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# Anthocyanin-rich *Mulberry* extract inhibit the gastric cancer cell growth *in vitro* and xenograft mice by inducing signals of p38/p53 and c-jun

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ABSTRACT

Anthocyanins have been well characterized by various bioactive properties. In previous studies, *Mulberry* anthocyanins (MACs) have proven to prevent atherosclerosis and inhibit melanoma metastasis. Here, AGS cells demonstrated an increase in the distribution of hypodiploid phase (apoptotic peak) after treatment with MACs. Further investigation revealed that MACs exerted their influence by inducing intrinsic and extrinsic apoptosis through p38/p53 and p38/c-jun signaling pathways. In addition, the caspase-related protein, such as caspase-3, was activated from pro-caspase to cleaved-caspase by treating MACs to AGS cells. We also used the experimental AGS gastric cancer xenograft model to verify the inhibitory effect of MACs. These findings suggest that, by targeting p38/p53 and the c-jun pathways, MACs suppressed cell survival and tumorigenesis, but induced apoptotic death in AGS cells. MACs can potentially prevent the growth of AGS cells for ineffective conventional chemotherapy of gastric carcinoma.



## 1. Introduction

Cancers of the gastrointestinal (GI) tract remain to be the second most common type of cancer and cause nearly half of the cancer-related deaths in the world (Jemal et al., 2005). In Asian, patients receiving conventional therapies including surgery, chemotherapy, and radiotherapy for gastric cancer have poor prognosis, with 5-year survival rate of less than 20% (Parkin, Bray, Ferlay, & Pisani, 2005). Recent studies attempting to understand the molecular changes in cancer, have expedited the development of targeted therapy specific to cancer cells. Among the environmental factors, salted foods and infection with *Helicobacter pylori* are the most common suspects in intestinal and diffuse gastric carcinogenesis (Strumylaite, Zickute, Dudzevicius, & Dregval, 2006). Loss of E-cadherin, ELF (a β-Spectin adaptor protein), and RUNX function results in diffuse gastric cancer (Li et al., 2002a, 2002b; Mishra et al., 1998). The carcinogenesis of intestinal gastric carcinoma appears to be more intricate. *H. pylori* infection leads to the abnormal expression of ELF, Ras, Smad4, RUNX, CDX2 and TGF-β (Kretzschmar, Doody, Timokhina, & Massague, 1999; Li et al., 2002b; Mishra et al., 1998; Xu et al., 2000). Furthermore, p53 alteration could be involved in the development of both types of gastric carcinomas (Qiao & Wong, 2009).

Early research has revealed that avoidance of apoptosis is another essential factor in the development of gastric carcinoma (Lee et al., 2005). Fas (CD95) and its ligand (FasL) are constitutively expressed in the GI tract, whereas mice lacking Fas have a limited life span after *H. pylori* infection (Cai et al., 2005). In GI cancer, Bcl-2 family proteins are highly expressed (de Bono & Rowinsky, 2002). Soung et al. (2005) have suggested that caspase-8 gene mutation could affect the pathogenesis of gastric cancers. Therefore, selective targeting of gastric cancer cells by inducing cancer cells apoptosis may be another way for inhibiting carcinogenesis (Lee et al., 2005).

Numerous studies have demonstrated the effects of several pure anthocyanins and anthocyanin-rich extracts from berries and grapes have exhibited pro-apoptotic effects in multiple cell types through both apoptotic pathways (Afaq et al., 2007; Seeram et al., 2006). Our recent studies have found that *Hibiscus* anthocyanin extracts mediated the apoptosis of human promyelocytic leukemia cells via the p38/Fas and Bid pathway (Chang, Huang, Hsu, Yang, & Wang, 2005). *Mulberry*, or *Morus alba* L., is a traditional Chinese edible fruit that is used effectively in folk medicine

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because of its antibacterial, antibiotic, anti-inflammatory, antioxidative, and immune system-stimulating properties. Like those extracted from *Hibiscus*, *Mulberry* extracts contain high amounts of anthocyanins, which have been reported to have antitumour effects *in vitro* and *in vivo* (Huang, Shih, Chang, Hung, & Wang, 2008). Here, and our findings suggest that MACs may be active agents against gastric carcinoma in terms of proliferation, survival, and tumourigenesis under both *in vitro* and *in vivo* conditions by inducing apoptosis via the activation of p38/jun/Fas/FasL and p38/p53/Bax cell death pathways.

## 2. Materials and methods

### 2.1. Cell culture and reagents

The AGS cell line was obtained from the Bioresource Collection and Research Center (BCRC), and maintained in F-12 medium containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. SB203580, PD098059 and wortmannin were purchased from Sigma (St. Louis, MO, USA). These inhibitors were stored in dimethyl sulfoxide (DMSO) and added to the culture medium to a final concentration as described in the figure legends. Polyclonal antibody against phospho-p38 MAP kinase (Thr180/Tyr182) and phospho-c-Jun (Ser-73) were purchased from Cell Signaling Technology (Beverly, MA, USA), and phospho-p53 (Ser392) was obtained from Transduction Lab (Lexington, KY). Antibodies against FAS (FL-335), FAS-L (C-178), caspase-8 (H-134), caspase-3 (H-277), BID (C-20), Bcl-2 (N-19), Bax (P-16), and cytochrome *c* (A-8) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal mouse antibody with reactivity to human cytochrome oxidase subunit IV was purchased from Molecular Probes (Eugene, OR, USA). Horseradish peroxidase-conjugated anti-mouse secondary antibodies were purchased from NEN Life Science Products, Inc. (Boston, MA, USA). Anti-rabbit, anti-β-actin, and anti-α-tubulin secondary antibodies were purchased from Sigma.

