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Inhibition of CHOP accentuates the apoptotic effect of α-mangostin from the mangosteen fruit (*Garcinia mangostana*) in 22Rv1 prostate cancer cells

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ABSTRACT

The mangosteen (*Garcinia mangostana*) fruit has been a popular food in Southeast Asia for centuries and is increasing in popularity in Western countries. We identified α-Mangostin as a primary phytochemical modulating ER stress proteins in prostate cancer cells and propose that α-Mangostin is responsible for exerting a biological effect in prostate cancer cells. Two human prostate cancer cell lines, 22Rv1 and LNCaP, and prostate epithelial cells procured from two patients undergoing radical prostatectomy were treated with α-Mangostin and evaluated by RT-PCR, Western blot, fluorescent microscopy and siRNA transfection to evaluate ER stress. Next, we evaluated α-Mangostin for microsomal stability, pharmacokinetic parameters, and anti-cancer activity in nude mice. α-Mangostin significantly upregulated ER stress markers in prostate cancer cells. Interestingly, α-Mangostin did not promote ER stress in prostate epithelial cells (PrECs) from prostate cancer patients. CHOP knockdown enhanced α-Mangostin-induced apoptosis in prostate cancer cells. α-Mangostin significantly suppressed tumor growth in a xenograft tumor model without obvious toxicity. Our study suggests that α-Mangostin is not the only active constituent from the mangosteen fruit requiring further work to understand the complex chemical composition of the mangosteen.

1. Introduction

The purple mangosteen (*Garcinia mangostana*) is a medicinal plant native to Southeast Asia. The major class of secondary metabolites are xanthones, of which alpha-mangostin is the most abundant in the mangosteen. Several xanthones including α-Mangostin have been reported to contain a variety of health promoting properties [1–3]. α-Mangostin has been shown to induce apoptosis on various cancer cells and modulate intracellular signaling pathways, such as Ca(2+)-ATPase [4], signal transducer and activator 3 (Stat3) [5], mitogen-activated protein kinase (MAPK) [6], phosphatidylinositide 3-kinase (PI3K) [7] and cyclin-dependent kinase-4 (CDK4) [8]. Recently, we reported on the endoplasmic reticulum as a potential target of a highly characterized mangosteen extract (>35% α-Mangostin) [9]. We observed mangosteen fruit extract to induce ER stress chaperones along with the induction of ER stress markers PERK, CHOP, and caspase-4 in prostate cancer cells.

Endoplasmic reticulum stress (ER stress) occurs when unfavorable changes in ER lumen environment decrease ER's protein-folding capacity or increasing protein synthesis overwhelms ER's protein-folding machinery. As a result, several cell signaling pathways collectively referred to as the unfolded protein response (UPR) are triggered to overcome these disturbances and restore cell homeostasis. Some well-established events during ER stress include: upregulation of ER chaperones, for example, Bip (immunoglobulin-binding protein); activation of ER membrane proteins, for example, PERK (protein kinase RNA-like ER kinase) phosphorylation; phosphorylation of eIF2 (eukaryotic translation initiation factor-2); formation of spliced transcription factor XBP-1. These events are aimed at decreasing ER protein folding burden and increasing ER protein folding capacity through mRNA degradation, global translation inhibition and UPR proteins upregulation. Despite all the efforts to reestablish cell homeostasis, however, prolonged ER stress will result in cellular apoptosis emphasizing the delicate balance of ER homeostasis [10–13].

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Based on our previous evaluation of mangosteen fruit, we investigated if the most abundant phytochemical, alpha-mangostin is sufficient to modulate ER stress chaperones and proteins. Also we did side by side comparison of alpha-mangostin and MFE to evaluate the efficacy of the individual phytochemical.

