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## **$\alpha$ -Mangostin, a Natural Agent, Enhances the Response of NRAS Mutant Melanoma to Retinoic Acid**

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Data Collection B  
Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
Literature Search F  
Funds Collection G

ABCDEF 1 **Yun Xia**  
AB 2 **Jing Chen**  
DF 2 **Chongwen Gong**  
AG 3 **Hongxiang Chen**  
AGF 1 **Jiaming Sun**

1 Department of Plastic Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, P.R. China  
2 Department of Oncology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, P.R. China  
3 Department of Dermatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, P.R. China

**Corresponding Authors:** Jiaming Sun, e-mail: sunjm1592@sina.com, Hongxiang Chen, e-mail: hongxiangchen@hotmail.com  
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**Background:** The identification and use of novel compounds alone or in combination hold promise for the fight against NRAS mutant melanoma.





**Material/Methods:** We screened a kinase-specific inhibitor library through combining it with  $\alpha$ -Mangostin in NRAS mutant melanoma cell line, and verified the enhancing effect of  $\alpha$ -Mangostin through inhibition of the tumorigenesis pathway.

**Results:** Within the kinase inhibitors, retinoic acid showed a significant synergistic effect with  $\alpha$ -Mangostin.  $\alpha$ -Mangostin also can reverse the drug resistance of retinoic acid in RARA siRNA-transduced sk-mel-2 cells. Colony assay, TUNEL staining, and the expressions of several apoptosis-related genes revealed that  $\alpha$ -Mangostin enhanced the effect of retinoic acid-induced apoptosis. The combination treatment resulted in marked induction of ROS generation and inhibition of the AKT/S6 pathway.

**Conclusions:** These results indicate that the combination of these novel natural agents with retinoid acid may be clinically effective in NRAS mutant melanoma.

**MeSH Keywords:** **Antineoplastic Agents • Melanoma • Tretinoin**

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## Background

Melanoma is a potentially fatal cutaneous cancer, with a significant increase in the number of cases diagnosed in the world in the past few years [1]. Due to its poor response to standard chemotherapy, the primary treatment for melanoma is surgical excision [2]. NRAS is an important oncogene mutated in 15% to 20% of melanoma cases compared to BRAF mutants in approximately 50% of cases. NRAS mutants are correlated with a higher rate of mitosis and no targeted therapies are currently available [3,4]. The progress of research on treatments for melanoma harboring NRAS mutation has been slow [5], and effective single-agent chemotherapies are currently limited [6]. Thus, for long-term response rates and minimizing drug toxicity and adverse effects, combining inhibition and multiple treatments are already underway [7].

There are extensive studies showing that retinoic acid can inhibit the cell proliferation and induce cell differentiation in a variety of human cancers [8]. The anti-proliferation effect of retinoic acid in the B16 murine melanoma cell model has been reported [9], however, the *in vitro* response of melanoma cell lines to retinoic acid is quite variable [10]. Resistance to retinoid treatment and the adverse effects limit the use of retinoic acid as a single-agent chemotherapy [11,12]; therefore, it is mainly used in combination with other antitumor agents for the treatment of cancer [13,14].

$\alpha$ -Mangostin is a natural product, been widely studied as a candidate treatment of various cancers [15,16]. The cytotoxic activity of  $\alpha$ -Mangostin also has been demonstrated in melanoma cell lines [17]. The use of these novel compounds alone or in combination therapies may greatly help in the fight against NRAS mutant melanoma.

## Material and Methods

### Human melanoma cell lines culture

Sk-mel-2 cell (human NRAS mutant melanoma cell line) was kept in our laboratory. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; fetal calf serum, 10%; penicillin, 100 000 U/l; streptomycin sulphate, 100 mg/l; Life Technologies, Grand Island, NY). Cells were incubated at 37°C in 5% CO<sub>2</sub>, 5% humidity, and passaged at 2×10<sup>4</sup> cells/ml when near-confluent monolayers were achieved. Cells were free from Mycoplasma contamination.

### Drugs and treatment

$\alpha$ -Mangostin was purchased from Sigma, and retinoic acid was purchased from LC Laboratories (MA). All the drugs were dissolved in dimethyl sulfoxide (DMSO) and were added directly to the culture medium of melanoma cells at the concentrations

to be tested. Melanoma cells incubated with culture medium with DMSO served as controls. The stock solutions of the drugs were prepared according to the manufacturer's specification and stored at -20°C.