### 2.2. Preparation of Mulberry anthocyanins

MACs were prepared from the lyophilized fruit of *Mulberry* (100 g), with a 3-fold volume of methanol containing 1% HCl for 1 day at 4 °C. The extract was filtered and then concentrated under reduced pressure at 30 °C. The precipitate was collected and stood on an Amberlife Diaion HP-20 resin column for 24 h. It was then cleaned in distilled water (5 L) containing 0.1% HCl solution and eluted with methanol. The filtrate was collected and lyophilized to obtain 5 g of MACs and stored at 4 °C before use (Huang et al., 2008). The use of standards for HPLC was cyanidine-3-glucoside and cyanidine-3-rutinoside. The total anthocyanin content was determined using the pH differential method. The yield of *Mulberry* extracts and total anthocyanin content of the extracts are summarized in Meng et al. (2008) (Liu, Lee, Shih, Chyau, & Wang, 2008).

### 2.3. Cell viability and apoptosis assay

Cells were seeded at a density of $1 \times 10^5$ cells/mL in a 24-well plate for 24 h. Then they were treated with MACs at various concentrations (0, 1, 2, 3, 4, and 5 mg/mL) for various durations (1 day, 2 days, and 3 days). To evaluate the cytotoxicity of MACs, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed to determine cell viability. After the addition of MTT solution (5 mg/mL) to the medium for 4 h, the formazan was dissolved in isopropanol and measured spectrophotometrically at 563 nm. To quantify apoptosis, the ApopTag™ in situ apoptosis detection kit (Oncor, Gaithersburg, MD) was used as per the instructions of the manufacturer. The ApopTag kit detects DNA strand breaks in single cells by terminal transferase-mediated dUTP-digoxigenin-end labeling (TUNEL).

### 2.4. Cell cycle analysis

Flow cytometric analysis of AGS cells was performed using a FAScan (Becton Dickinson Immunocytometry System, UK) after 24 h of culturing. Cell suspension was centrifuged at 1500 rpm for 5 min after two washes with PBS solution. By adding 1 mL of 70% methanol to the pellet and incubating at −20 °C for at least 24 h, 1 mL of cold propidium iodide (PI) stain solution (20 mg/mL PI, 20 mg/mL RNase A, and 0.1% Triton X-100) was added to the cells and incubated for 15 min in the dark. PI was excited at 488 nm and the fluorescence signal was subjected to logarithmic amplification with PI fluorescence (red) being detected above 600 nm. Cell cycle distribution is presented as the number of cells versus the amount of DNA as indicated by the intensity of fluorescence, and the percentage of apoptotic cells (sub-G1 phase) was determined with CELLQuest Version 3.3 software.

### 2.5. Western blotting analysis

Whole-cell or tumor lysates (50 μg purified protein) were mixed with a 5X sample buffer and boiled for 10 min. Then, an equal protein content of total cell lysate was resolved on 10% or 12% SDS–PAGE gels. Separated proteins were then transferred onto nitrocellulose membranes (Millipore, Bedford, MA) by electroblotting using an electroblotting apparatus (Bio-Rad). Nonspecific binding of the membranes was blocked with Tris-buffered saline (TBS) containing 1% (w/v) nonfat dry milk and 0.1% (v/v) Tween-20 (TBST) for more than 2 h, and then incubated with specific primary antibodies and with an appropriate secondary antibody (horseradish peroxidase-conjugated goat antimouse or antirabbit IgG). After washing the membrane, band detection was conducted by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed ECL hyperfilm in FUJFILM Las-3000 (Tokyo, Japan). Protein quantitative was determined by densitometry using FUJFILM-Multi Gauge V2.2 software.

### 2.6. Animal and tumor xenograft study

Balb/c nude mice (male, 5 weeks old) were purchased from the National Taiwan University Animal Center, Taiwan. Mice were randomly selected to be placed in one of the five treatment groups (four mice in each group). Mice were s.c. injected with $2 \times 10^6$ AGS cells mixed with equal volume of matrigel (BD Biosciences) on the right groin of each mouse and with matrigel alone on the left groin as control. The first group served as control and was fed with autoclaved drinking water. Meanwhile, the second and third groups (shown as 0.1IM and 0.2IM, respectively) were injected with 0.1% or 0.2% MACs at the right groin near the tumor cells (IM: Injected MACs). The fourth and fifth groups (shown as 0.1OM and 0.2OM, respectively) were fed with 0.1% MACs or 0.2% MACs every day using an oral gavage (OM: Oral MACs). The last control group with no inoculation of AGS cells was fed with 0.2% MACs upon completion of the experiment. During the 7-week feeding period, all mice used were handled according to the guidelines of the Instituted Animal Care and Use Committee of Chung Shan Medical University (IACUC, CSMC) for the care and use of laboratory animals. Mice were housed with a regular 12 h light/12 h dark cycle. After 49 days, the mice were sacrificed for the assay of tumorigenicity (e.g., body weight and tumor volume) and related proteins (e.g., Western blotting).

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### 2.7. Statistical analysis

Data were expressed as means ± SD of three independent experiments which were performed by Student's *t*-test (Sigmaplot 2001). Significant differences were established at $p \leqslant 0.05$.