2. Materials and methods

2.1. Materials

α-Mangostin was obtained from Avesthagen, Inc. (Chatsworth, CA). Antibodies for Western blot, BrdU cell proliferation assay kit, and cleaved caspase-3 ELISA kit were obtained from Cell Signaling Technology (Danvers, MA). BCA Protein assay kit and chemiluminescent substrates were obtained from Pierce (Rockford, IL). siRNA were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). TransIT-siQUEST transfection reagents were obtained from Mirus Bio (Madison, WI). One-Step RT-PCR kit, Opti-MEM medium and Alexa Fluor 568 goat anti-mouse IgG were obtained from Life Technologies (Grand Island, NY). RNeasy mini kit and RNase-Free DNase set were obtained from QIAGEN (Santa Clarita, CA). RT-PCR Primers were obtained from IDT (Coralville, IA). Sequences were, CHOP forward 5′-AGC TGG AAC CTG AGG AGA GA-3′, CHOP reverse 5′-GTG ACC TCT GCT GGT TCT GG-3′, GAPDH forward 5′-ACC ACA GTC CAT GCC ATC AC-3′, GAPDH reverse 5′-TCC ACC ACC CTG TTG CTG TA-3′, XBP-1 forward 5′-TTA CGA GAG AAA ACT CAT GGC C-3′, XBP-1 reverse 5′-GGG TCC AAG TTG TCC AGA ATG C-3′.

2.2. Cell culture and treatment

LNCaP and 22Rv1 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained under standard cell culture conditions as described previously [8]. Primary prostatic epithelial cells (PrECs) were established from radical prostatectomy tissue at the University of Illinois at Chicago Medical Center as described previously [14]. Cells were treated with desired doses of α-Mangostin for desired times followed by downstream experiments. In some experiments, MFE containing the same amounts of α-Mangostin were used for comparison purpose (e.g. 17 μg/ml MFE containing 15 μM α-Mangostin; 8.6 μg/ml MFE containing 7.5 μM α-Mangostin).

2.3. Cell proliferation

Cell proliferation was determined by BrdU assay according to the manufacturer's manual.

2.4. Western blots

Western blots were performed as previously described [9].

2.5. siRNA transfection

22Rv1 cells were transfected with control or CHOP siRNA according to manufacturer's manual. After 24 h, transfected cells were treated with 15 μM α-Mangostin for 15 h. Then cell lysates were prepared, quantified and ready for Western blots or ELISA.

2.6. RT-PCR

22Rv1 and LNCaP cells were treated with 15 μM α-Mangostin or DMSO for 24 h. RT-PCR was performed as previously described [9].

2.7. Fluorescence microscopy

Fluorescence microscopy was performed as previously described [15].

2.8. ELISA

Cleaved Caspase-3 levels in same amounts of different cell lysates were detected with ELISA. The manufacturer's manual was followed.

2.9. Pharmacokinetic analysis of α-Mangostin in athymic nude mice

Athymic nude mice were administered 35 mg/kg of α-Mangostin dissolved in cottonseed oil (1 mg/200 μL) by IP injection at time zero. Plasma samples of α-Mangostin treated mice were collected at 30 ($n = 3$), 60 ($n = 3$), and 120 ($n = 2$) minutes after drug administration as previously described [16,17]. Pharmacokinetic variables were determined by non-compartmental methods WinNonlin Pro version 5.2 (Pharsight Corporation, Cary, N.C.). Area under the plasma concentration–time curve (AUC) was estimated using the trapezoidal rule from time 0 to peak concentration (C_{max}) and the log-trapezoidal rule from the peak concentration to the last measurable plasma concentration (AUC_{last}).

2.10. Stability of α-Mangostin in liver microsomes

This was performed as previously described [9].