### Screening assay

The kinase inhibitor library was screened in sk-mel-2 cell line alone or in combination with  $\alpha$ -Mangostin to determine the synergistic effect. The screening was performed in sk-mel-2 cell line plating in 384-well clear bottom plates (Corning, Tewksbury, MA) with 20 000 cell/ml density. Kinase inhibitors were pin-transferred into each well at the original concentration of 10 mM with or without  $\alpha$ -Mangostin (5  $\mu$ M). The cell proliferation and cytotoxicity were measured using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI) at 48 h using automated high-throughput analysis.

### Cell proliferation assay

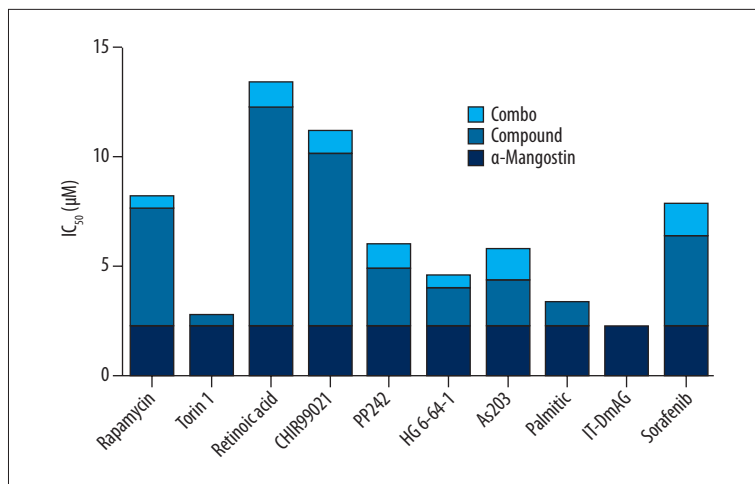
Drug cytotoxicity was determined using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI) as previously described. Briefly, cells were plated in a 96-well clear bottom plate (Corning, Tewksbury, MA) at the density of 20 000 cells/ml in culture medium (DMEM supplemented with 10% FBS and penicillin/streptomycin), drugs were added to the first well of each row at the concentration of 10  $\mu$ M alone or in combination with  $\alpha$ -Mangostin (5  $\mu$ M), then double-diluted by more than 10 times. After 48 h of culture, 8  $\mu$ l of CellTiter-Glo (10 $\mu$ M, in PBS) was added into each well and incubated for 10 min at room temperature. The absorbance at a wavelength of 490 nm was read using a EnVision® Multilabel Plate Reader (Envision, Perkin Elmer) using ultrasensitive luminescence. The experiments were done in triplicate.

### Colony formation assay

Sk-mel-2 cells were seeded in 6-well plates at the density of 100 cells/well, and drugs were added into each well at the concentration of 5  $\mu$ M alone or with  $\alpha$ -Mangostin (2  $\mu$ M) for 6 days. Cells were fixed with 4% paraformaldehyde, and colonies were stained with 0.1% crystal violet.

### Immunofluorescence microscopy

Sk-mel-2 cells mounted on glass slides were fixed with 4% paraformaldehyde for 20 min, and permeabilized with PBS containing 0.1% Triton X-100 and 0.1% glycine for 2 min on ice. The TUNEL staining was performed using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Germany). Cells were co-stained with 4'-6'-diamidino-2-phenylindole (DAPI) to visualize nuclei. The images were taken using a FSX100 all-in-one microscope (Olympus Corporation, Japan).



**Figure 1.** Screening of kinase-specific inhibitors with  $\alpha$ -Mangostin. The dosage-dependent cell proliferation ratio assay of drugs and drug combination in sk-mel-2 cells was performed with or without the presence of  $\alpha$ -Mangostin (5  $\mu$ M). The synergistic effect of drug combination was shown in the form of half-lethal dosage  $IC_{50}$ . Ten compounds were selected, and strong synergistic effects of those compounds combined with  $\alpha$ -Mangostin respectively were found. A marked decrease is seen in the half-lethal dosage  $IC_{50}$  in combination groups compared to the single-agent groups.