## 3. Results

### 3.1. Characterization of anthocyanin compounds of MACs

To establish the ingredients of MACs from *Mulberry*, the anthocyanin contents were determined by HPLC and spectrophotometer. As seen in Fig. 1, HPLC analysis of the identified anthocyanic compounds showed that the retention times of cyanidins-3-glucoside and cyanidins-3-rutinoside were 29.9 and 31.5 min, respectively. According to our previous study, the anthocyanin purity of MACs was approximately 89% (Liu et al., 2008). After characterized by HPLC, 1 g MACs included 301.75 ± 7.96 mg cyanidine-3-glucside and 108.79 ± 3.35 mg cyanidine-3-rutinoside (Fig. 1). Conversion of the above results revealed that 46.13% of cyanidine content was obtained in MACs.

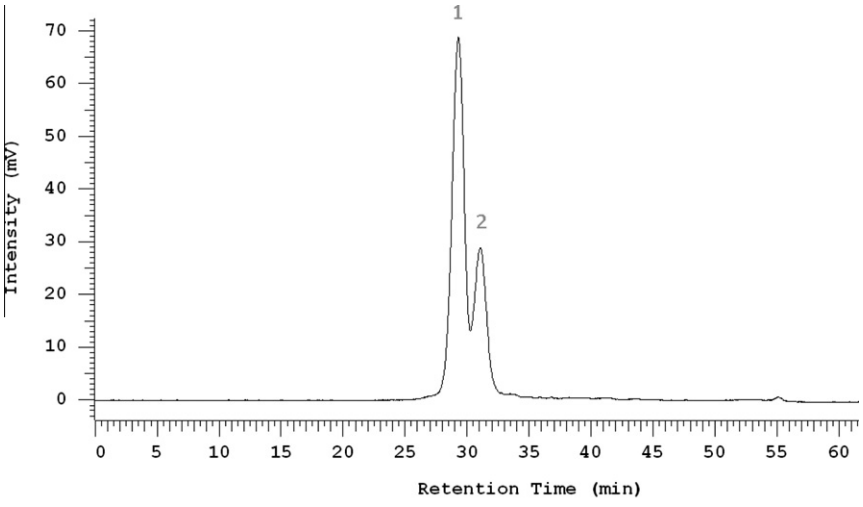
### 3.2. MACs induce apoptosis in human gastric carcinoma AGS cells

We first investigated whether MACs could reduce tumorigenicity through inhibiting tumor cells survival. According to the absorbance in the MTT assay, Fig. 2A displayed the cell viability of MACs to AGS cells at different concentrations (0–3 mg/ml) for 12, 24 and 48 h. The concentration of MACs inhibiting 50% of AGS cell viability ($IC_{50}$) after MACs exposure for 24 h was 2.88 mg/ml. The membranes of the cells treated with MACs were blurry and less tentacle (data not shown), indicating the possibility that MACs induced degeneration, apoptosis, necrosis or transformation of the cells. Accordingly, the doses of 1–3 mg/ml for 24 h were selected for the following *in vitro* experiments.

Apoptosis is a form of programmed cell death characterized by cytoplasmic condensation, plasma membrane blebbing and nuclear pycnosis. It leads to nuclear DNA breakdown into multiples of ~200–500 bp oligonucleosomal size fragments. The examination of apoptosis in cultured cells relies on techniques involving the detection of DNA damage by end labeling using TUNEL assay and the analysis of apoptotic states using flow cytometry. After treatment of MACs for 24 h, AGS cells demonstrated a concentration-dependent increase in DNA damage (Fig. 2B). To further illustrate the possible mechanism of MACs-mediated apoptosis, AGS cells were preincubated with 5 nM wortmannin (PI3K inhibitor), 25 μM PD98059 (MEK1 inhibitor) and 20 μM SB203580 (p38 inhibitor), respectively. As shown in Fig. 2C, in AGS cells treated with SB203580 followed by a 24 h exposure to 3 mg/ml MACs, the level of sub-G1 phase exhibited DNA content similar to the exposure of AGS cells to SB203580 alone. Together, these observations strongly suggest that MACs are involved in the p38 signaling and induce apoptosis in AGS cells.

### 3.3. MACs induce cell death through both extrinsic and intrinsic apoptosis pathways

Next, the effect of MACs on p38-related pathways was analyzed using Western blotting. Phosphorylated levels of p38, c-jun and p53 were raised in a time-dependent manner, suggesting that intrinsic and/or extrinsic-related apoptosis were switched (Fig. 3A). To further confirm whether the MACs-induced apoptosis is regulated by the activation of the p38 pathway, AGS cells were treated in the absence or presence of SB203580 with MACs for 2 h. Indeed, the phosphorylated levels of p38, c-jun and p53 were significantly restored (Fig. 3B). Consistent with these results, immunoblot analysis showed a strong dose-dependent increase in Fas and FasL levels as well as prominent upregulation of cleaved caspase-8 and caspase-3 in AGS cells treated with MACs for 24 h (Fig. 4A and B). Moreover, the expression of phosphorylated p53 prompted us to assess the effect of MACs on Bcl-2 family proteins, such as Bcl-2, Bax and t-Bid, because these proteins activated by p53 have been shown to play a role in the intrinsic apoptosis of cells. MACs triggered the intrinsic apoptotic pathway by attenuating the expression of Bcl-2 and increasing the levels of Bax and t-Bid to induce the cytochrome *c* release from mitochondria to the cytoplasm (Fig. 4B). Fig. 4C revealed that MACs and SB203580 co-treatment were significantly decreased FasL expression. These




| Peak | Anthocyanic composition | Content (mg/g sample) |
|---|---|---|
| 1 | cyanidine-3-glucoside chloride | 301.75±7.96 |
| 2 | cyanidine-3-rutinoside chloride | 108.79±3.35 |

**Fig. 1.** HPLC chromatogram of MACs. Chromatograms monitored at 518 nm, which corresponded to identify anthocyanic compositions. Anthocyanic compositions corresponding to peaks are marked. Peaks: 1. Cyanidine-3-glucoside; 2. Cyanidine-3-rutinoside.