2.11. In vivo 22Rv1 tumor xenograft model

22Rv1 xenograft model was constructed as previously described [9]. Twenty-one mice were divided into 3 cohorts each

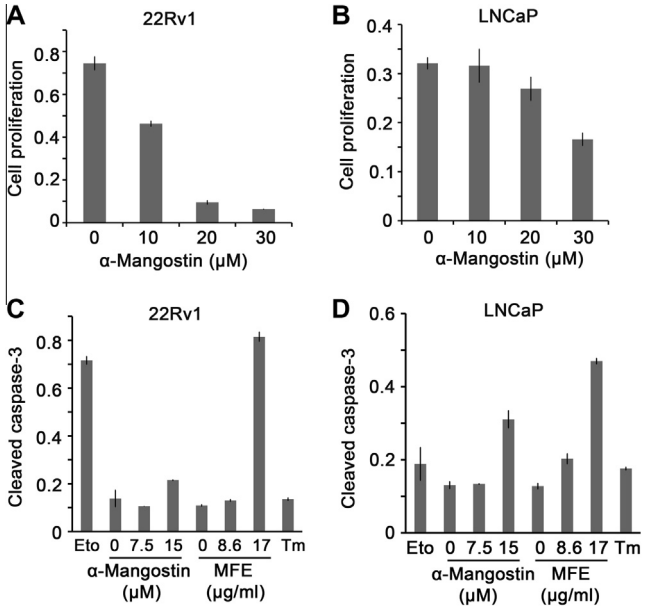

Fig. 1. α-Mangostin decreased cell proliferation and increased cleaved caspase-3 on prostate cancer cells. For cell proliferation evaluation, 22Rv1 and LNCaP cells were treated with 0, 10, 20 and 30 μM of α-Mangostin for 24 h, and then subjected to BrdU assay. For cleaved caspase-3 ELISA, cells were treated with desired doses of α-Mangostin or mangosteen fruit extract (MFE) for 24 h. Cell lysates were prepared and cleaved caspase-3 levels were evaluated by ELISA. Tunicamycin (Tm) and etoposide (eto) were included as controls. (A) Cell proliferation for treated 22Rv1 cells. (B) Cell proliferation for treated LNCaP cells. (C) Cleaved caspase-3 levels for treated 22Rv1 cells. (D) Cleaved caspase-3 levels for treated LNCaP cells. These experiments are represented by the mean along with standard deviation, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