### Western blotting

Cells were lysed in 1 $\times$  radioimmunoprecipitation assay (RIPA) lysis buffer (Life Technologies, Grand Island, NY), and the protein concentration was determined by BCA protein Assay Kit (Thermo Scientific). Equal amounts of the proteins were electrophoresed on 4–15% Bis-Tris Gels (BioRad, USA) and transferred onto polyvinylidene difluoride membranes. Membranes were blocked for 1 h at room temperature with 5% BSA in Tris-Buffered Saline and Tween 20, incubated with various primary antibodies diluted in blocking buffer at 4°C overnight, and detected with either anti-rabbit (1:5000) or anti-mouse (1:5000) secondary antibodies for 1 h at room temperature. The final immunoreactive products were detected by enhanced chemiluminescence (ECL) system (Promega, Madison, WI). The human anti-S6, anti-phospho-S6, anti-AKT, anti-phospho-AKT, anti-Mcl-1, anti-P21, anti-cleaved PARP, anti-PARP, and anti-beta-actin antibodies were purchased from Cell Signaling Technologies (Dedham, MA).

### Determination of ROS

The CellROX Oxidative Stress Reagents (Life Technology, Grand Island, NY, USA) provides a cell-based assay for measuring primarily hydrogen peroxide, along with hydroxyl, peroxy, and other ROS levels within a cell. Cells were incubated with 5  $\mu$ M CellROX Oxidative Stress Reagents for 30 min, after which they were washed with PBS, cells were co-stained with 4'6'-diamidino-2-phenylindole (DAPI) to visualize nuclei, and the amount of fluorescence was determined using an FSX100 all-in-one microscope (Olympus Corporation, Japan).

### RNA interference studies

Transfection of SK-mel-2 cells was performed with Attractgene Transfection Reagent according to the manufacturer's protocol (Qiagen, Germany) with siRNAs against RAR $\alpha$  and control siRNA (Santa Cruz, CA). Transfected cells were treated with

$\alpha$ -Mangostin (2 $\mu$ M), retinoic acid (2  $\mu$ M), or their combination for 48 h for further studies.

### Statistical analysis

The differences and variances were analyzed using the *t* test for unpaired observations. The results are presented as mean  $\pm$ SEM and  $P < 0.05$  was considered statistically significant.