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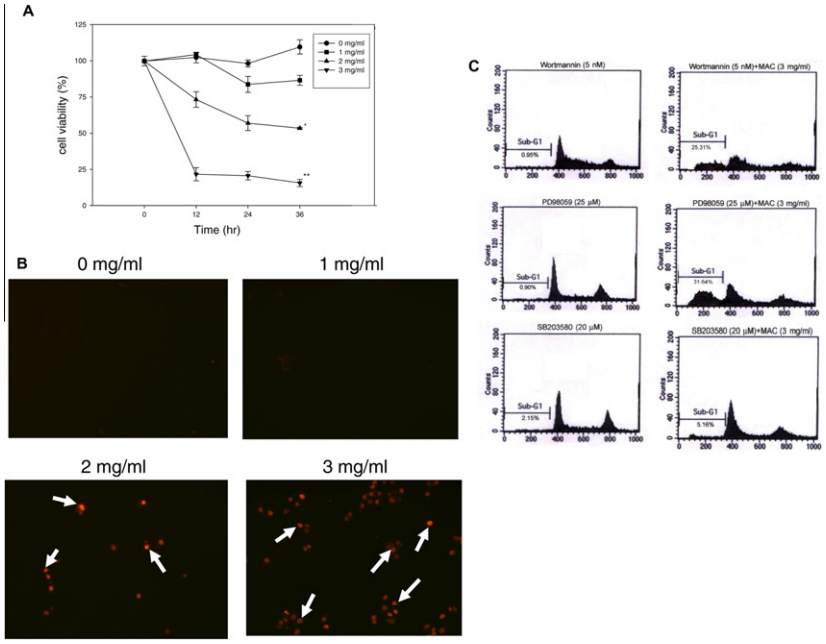


**Fig. 2.** Effect of MACs induced apoptosis in AGS cells. AGS cells were treated with different concentration for 0, 12, 24, 36 h. Cells viability at each time point was assessed (A). Cultured cells were treated with or without MACs for 24 h, the apoptotic cells were studied by TUNEL assay (B). The arrows indicated the apoptotic cells. Cell cycle distribution of AGS preincubated for 1 h with various MAP kinase inhibitors, including wortmannin (5 nM), PD98059 (25 μM) and SB203580 (20 μM), and then incubated for 24 h with MACs (3 mg/ml) (C). The DNA content was analyzed using flow cytometry. The values are means ± SD, $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.005$ was compared with control.

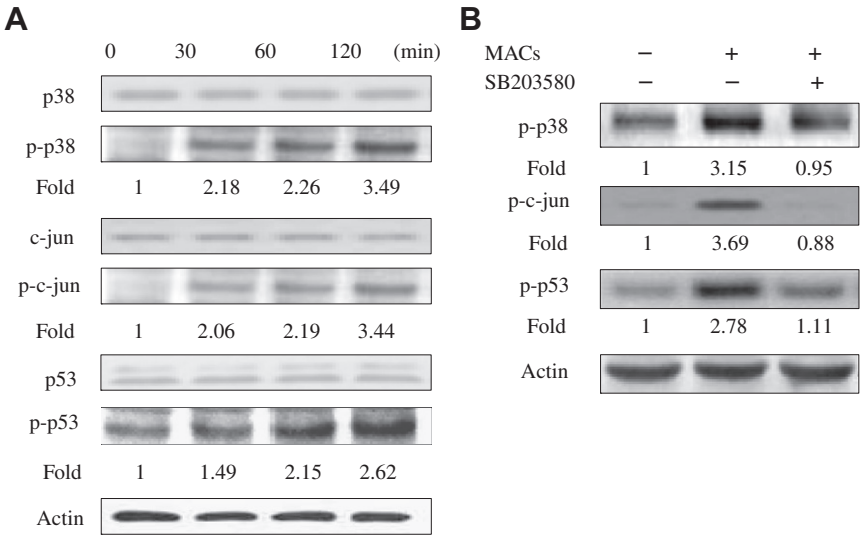


**Fig. 3.** Time course of MACs-induced MAP kinases activation in AGS cells. AGS cells were treated with MACs (3 mg/ml) for the time indicated and assayed for MAP kinases activation. Proteins extracts were prepared at the incubated time points to assess the activation of p-p38, p-c-jun, and p-p53 (A). The cells were treated in the absence or presence of p38 MAPK inhibitor (SB203580) (20 μM, 1 h) with MACs (3 mg/ml) for indicated times (B). The levels of proteins were analyzed using Western blotting. The figure is a representative of three independent experiments with similar results.

results hint that MACs induce apoptosis via the activation of p38/jun/Fas/FasL and p38/p53/Bax signaling mechanisms.