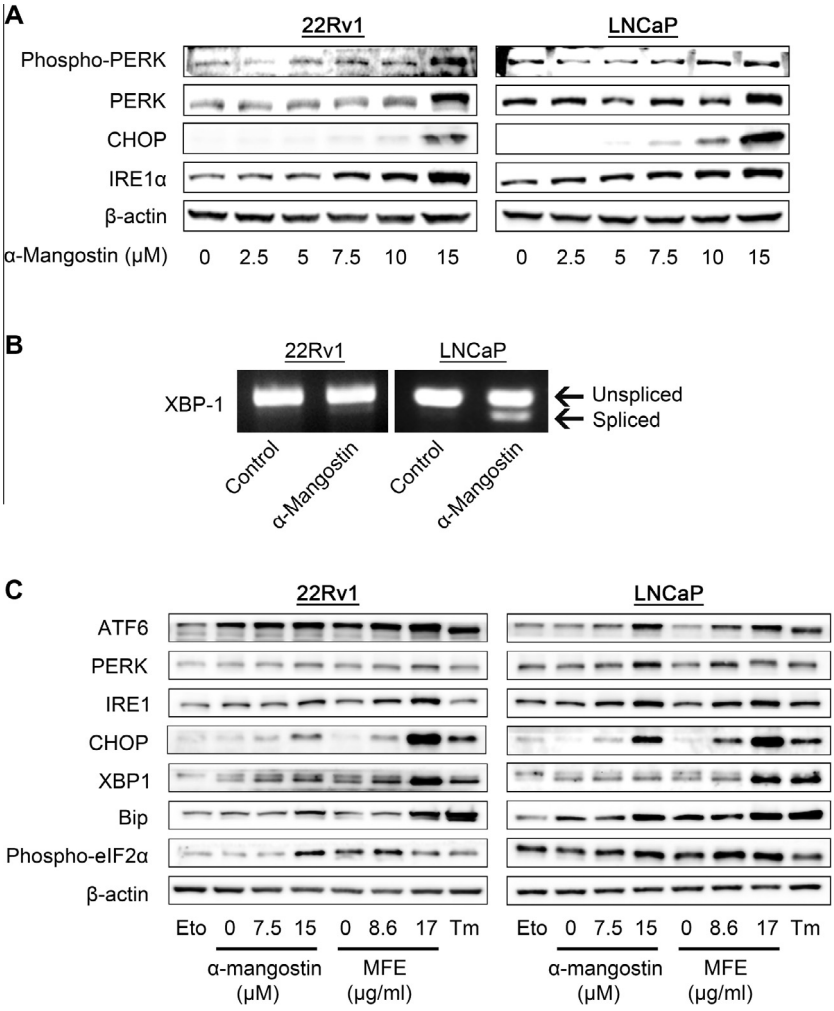

Fig. 2. α-Mangostin promoted endoplasmic reticulum (ER) stress in prostate cancer cells. 22Rv1 and LNCaP cells were treated with increasing doses of α-Mangostin for 24 h. Cell lysates were prepared and subjected to Western blot for detecting the expression of ER stress proteins. (A) Expression of ER stress proteins in increasing doses of α-Mangostin-treated 22Rv1 and LNCaP cells. (B) Agarose gel pictures of RT-PCR products. 22Rv1 and LNCaP cells were treated with 15 μM α-Mangostin for 24 h. Total RNA was extracted and subjected to RT-PCR. Primers spanning the spliced sequence were used. RT-PCR products were run on a 2% agarose gel. These results are representatives from three independent experiments. (C) Side-by-side comparison of α-Mangostin and MFE on inducing ER stress proteins by Western blot analysis. 22Rv1 and LNCaP cells were treated with desired doses of α-Mangostin or MFE for 24 h. Cell lysates were prepared and subjected to Western blot analysis. For comparison purpose, 8.6 μg/ml MFE contains around 7.5 μM α-Mangostin, and 17 μg/ml MFE contains around 15 μM α-Mangostin. ER stress inducer tunicamycin was used as a positive control. β-actin was used as a loading control. These results are representatives from three independent experiments.

receiving vehicle (100 μL) as control (Group 1), α-Mangostin 35 mg/kg (Group 2), or α-Mangostin 70 mg/kg (Group 3) by intraperitoneal administration two times weekly. Body weights were recorded throughout the study. All animal experiments were performed in accordance with the guidelines approved by the Animal Care and Use Committee of the University of Illinois at Chicago. The protocol was approved by the animal care committee at the University of Illinois at Chicago (Protocol Number: ACC-11-019).

2.12. Statistical analysis

All statistical analysis was performed by using VassarStats software. Data are expressed as mean with standard deviation for all groups. Statistical significance of differences in all measurements between control and treated groups was determined by one-way ANOVA followed by Tukey's HSD test for multiple comparisons. Student's paired *t* test was used for pair wise group comparisons, as needed. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. α-Mangostin inhibited proliferation and increased apoptosis marker on prostate cancer cells

22Rv1 and LNCaP cells were treated with α-Mangostin and proliferation and cleaved caspase-3 evaluated. α-Mangostin decreased cell proliferation in a dose-dependent manner (Fig. 1A and B). Cleaved capase-3 significantly increased at 15 μM α-Mangostin in 22Rv1 ($P = 0.0015$) and LNCaP ($P = 0.0103$) (Fig. 1C and D). As a comparison, MFE induced more cleaved caspase-3 than pure α-Mangostin (Fig. 1C and D), suggesting MFE is more effective in promoting cleavage of caspase 3 in prostate cancer cells.

3.2. α-Mangostin promoted endoplasmic reticulum (ER) stress in prostate cancer cells

α-Mangostin treatment increased ER stress markers including phosphorylated PERK, IRE1, CHOP and spliced XBP-1 at 15 μM (Fig. 2A and B). These data demonstrated that α-Mangostin