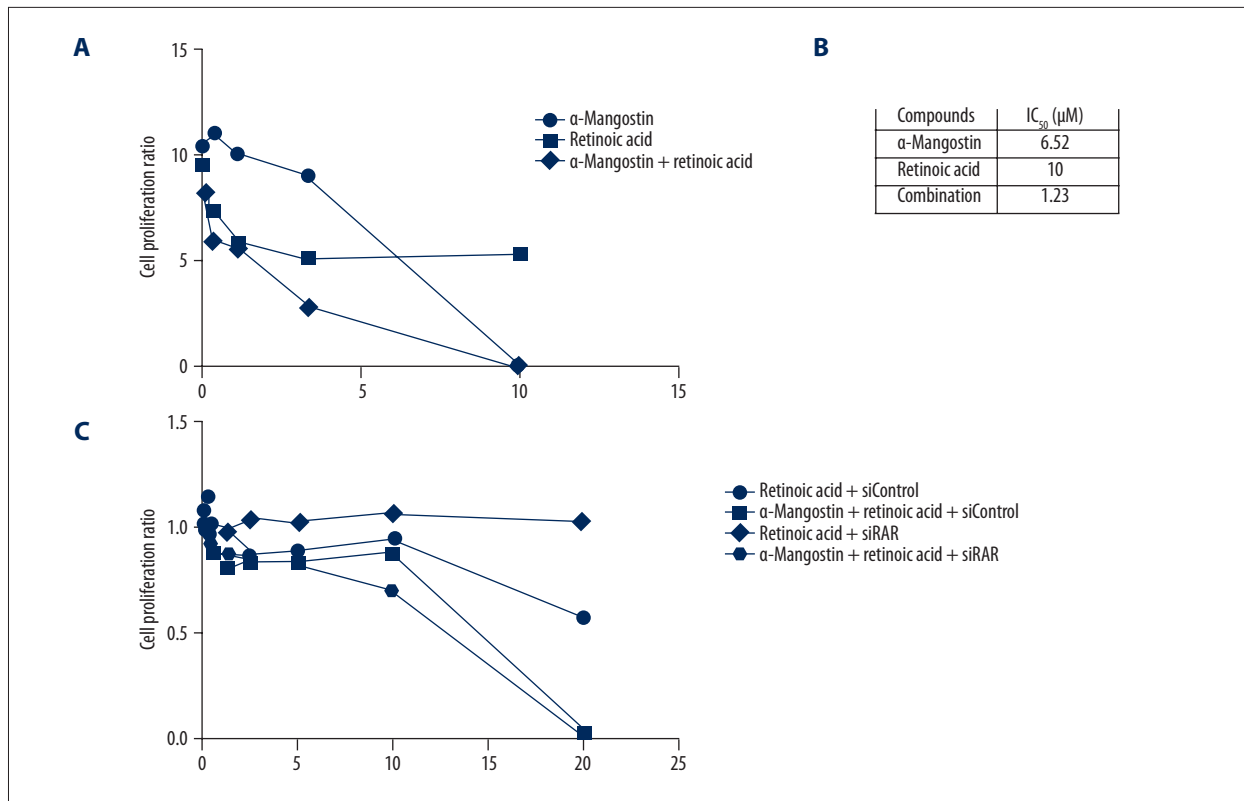
## Results

### Screening of the kinase inhibitors and primary hit selection

A total of 2000 compounds in the kinase-specific inhibitor library were screened with or without the combination of  $\alpha$ -Mangostin, and 10 compounds were identified to be significantly inhibiting cell proliferation and inducing cell death after combined with  $\alpha$ -Mangostin in sk-mel-2 cells (Figure 1). Several kinase inhibitors, such as Rapamycin, retinoic acid, and Torin1, were previously reported to have the ability to inhibit tumor growth in various cancers [18–20]. Some of them were being studied in treating melanoma alone or with other combinations. These compounds include inhibitors targeting mTOR (Rapamycin, Torin1), GSK3 (CHIR99021), and VEGFR (Sorafenib) which are closely connected with cancer formation, growth, and metastasis [21–23].

### Secondary hit selection and verification of the combination effect

We further verified the efficacy of 5 lead compounds with  $\alpha$ -Mangostin by CellTiter-Glo Luminescent Cell Viability Assay. Improved  $IC_{50}$  was shown in combination groups. Within all these compounds, we found that retinoic acid showed a significant inhibition effect after combining with  $\alpha$ -Mangostin. The  $IC_{50}$  of



**Figure 2.** The combination effect of  $\alpha$ -Mangostin and retinoic acid was verified. (A, B) Within all these compounds, we found the effect of retinoic acid (10  $\mu$ M) and verified its synergistic effect with  $\alpha$ -Mangostin (5 $\mu$ M) in sk-mel-2 cells. The half-lethal dosage IC<sub>50</sub> was decreased to 1.23  $\mu$ M, nearly 8 times higher than the 10  $\mu$ M in the single-agent group. (C) We determined the correlation of the synergistic effect with RAR level in RAR $\alpha$  siRNA-transduced sk-mel-2 cells, showing that cells without RAR expression were not sensitive to retinoic acid, but the synergistic effect of retinoic acid and  $\alpha$ -Mangostin was significantly improved.

retinoic acid was decreased nearly by 8-fold in sk-mel-2 cells in combination with  $\alpha$ -Mangostin (Figure 2A, 2B). To evaluate the correlation of RAR (retinoic acid receptor) level with retinoic acid resistance in NRAS mutant melanoma, we inhibited the RAR $\alpha$  expression in sk-mel-2 cells using RAR $\alpha$  siRNA, and determined the sensitivities of retinoic acid,  $\alpha$ -Mangostin, and their combination by CellTiter-Glo Luminescent Cell Viability Assay in RAR $\alpha$  siRNA-transduced cells and control cells. The results showed that RAR $\alpha$  siRNA-transduced sk-mel-2 cells were more sensitive to retinoic acid combined with  $\alpha$ -Mangostin, which means that  $\alpha$ -Mangostin can significantly reverse the retinoic acid resistance in RAR $\alpha$  siRNA-transduced sk-mel-2 cells (Figure 2C).

