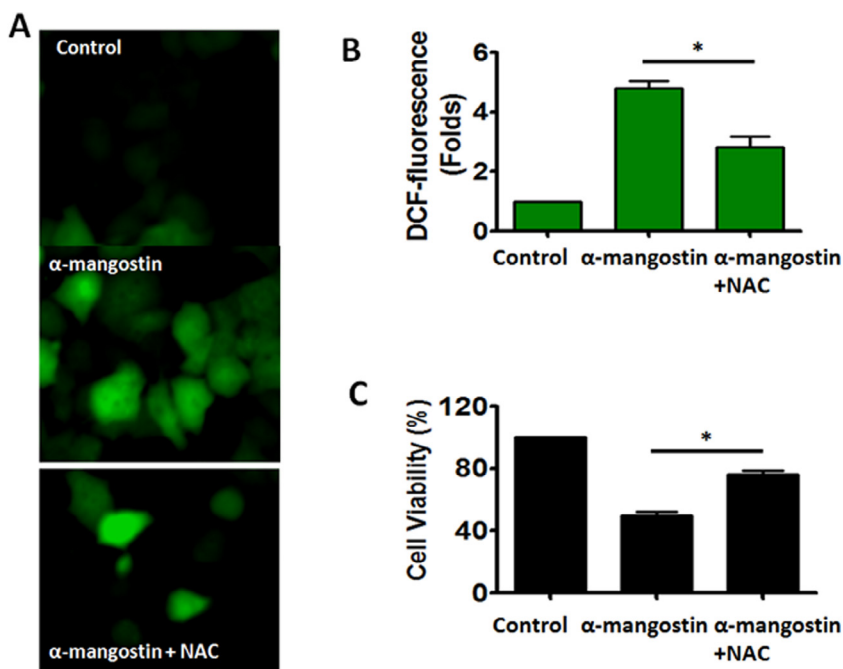Fig. 4. Effect of xanthone α-mangostin on ROS-generation in NSCLC cell.

Fig. 5. Effect of N-acetyl cysteine (NAC) attenuates ROS-mediated cytotoxicity.

3.6. Antioxidant N-acetyl cysteine (NAC) attenuates ROS-mediated cytotoxicity in α-mangostin -treated NSCLC cells

Since α-mangostin -treatment resulted in significant ROS generation in A549 cells, we further investigated whether ROS is responsible for α-mangostin -induced cell death A549 cells. Co-treatment of α-mangostin -treated A549 cells with the antioxidant NAC significantly diminished ROS generation as indicated by the decrease in DCF-fluorescence (Fig. 5). Moreover, administration of NAC significantly restored the cell viability in α-mangostin -treated A549 cells (Fig. 5C). Thus we might conclude that ROS is a key player in α-mangostin -mediated cytotoxicity in NSCLC cells.

4. Discussion

The naturally occurring xanthone α-mangostin is well known for diverse biological benefits (Jindarat, 2014). The anticancer properties of α-mangostin against various malignancies are also well documented (Verma et al., 2016). It has been reported that reactive oxygen species (ROS) are known to play important roles in drug induced apoptosis in cancer cells (Forman et al., 2009; Jeong and Joo, 2016; Liou and Storz, 2010). Hence in the present study we have investigated whether oxidative stress plays any role in α-mangostin induced cytotoxicity, in NSCLC cells.

Treatment of A549 cells with α-mangostin resulted in a dose-dependent loss of cell viability with a limited toxicity towards the noncancerous cells such as WI-38 and *h*PBMC cells. Furthermore, we observed that the mode of cell death by α-mangostin is apoptosis, as confirmed by annexin V/PI staining and monitoring Bax/Bcl-2 ratio. α-Mangostin -treatment also resulted in the significant inhibition of the migratory properties of A549 cells, as determined by Boyden camber assay. To determine the involvement of ROS, we monitored ROS-generation in α-mangostin treated A549 cells by DCF-DA staining. We observed that, there is significant increase in ROS-generation in α-mangostin treated A549 cells, in a dose-dependent fashion. There has been several reports which suggest that α-mangostin can modulate antioxidant enzymes (Lei et al., 2014; Fang et al., 2016). Hence we estimated the enzymatic activities of catalase and superoxide dismutase, and also the total GSH levels in α-mangostin treated A549 cells. We observed that α-mangostin imparts dual effect on the antioxidant markers. At lower doses, α-mangostin acts as an antioxidant and enhances the activities of these enzymes, while at near IC50 doses, the pro-oxidant effect of α-mangostin was more prominent. In presence of 5 μM and 10 μM α-mangostin, the activities of catalase and GPx, and also the levels of reduced glutathione was found to be significantly reduced. Finally the involvement of ROS in α-mangostin -mediated cytotoxicity was confirmed, when we observed that administration of NAC significantly restored the viability in α-mangostin treated cells. Thus we can conclude that ROS plays an important role in α-mangostin -mediated cytotoxicity in NSCLC cells.

Conflict of interest

The authors declare that they have no conflicts of interest.

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