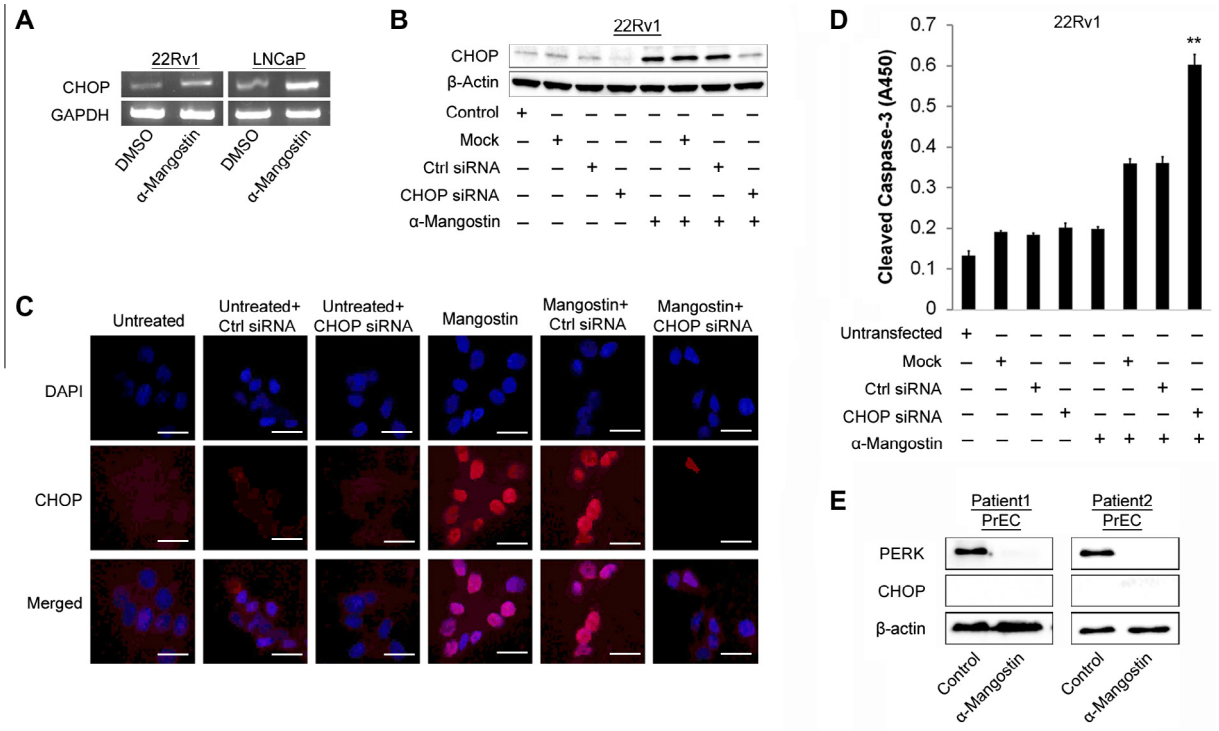

Fig. 3. The effects of CHOP knockdown on α-Mangostin-treated 22Rv1 cells and the effects of α-Mangostin treatments on ER stress in PrECs from prostate cancer patients. For CHOP knockdown, cells were mock transfected or transfected with control siRNA or CHOP siRNA. After 19 h, cells were then treated with 15 μM α-Mangostin for 15 h. Cell lysates were prepared and subjected to Western blot analysis. For fluorescence microscopy, cells were plated in culture slides, transfected and treated as above-described followed by immunofluorescent staining. (A) RT-PCR analysis of CHOP mRNA levels in DMSO- or α-Mangostin-treated cells, GAPDH was used as an internal control. (B) CHOP expression in different groups of treated cells. (C) Cleaved caspase-3 levels in differently treated cells, $^{**}P < 0.01$. (D) Intracellular staining of CHOP (Alexa Fluor 568, red) and nucleus (DAPI, blue) in differently treated cells. Bar scale represents 50 px (E) CHOP and PERK expressions in differently treated cells. PrECs from two different prostate cancer patients were treated with 15 μM α-Mangostin for 24 h and then subjected to Western blots. β-Actin was used as a loading control. Minus and plus symbols respectively represent absence and presence of indicated agents. These results are representatives from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

promoted ER stress in prostate cancer cells. In a side-by-side comparison, MFE induced more ER stress proteins such as BiP, XBP-1 and CHOP than pure α-Mangostin (Fig. 2C) at 17 μg/ml, suggesting that MFE is a more potent ER stress inducer.