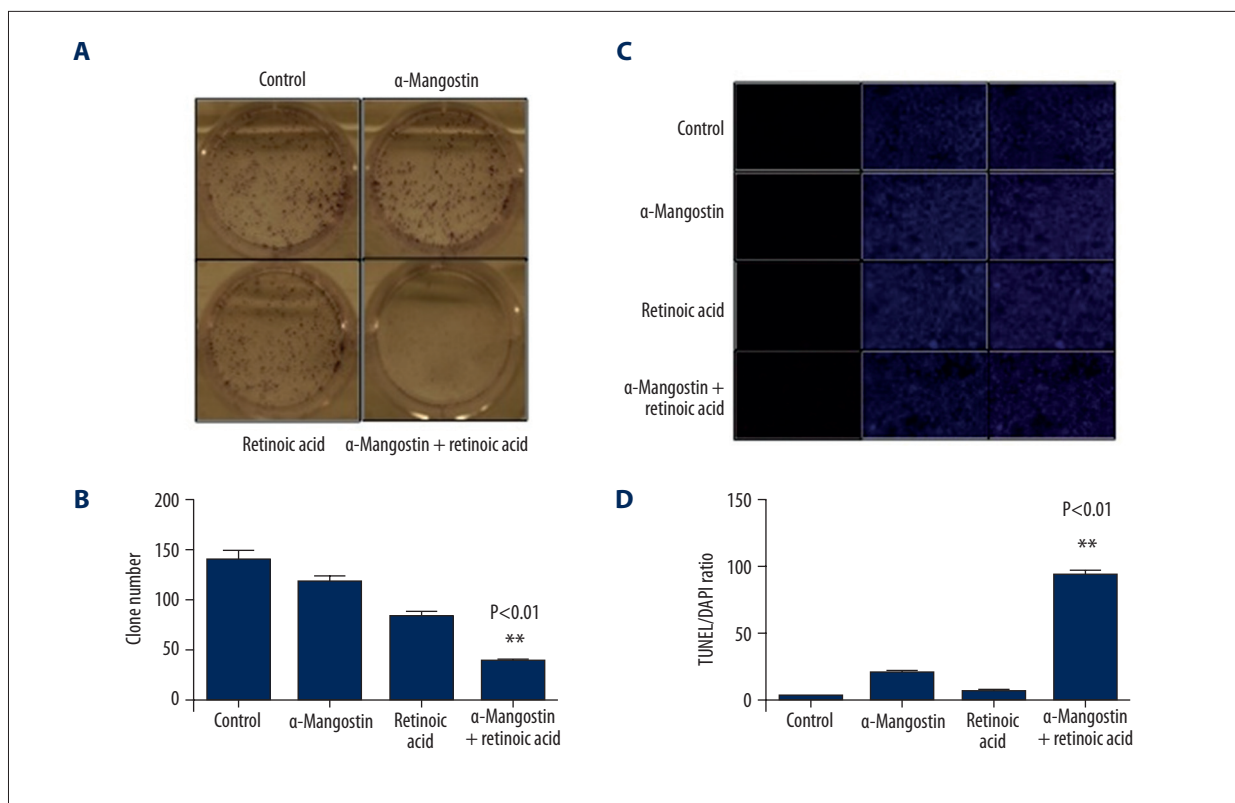
#### $\alpha$ -Mangostin activates retinoic acid-induced apoptosis

We examined the effect of retinoic acid,  $\alpha$ -Mangostin, and their combination on the survival of sk-mel-2 cells by colony survival assay. Colony formation was completely inhibited in cells treated with retinoic acid combined with  $\alpha$ -Mangostin for 5 days. In contrast, retinoic acid or  $\alpha$ -Mangostin alone showed little effect under the same condition (Figure 3A, 3B). To study whether

retinoic acid combined with  $\alpha$ -Mangostin could result in increased apoptosis compared to being used alone, we further examined the growth-inhibitory effects in sk-mel-2 cells via TUNEL (Terminal deoxynucleotidyl transferase dUTP nick-end labeling) staining assay. As shown in Figure 3C and 3D, apoptotic bodies, as an indication of DNA fragmentation and activated apoptotic cascades, were markedly increased in the retinoic acid combined with  $\alpha$ -Mangostin treatment group. Thus, the enhancing effect of  $\alpha$ -Mangostin on retinoic acid-induced cell apoptosis was determined. We also noticed that the expression of cleaved PARP, which is an apoptotic marker [24], was increased with the treatment of retinoic acid and  $\alpha$ -Mangostin (Figure 4A). P21 and Mcl-1, which are anti-apoptotic proteins [25, 26], were significantly down-regulated with 2  $\mu$ M of retinoic acid and  $\alpha$ -Mangostin treatment for 8 h in sk-mel-2 cells (Figure 4B).