### *3.4. MACs inhibit AGS tumor xenograft growth in nude mice with in vivo*

A modified *in vivo* xenograft model served as a reliable tool for examining the presence of tumorigenesis (Szepeshazi et al., 2003). Experimental results showed no significant changes in body weight, diet consumption, and water drinking (data not shown) in both experimental and control animals. Moreover, we did not find any adverse effects in terms of general behavior of the animals fed or injected with MACs, as compared with the control animals throughout the experiment. Regarding the anticancer efficacy of MACs, all mice inoculated with AGS cells started growing tumors of 2 $mm^3$ after 3 weeks of treatment, and reduced tumor volumes were observed in groups receiving 0.1IM, 0.2IM, 0.1OM and 0.2OM treatment in week 5 (Fig. 5A). By the end of the study, as shown in Fig. 5, tumorigenicity was inhibited by 0.2IM and 0.2OM treatment, whereas the low-dose MACs treatments showed no effect on

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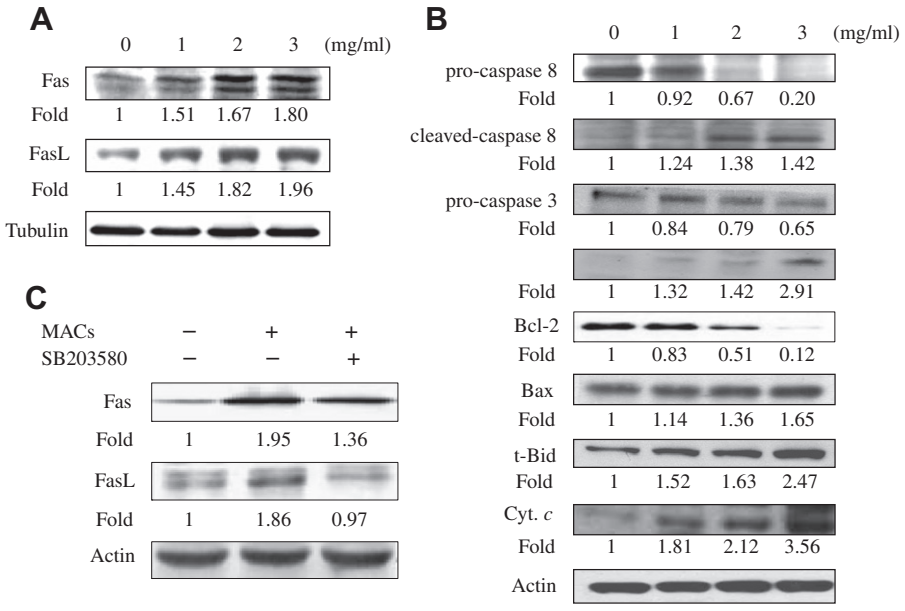


**Fig. 4.** Involvement of proteins related to the apoptotic signaling in MACs-induced apoptosis. Cells were treated with as described in the section 2 and analyzed using immunoblotting with Fas, FasL (lower band) (A), cleaved-caspase-8/−3 and pro-caspase-8/−3, Bax, t-Bid and cytochrome *c* (Cyt. *c*) (B). The cells were treated in the absence or presence of SB203580 (20 μM, 1 h) with MACs (3 mg/ml) for indicated times, then Fas and FasL (lower band) expressions were analyzed by Western blotting (C). Actin was the loading control. The data were representative of three independent experiments with comparable observations.