3.3. CHOP knockdown increased apoptotic marker in α-Mangostin-treated prostate cancer cells

Our results suggest that α-mangostin upregulates CHOP mRNA (Fig. 3A). As CHOP plays an important role in ER stress-induced apoptosis, we performed CHOP siRNA transfection (Fig. 3B and D) and evaluated its effects on apoptosis marker change in α-Mangostin-treated prostate cancer cells. Surprisingly, CHOP knockdown significantly increased cleaved caspase-3 in α-Mangostin-treated 22Rv1 cells, as if CHOP siRNA has a synergistic effect with α-Mangostin. Knowing the role of CHOP in ER stress this may suggest that silencing of CHOP siRNA resulted in destabilization of the ER resulting in α-mangostin being more effective in promoting the cleavage of caspase-3. The data seems to suggest that in 22Rv1 cells CHOP prevents the action (i.e. is a defense mechanism) of ER stress modulator α-mangostin. However, this effect was not observed on α-Mangostin-treated LNCaP cells (data not shown). This might due to some fundamental differences between these two cells lines including androgen dependent (LNCaP) versus androgen independent (22Rv1) and mutated androgen receptor (LNCaP at 877). We actually found that CHOP siRNA increased AR in LNCaP but not in 22Rv1 (unpublished data), which might help LNCaP resist α-Mangostin induced apoptosis. Our data suggest that ER stress/UPR plays a pro-survival role in prostate cancer cells in the absence of additional ER stress activator. During the early stage dealing with the stress activator α-Mangostin, the cancer cells resort to a sustained UPR. This makes the cancer cells vulnerable to apoptosis. With a further push from α-Mangostin, apoptosis is induced through ER stress/UPR or other apoptotic pathways. Our data suggests that modulation of ER stress proteins, whether positive or negative, using a small molecule and/or siRNA represents a unique therapeutic strategy to control cancer. However, this should be cautious as ER stress plays paradoxical roles and many fundamental questions of ER stress are still unclear.

3.4. α-Mangostin did not increase ER stress proteins in prostate epithelial cells

Next, our objective was to characterize the potential of α-Mangostin to promote ER stress in non-tumorigenic cancer cells. We found that 15 μM of α-Mangostin decreased PERK and did not increase CHOP in prostate epithelial cells (Fig. 3E), suggesting that α-Mangostin might have a differential effect on modulating ER stress proteins in cancer cells versus non-cancerous cells. This may be partly explained by the intrinsic characteristics and external environment of cancer cells. Cancer cells by their very nature are under increased demands to synthesize more proteins to help fulfill their seemingly unlimited proliferation. Also, they are often under a hypoxia, low PH and nutrient-lacking environment, which is known to promote ER stress/UPR [18]. As a result, cancer cells undergo ER stress more often than normal cells. This was also confirmed by the fact that many ER-residing proteins show an increased expression pattern in cancers [19]. For example, ER