#### $\alpha$ -Mangostin is synergistic with retinoic acid on pAKT down-regulation in SK-mel-2 cell line

To further explore the underlying mechanisms of the effect, we next analyzed the expression of a variety of proteins involved



**Figure 3.**  $\alpha$ -Mangostin activates retinoic acid-induced apoptosis. (A) Clonogenic survival assay was performed to examine the synergistic effect of the combination of retinoic acid and  $\alpha$ -Mangostin on the survival of sk-mel-2 cells. Cell colonies were stained blue. (B) Colony numbers were counted. The paired *t* test was used to check the statistical significance ( $P < 0.001$ ). (C) The growth-inhibitory effects were examined via TUNEL staining. Slices were cultured in medium containing retinoic acid (2  $\mu$ M),  $\alpha$ -Mangostin (2  $\mu$ M), or their combination for 3 h and stained for TUNEL (red) or 4',6-diamidino-2-phenylindole (DAPI; blue) to label all cells. Representative images of 3 independent experiments are shown. D. The numbers of positive cell were counted. The paired *t* test was used to assess the statistical significance ( $P < 0.001$ ).

in the regulation of cell growth and vitality in sk-mel-2 cells after single-agent and combination therapy with retinoic acid and  $\alpha$ -Mangostin. The AKT survival pathway has been shown as an important pathway involved in cancer growth [27]. The effects of retinoic acid with or without  $\alpha$ -Mangostin on the expression of pAKT, AKT, pS6, and S6 in sk-mel-2 cells were analyzed by Western blot. It revealed that their combination could inhibit the phosphorylation of AKT and S6, with no effect on the total expression of AKT and S6. Time course analysis showed that the inhibition of phospho-AKT by  $\alpha$ -Mangostin was observed within 2 h after the treatment (Figure 4C). Thus,  $\alpha$ -Mangostin may sensitize NRAS mutant melanoma to the apoptotic effect of retinoic acid through its inhibition of the AKT pathway.

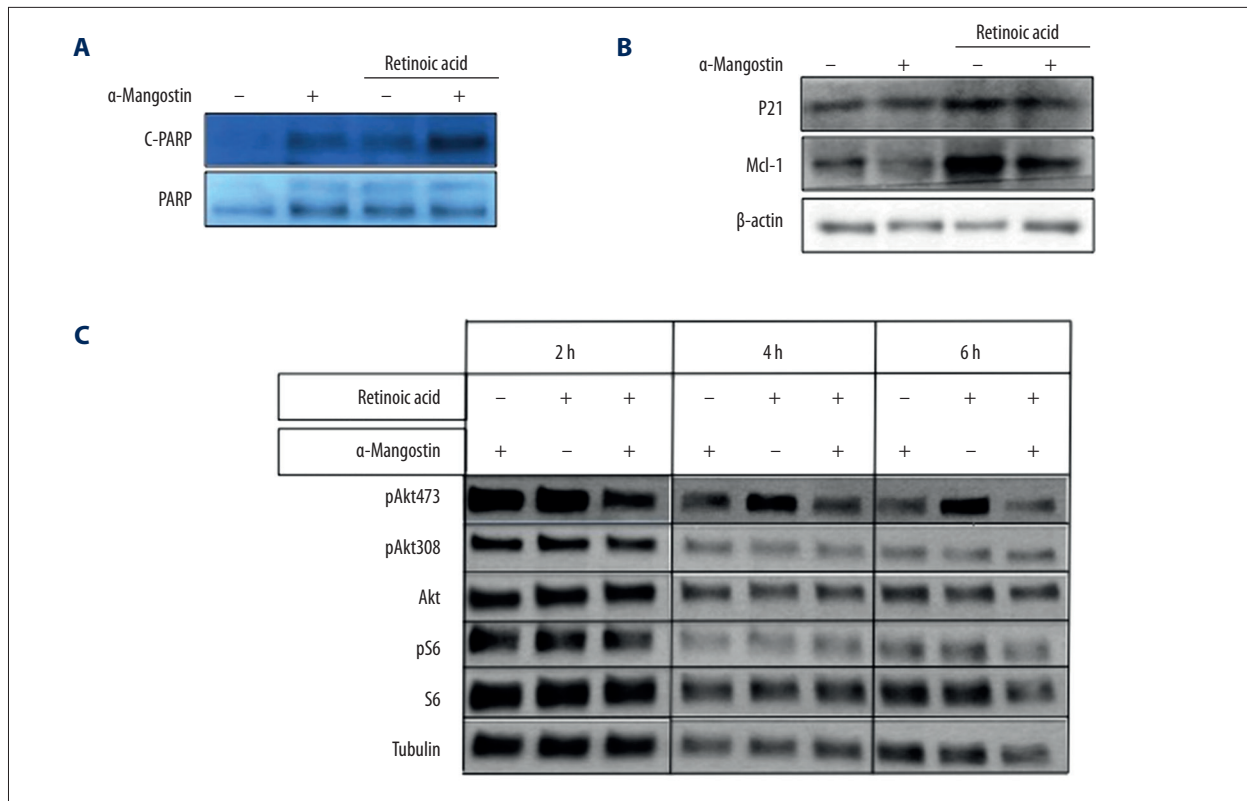
#### The combined effect of $\alpha$ -Mangostin and retinoic acid on ROS level