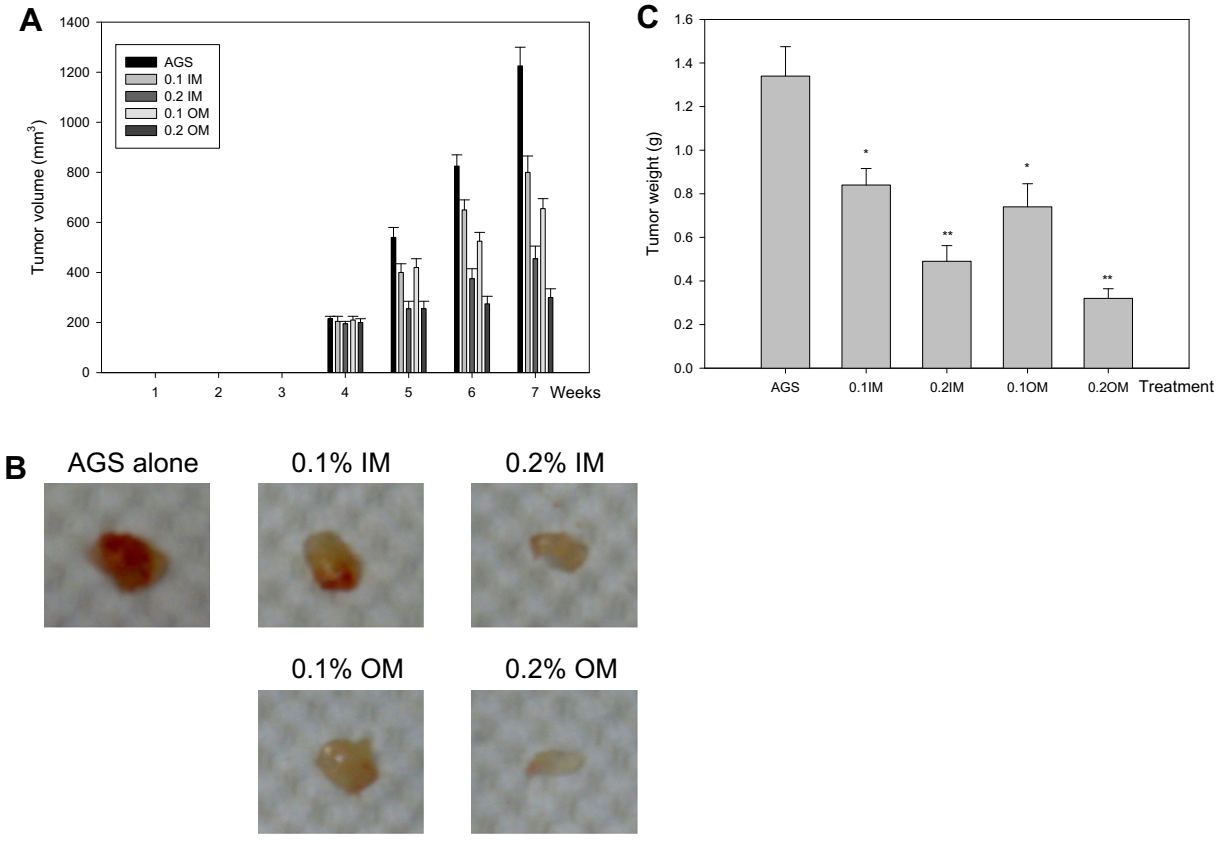


**Fig. 5.** Effect of MACs on inhibiting tumorigenesis *in vivo*. Tumorigenicity of the AGS cells as above in Balb/c nude mice. The volumes of tumors were monitored at the indicated time (A). Photographs of individual mice inoculated with AGS cells (B). The tumor weights were detected after sacrificed (C). Data were presented as mean ± SD from four mice. $^{*}p < 0.001$; $^{**}p < 0.0001$ versus AGS group.

tumor inhibition. Tumor weight per mouse decreased from 1.34 ± 0.135 g in the AGS group and to 0.49 ± 0.072 g and 0.32 ± 0.044 g in the 0.2IM and 0.2OM groups, respectively (Fig. 5B and C).

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## 4. Discussion

Anthocyanins occur ubiquitously in the plant kingdom and are responsible for most of the bright red, blue, and purple colors of vegetables and fruits. The amount of anthocyanins in the US diet is estimated to be between 180 and 215 mg per day (Hertog, Hollman, Katan, & Kromhout, 1993). However, there is great difference in dosage tolerance between humans and animals. Briefly, animals have greater dosage tolerance than humans. Based on the National Health Administration applies for the norm of health food in Taiwan, the ingestion of dried food for an adult is 500 g per day. After calculation, the effective dose of MACs to an individual is 1 g per day, which serves 410.54 mg cyanidine. In addition, Frank and his work partners studied the absorption of *Hibiscus sabdariffa's* anthocyanidin in humans (Frank et al., 2005). Subjects were given a single oral dose of 10 g *H. sabdariffa* L. extract in 150 ml water. After 3 h, the plasma concentration of total anthocyanins under the curve estimate was 7.37 ng/ml. No side effect was found in subjects, indicating that humans have high tolerance to anthocyanins. Our study has demonstrated that MACs could reduce tumor growth and tumor volume of AGS-bearing Balb/c nude mice. In the future clinical study, the 1 g/ day MACs that applies in human is workable.

Epidemiologic investigations suggest that the consumption of anthocyanins lowers the risk of cardiovascular disease, diabetes, arthritis and cancer due to their anti-oxidant and anti-inflammatory activities by reducing the deleterious effects of reactive oxygen species (Prior & Wu, 2006). Therefore, anthocyanins are not only food products but also therapeutic agents. In our previous studies, MACs showed strong anti-oxidative ability against LDL-oxidation and macrophage-derived foam cell formation (Liu et al., 2008), and inhibited melanoma metastasis by triggering Ras/PI3K pathway (Huang et al., 2008). In the present study, we demonstrated that anthocyanins extracted from *Mulberry* had notable promotive effects on p38/jun/Fas/FasL and p38/p53/Bax signaling pathways, which accounted for its *in vitro* and *in vivo* growth-inhibitory and apoptotic responses in AGS cells.

In a stable mature tissue, the rates of replication and cell death are balanced and regulated by checkpoints at the major stages of the cell cycle. If any one of the checkpoints is annulled, cells can be prone to natural or induced mutations and the damaged DNA cannot be repaired. With accumulated genetic alterations, mutated cells escape apoptotic control and become the progeny of neoplastic cell population through multistage processes. This is referred to as carcinogenesis (Coleman & Tsongalis, 2006). Thus, an agent that can selectively induce apoptosis in cancer cell is potentially useful in cancer therapy. Fimognari et al. demonstrated that cyaniding-3-glucoside induced apoptosis in two human leukemia cells by increasing the levels p53 and Bax and reducing Bcl-2 expression (Fimognari, Berti, Nusse, Cantelli-Forti, & Hrelia, 2004). The induction of apoptosis by resveratrol has been reported to be associated with increased caspase activity, decreased Bcl-2 and Bcl-XL levels, and increased Bax levels (Billard et al., 2002). Interestingly, these pro-apoptotic actions are associated with the activation of p53. Consistent with these, MACs-induced apoptosis was found to be related to the downregulation of Bcl-2 and overexpression of Bax and t-Bid after p53 phosphorylation. To evaluate the role of MAPKs in the apoptotic receptor pathway, we provided proof of the involvement of p38 in the signaling pathway of apoptotic activity induced by MACs using wortmannin, PD98059, and SB203580 MAPK inhibitors for PI3K, ERK-1/2, and p38. In addition, we also observed an increase in phosphorylation of c-jun in exposure of AGS cells to MACs. Both activation of p53 and c-jun led to the activation of Fas/FasL and promoted the recruitment of initiator procaspase-8 (Lin, Huang, Huang, Chen, & Wang, 2005; Xia, Dickens, Raingeaud, Davis, & Greenberg, 1995). Released cytochrome *c* induced by Bax and activated caspase-8 resulted in an apoptotic death pathway. Therefore, in contrast to several recently developed proapoptotic receptor agonists (PARAs), such as mapatumab, sorafenib, cisplatin and doxorubicin, which have specific targets (Lacour et al., 2001; Meng et al., 2007; Tolcher et al., 2007), MACs affect multiple apoptotic signaling, such as the induction of intrinsic, extrinsic and caspase cascade pathways (Figs. 2–4).