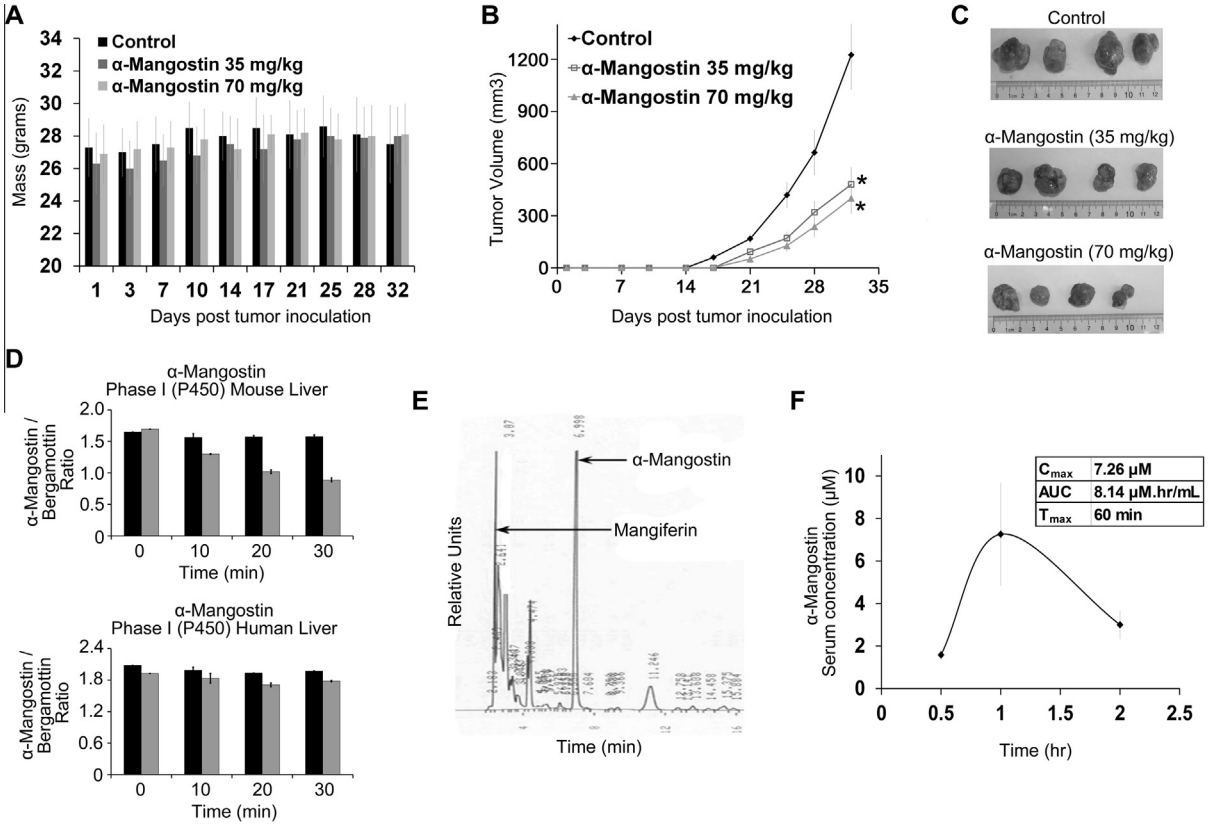

Fig. 4. *In vivo* study and pharmacokinetics analysis. (A) Body weights of athymic nude mice treated with control vehicle or α-Mangostin (35 or 70 mg/kg) intraperitoneally twice per week were measured two times weekly. (B) Eighteen animals were subcutaneously injected in each flank of the mouse (i.e. two tumors per mice) with $\sim 1 \times 10^6$ 22Rv1 cells to initiate tumor growth. Twenty-four hours after cell implantation, the animals in each cohort received by intraperitoneal administration vehicle or α-Mangostin (35 mg/kg and 70 mg/kg). The average tumor volume of control and α-Mangostin treated mice was plotted over days after tumor cell inoculation. Data points represent the mean of 12 tumors from six mice; bars represent standard deviation of the mean, $^*P < 0.01$. (C) Two representative tumors from each cohort (i.e. control, α-Mangostin 35 mg/kg, and α-Mangostin 70 mg/kg) at the end of the study are shown comparing control mice to mice treated with a-mangostin (35 and 70 mg/kg). (D) α-Mangostin was incubated with liver microsomes to mimic P450 Phase I metabolism in the mouse or human liver. Human and mouse liver microsomes were prepared as described in Section 2. Black bars represent the respective control for that individual time point. Gray bars represent the microsomal stability of α-Mangostin in Phase I enzyme system in both mouse and human liver microsomes. A 'no preincubation' was performed (data not shown) and did not have any impact on the interpretation of this data. (E) Representative HPLC chromatograph, plasma samples were spiked with Mangostin RT of 6.998 and internal standard (Mangiferin) RT of 2.469. (F) A concentration versus time profile was determined following intraperitoneal administration of α-Mangostin (35 mg/kg).