Since the generation of ROS is known to induce apoptosis in melanoma cells [28], we investigated whether the combination

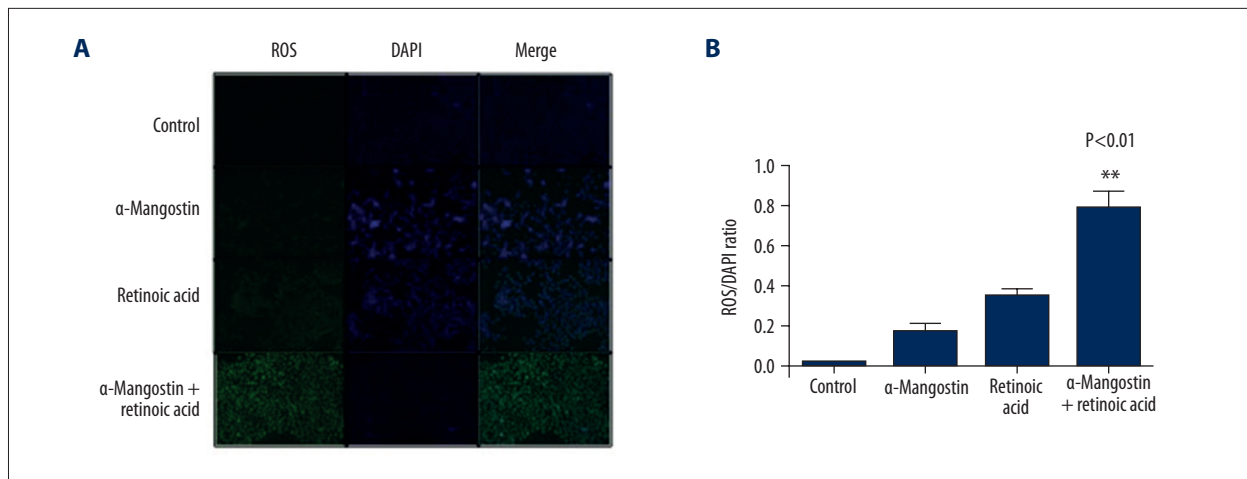
of  $\alpha$ -Mangostin and retinoic acid-induced apoptosis is related to ROS generation. For this, we treated sk-mel-2 cells with  $\alpha$ -Mangostin, retinoic acid, or their combination. The combination treatment resulted in marked induction of ROS generation at 8 h, signifying that the combination-induced apoptosis is mediated through ROS generation (Figure 5). Therefore,  $\alpha$ -Mangostin might sensitize melanoma to the apoptotic effect of retinoic acid through its induction of ROS level.

## Discussion

The incidence of melanoma is rapidly increasing around the world [29]. BRAF mutations contribute to ~50% of cutaneous melanomas, which is the most common type [30]. In contrast, NRAS mutations are detected in ~20% of cutaneous melanomas, with advanced high-risk tumor features and low survival rate in clinical melanoma patients. Rapid and successful advances have been made in targeted therapies of BRAF mutations; however, there are still no effective therapeutics against



**Figure 4.** α-Mangostin is synergistic with retinoic acid on inhibiting apoptosis-related genes and AKT signaling pathway. **(A)** The expression of cleaved PARP and PARP were analyzed by Western blot in sk-mel-2 cells exposed to retinoic acid (2 μM) in either the presence or absence of α-Mangostin (5 μM) for 8 h. The expression of cleaved PARP was increased with the combination of retinoic acid and α-Mangostin. **(B)** As anti-apoptotic proteins, MCL-1 and P21 expression levels were suppressed with retinoic acid (2 μM) and α-Mangostin (5 μM) treatment for 8 h in sk-mel-2 cells. **(C)** Down-regulation of phosphorylation of AKT and S6 is much more significant within 2 h after the treatment with the combination of retinoic acid (2 μM) and α-Mangostin (5 μM) than by single agents.