Generally speaking, patients receiving conventional therapies, including surgery, radiotherapy and chemotherapy, for gastric cancer have poor prognosis (Khan & Shukla, 2006). Traditional cancer therapy, which predominantly involves cytotoxic chemotherapeutic agents, is often accompanied by substantial adverse effects. Targeting apoptosis or angiogenesis as therapeutic approaches to gastric cancer has recently provided a new strategy for gastric cancer therapy. Wang and Stoner pointed out that anthocyanins display a wide range of biological activities, including antioxidant, anti-inflammatory, anti-cell proliferative, anti-angiogenic, and anti-invasive activities, as well as the induction of apoptosis and chemopreventive effects (Wang & Stoner, 2008). We have shown that MACs help prevent gastric tumorigenesis in a mouse model, suggesting that increasing the oral intake of natural MACs provides protection against gastric carcinoma in humans and that direct delivery of MACs may be useful in gastric cancer therapeutics.

## 5. Conclusion

In the past decade, fruits and vegetables enriched anthocyanins were demonstrated the effects of anti-tumorigenesis in different mechanisms. Here, this study revealed that the extracts of Mulberry, MACs, inhibited the growth of human gastric carcinoma cells. MACs induced apoptosis in AGS cells through not only extrinsic (p38/Fas/FasL/caspase 8 signaling) but also intrinsic (p38/p53/Bax signaling) apoptotic pathway. The phosphorylated levels of p38, c-jun, p53, Fas and FasL were significantly restored after treatment SB203580, a p38 protein inhibitor, further confirmed that the MACs-induced apoptosis is regulated by the activation of the p38 pathway. Moreover, 0.2% MACs by both injection and oral gavage showed the effect on tumor inhibition. This study suggests that the Mulberry anthocyanins can be a potentially therapy agent in preventing the gastric carcinoma formation related to inducing various apoptotic pathways.

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## References

Afaq, F., Syed, D. N., Malik, A., Hadi, N., Sarfaraz, S., Kweon, M. H., et al. (2007). Delphinidin, an anthocyanidin in pigmented fruits and vegetables, protects human HaCaT keratinocytes and mouse skin against UVB-mediated oxidative stress and apoptosis. *The Journal of Investigative Dermatology, 127*(1), 222–232.

Billard, C., Izard, J. C., Roman, V., Kern, C., Mathiot, C., Mentz, F., et al. (2002). Comparative antiproliferative and apoptotic effects of resveratrol, epsilon-viniferin and vine-shots derived polyphenols (vineatrols) on chronic B lymphocytic leukemia cells and normal human lymphocytes. *Leukemia and Lymphoma, 43*(10), 1991–2002.

Cai, X., Stoicov, C., Li, H., Carlson, J., Whary, M., Fox, J. G., et al. (2005). Overcoming Fas-mediated apoptosis accelerates Helicobacter-induced gastric cancer in mice. *Cancer Research, 65*(23), 10912–10920.

Chang, Y. C., Huang, H. P., Hsu, J. D., Yang, S. F., & Wang, C. J. (2005). Hibiscus anthocyanins rich extract-induced apoptotic cell death in human promyelocytic leukemia cells. *Toxicology and Applied Pharmacology, 205*(3), 201–212.

Coleman, W. B., & Tsongalis, G. J. (2006). Molecular mechanisms of human carcinogenesis. *EXS* (96), 321–349.

de Bono, J. S., & Rowinsky, E. K. (2002). Therapeutics targeting signal transduction for patients with colorectal carcinoma. *British Medical Bulletin, 64*, 227–254.

Fimognari, C., Berti, F., Nusse, M., Cantelli-Forti, G., & Hrelia, P. (2004). Induction of apoptosis in two human leukemia cell lines as well as differentiation in human promyelocytic cells by cyanidin-3-o-beta-glucopyranoside. *Biochemical Pharmacology, 67*(11), 2047–2056.

Frank, T., Janssen, M., Netzel, M., Strass, G., Kler, A., Kriesl, E., et al. (2005). Pharmacokinetics of anthocyanidin-3-glycosides following consumption of *Hibiscus sabdariffa* L. extract. *Journal of Clinical Pharmacology, 45*(2), 203–210.

Hertog, M. G., Hollman, P. C., Katan, M. B., & Kromhout, D. (1993). Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutrition and Cancer, 20*(1), 21–29.

Huang, H. P., Shih, Y. W., Chang, Y. C., Hung, C. N., & Wang, C. J. (2008). Chemoinhibitory effect of mulberry anthocyanins on melanoma metastasis involved in the Ras/PI3K pathway. *Journal of Agricultural and Food Chemistry, 56*(19), 9286–9293.