stress chaperone BiP (also known as GRP78 or Hsp70) has been found to have an increased expression in over 10 different cancers, including prostate, breast, lung, and colon cancers, or hepatocellular carcinoma [19,20]. In addition, ER stress may also contribute to neovascularization in cancers as suggested by some studies [21–24]. Taken together, it seems to be more natural for cancer cells to resort to ER stress/UPR as a pro-survival tool when facing an additional external stress, in our case, the α-Mangostin treatment. On the other hand, as our previous study suggested that α-Mangostin is a cell cycle inhibitor by binding cyclin-dependent kinase-4 [9], it is reasonable that α-Mangostin has a preferential inhibitory/stress-producing effect on fast-dividing prostate cancer cells instead of non-dividing prostate epithelial cells. This might also partly explain why α-Mangostin selectively induced ER stress in prostate cancer cells. A further and more detailed understanding of this differential effect will be required.

3.5. α-Mangostin significantly inhibited the growth of human prostate carcinoma 22Rv1 cells in athymic nude mice

To examine α-Mangostin's anticancer activity *in vivo*, we constructed xenograft mice models with 22Rv1 cells and then treated them with α-Mangostin or vehicle. No obvious difference in body weights was observed between α-Mangostin-treated groups and vehicle-treated groups (Fig. 4A). Compared to control, 35 or 70 mg/kg of α-Mangostin significantly inhibited tumor growth (Fig. 4B and C), suggesting that α-Mangostin inhibited prostate cancer cell growth *in vivo*. We have previously found that 35 mg/kg of MFE (contains approximately 12 mg/kg of α-Mangostin) inhibited tumor volume by 88% in 22Rv1 implanted mice [9]. These data, as well as our results from side-by-side comparison experiments, demonstrate that the pure phytochemical was not as potent as MFE, suggesting a synergistic effect among individual phytochemicals was occurring. Alternative explanations include the presence of other potent xanthones, or a longer half-life of α-Mangostin when administered in combination with other xanthones.

3.6. Stability of α-Mangostin in the presence of liver microsomes

To characterize the extent of P450 metabolism, α-Mangostin was incubated in the presence of liver microsomes from mouse and human sources. For this particular experiment we evaluated liver microsomes as the administration of study agent was by intraperitoneal administration. By 30 min, the stability of α-Mangostin was found to decrease in mouse and human liver microsomes by 47.6% and 7.5%, respectively (Fig. 4D). These results suggest that Phase I metabolism has a minimal role in the

metabolism in α-Mangostin. Phase II metabolism in microsomes appears to be the primary route of metabolism.

3.7. Pharmacokinetic profile of intraperitoneal α-Mangostin in athymic nude mice

To establish a maximum serum concentration following intraperitoneal treatment with α-Mangostin plasma was collected following a single dose of α-Mangostin 35 mg/kg. A maximum serum concentration (C_{max}) of 7.26 μM was achieved with a maximum time to concentration (T_{max}) of 1 h (Fig. 4E and F). The AUC was determined to be 8.14 μM h/mL. This is an important observation as the serum level is close to 7.5 μM, similar to the concentrations that achieved a biological response in *in vitro* prostate cancer cells.

In summary, our study found that α-Mangostin induced ER stress in prostate cancer cells which correlates with an increase in apoptotic indices. We also provided preliminary data that suggests a different response in normal prostate epithelial cells may be observed following treatment with α-Mangostin. In particular, these cells were from patients at high risk of developing prostate cancer will have to be studied more in the future. We also found that α-Mangostin is bioavailable at a concentration exerting biological functions. From our studies it is evident that α-Mangostin is not the only active constituent from the mangosteen fruit requiring further work to understand the complex chemical composition of the mangosteen. Our study provided a rationale for the mangosteen and its main phytochemical α-Mangostin exerting biological effects in prostate cancer research models.

Conflicts of Interest

The authors do not have any conflicts of interest.

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