**Figure 5.** α-Mangostin enhanced the apoptotic effect of retinoic acid through its induction of ROS level. **(A)** SK-MEL-2 cells were treated with retinoic acid (5 μM), α-Mangostin (5 μM), or their combination for 8 h. High levels of ROS were detected in the combination group. Reactive oxygen species (ROS) production was detected by staining with dichlorofluorescein diacetate (DCFDA), which turns into a fluorescent compound (green) upon oxidation and all the cells were stained for the nucleus (blue, DAPI). Representative images of 3 independent experiments are shown. **(B)** The numbers of positive cell were counted. The paired *t* test was used to assess statistical significance (P=0.005).

NRAS mutant melanomas [31]. NRAS is an important oncogene in the biology of melanomas that contain NRAS mutations [32]; therefore, it is necessary to understand the related mechanisms and the benefits of multiple treatments to guide therapeutics against different types of melanoma. Some studies have lighted the possibility that both the RAS/RAF/MEK/ERK and the PI3K/AKT pathways are necessary for inducing and maintaining the malignant phenotype in NRAS mutant melanomas [33,34]. To date, there is no targeted therapy that directly targets NRAS. Many approaches have been addressed and studied, including various kinds of combined inhibitors, such as the combined use of MEK inhibitors and RAF inhibitors, and the combination of PI3K pathway inhibitors with ERK pathway inhibitors, and some of them are currently being evaluated in clinical trials [7,35]. There is evidence that combined targeting of the PI3K and ERK pathways may enhance the responses in NRAS mutant melanoma [36], that the activation of AKT is required for melanoma initiation, and that the binding of RAS to PI3K p110 plays a critical role in the tumorigenesis of RAS mutant melanoma [37]. The above evidence suggests that combined therapeutics are necessary for the treatment of melanoma, especially that with high incidence of drug resistance.

Many studies have shown that retinoic acid can inhibit cell proliferation and induce cell differentiation in a variety of human cancers, and its use in cancer therapy has been extensively studied in recent decades [38,39]. Most retinoids show limited clinical efficacy in treatment of solid malignancies; they have adverse effects and are associated with frequent development of resistance [40]. Much effort has been spent on developing use of retinoic acid alone or in combination with other related chemotherapeutic agents to keep its antitumor activity, as well as to minimize the toxicity in the prevention and treatment of cancers [14]. There is evidence that retinoic acid can induce growth arrest and differentiation in a B16 murine melanoma cell model, and its derivatives have been used for treatment of primary melanoma; however, the response of different melanoma cell lines against retinoic acid is quite variable [41]. There is a growing need for novel therapeutics, such as enhancing antitumor activity with combined chemotherapeutic agents.

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Our studies addressed an innovative combination with  $\alpha$ -Mangostin, a natural product and the active component of some commercial nutraceuticals. Although it has been extensively studied for its wide range of biological activities, including antitumor, antioxidant, anti-inflammatory activities, the details of the mechanisms related in its activities are currently unknown [42]. Previous research has shown that  $\alpha$ -Mangostin can inhibit the proliferation of several melanoma cell lines *in vitro* [17], probably through the inhibition of the PI3K/mTOR pathway [43]. Our lab has studied melanoma for many years; we have identified  $\alpha$ -Mangostin as a candidate pigmentation inhibitor from a large numbers of compounds using a high-throughput, high-content screening approach, showing the possibility of  $\alpha$ -Mangostin as an anti-melanoma agent [44]. We therefore screened a kinase-specific inhibitor compound library in human melanoma cell lines, including BRAF mutant and NRAS mutant melanoma, to explore the effect of  $\alpha$ -Mangostin on enhancing inhibition and reversing chemo-resistance to the kinase-specific inhibitors. We found  $\alpha$ -Mangostin can significantly enhance the inhibition of retinoic acid in an NRAS mutant melanoma cell line and further verified this effect in terms of the decreasing phosphorylation levels of related proteins in the PI3K pathway, as well as through its induction of ROS level [45]. The specific mechanism is unclear, but there is evidence that CF31 can inhibit RXR (retinoid X receptor) signaling through its unique RXR- $\alpha$  binding mode [46]. We hypothesize that  $\alpha$ -Mangostin, as a kind of xanthone, may act synergistically with retinoic acid through targeting the binding or activity of retinoid-related receptors [47].

## Conclusions

The need for novel, natural, and effective chemotherapies for NRAS mutant melanoma is growing. Our studies on the details of the mechanism are underway, and a number of approaches are being developed. Our novel combination therapy with natural products holds promise.

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