Jemal, A., Murray, T., Ward, E., Samuels, A., Tiwari, R. C., Ghafoor, A., et al. (2005). Cancer statistics, 2005. *CA: A Cancer Journal for Clinicians, 55*(1), 10–30.

Khan, F. A., & Shukla, A. N. (2006). Pathogenesis and treatment of gastric carcinoma: An up-date with brief review. *Journal of Cancer Research and Therapeutics, 2*(4), 196–199.

Kretzschmar, M., Doody, J., Timokhina, I., & Massague, J. (1999). A mechanism of repression of TGFbeta/Smad signaling by oncogenic Ras. *Genes and Development, 13*(7), 804–816.

Lacour, S., Hammann, A., Wotawa, A., Corcos, L., Solary, E., & Dimanche-Boitrel, M. T. (2001). Anticancer agents sensitize tumor cells to tumor necrosis factor-related apoptosis-inducing ligand-mediated caspase-8 activation and apoptosis. *Cancer Research, 61*(4), 1645–1651.

Lee, D. S., Kang, S. B., Baek, J. T., Nam, S. W., Lee, K. M., Ahn, B. M., et al. (2005). Immunohistochemical expression of bcl-2, bcl-xL, bax, p53 proteins in gastric adenoma and adenocarcinoma. *The Korean Journal of Gastroenterology, 45*(6), 394–400.

Li, Q. L., Ito, K., Sakakura, C., Fukamachi, H., Inoue, K., Chi, X. Z., et al. (2002b). Causal relationship between the loss of RUNX3 expression and gastric cancer. *Cell, 109*(1), 113–124.

Li, C. C., Xu, B., Hirokawa, M., Qian, Z., Yoshimoto, K., Horiguchi, H., et al. (2002a). Alterations of E-cadherin, alpha-catenin and beta-catenin expression in neuroendocrine tumors of the gastrointestinal tract. *Virchows Archiv, 440*(2), 145–154.

Lin, H. H., Huang, H. P., Huang, C. C., Chen, J. H., & Wang, C. J. (2005). Hibiscus polyphenol-rich extract induces apoptosis in human gastric carcinoma cells via p53 phosphorylation and p38 MAPK/FasL cascade pathway. *Molecular Carcinogenesis, 43*(2), 86–99.

Liu, L. K., Lee, H. J., Shih, Y. W., Chyau, C. C., & Wang, C. J. (2008). Mulberry anthocyanin extracts inhibit LDL oxidation and macrophage-derived foam cell formation induced by oxidative LDL. *Journal of Food Science, 73*(6), H113–H121.

Meng, X. W., Lee, S. H., Dai, H., Loegering, D., Yu, C., Flatten, K., et al. (2008). Mcl-1 as a buffer for proapoptotic Bcl-2 family members during TRAIL-induced apoptosis: a mechanistic basis for sorafenib (Bay 43-9006)-induced TRAIL sensitization. *The Journal of Biological Chemistry, 282*(41), 29831–29846.

Mishra, L., Cai, T., Levine, A., Weng, D., Mezey, E., Mishra, B., et al. (1998). Identification of elf1, a beta-spectrin, in early mouse liver development. *The International Journal of Developmental Biology, 42*(2), 221–224.

Parkin, D. M., Bray, F., Ferlay, J., & Pisani, P. (2005). Global cancer statistics, 2002. *CA: A Cancer Journal for Clinicians, 55*(2), 74–108.

Prior, R. L., & Wu, X. (2006). Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. *Free Radical Research, 40*(10), 1014–1028.

Qiao, L., & Wong, B. C. (2009). Targeting apoptosis as an approach for gastrointestinal cancer therapy. *Drug Resistance Updates, 12*(3), 55–64.

Seeram, N. P., Adams, L. S., Zhang, Y., Lee, R., Sand, D., Scheuller, H. S., et al. (2006). Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *Journal of Agricultural and Food Chemistry, 54*(25), 9329–9339.

Soung, Y. H., Lee, J. W., Kim, S. Y., Jang, J., Park, Y. G., Park, W. S., et al. (2005). CASPASE-8 gene is inactivated by somatic mutations in gastric carcinomas. *Cancer Research, 65*(3), 815–821.

Strumylaite, L., Zickute, J., Dudzevicius, J., & Dregval, L. (2006). Salt-preserved foods and risk of gastric cancer. *Medicina (Kaunas), 42*(2), 164–170.

Szepeshazi, K., Schally, A. V., Nagy, A., Wagner, B. W., Bajo, A. M., & Halmos, G. (2003). Preclinical evaluation of therapeutic effects of targeted cytotoxic analogs of somatostatin and bombesin on human gastric carcinomas. *Cancer, 98*(7), 1401–1410.

Tolcher, A. W., Mita, M., Meropol, N. J., von Mehren, M., Patnaik, A., Padavic, K., et al. (2007). Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. *Journal of Clinical Oncology, 25*(11), 1390–1395.

Wang, L. S., & Stoner, G. D. (2008). Anthocyanins and their role in cancer prevention. *Cancer Letters, 269*(2), 281–290.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., & Greenberg, M. E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science, 270*(5240), 1326–1331.

Xu, X., Brodie, S. G., Yang, X., Im, Y. H., Parks, W. T., Chen, L., et al. (2000). Haploid loss of the tumor suppressor Smad4/Dpc4 initiates gastric polyposis and cancer in mice. *Oncogene, 19*(15), 1868–1874.

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