Mulberry Anthocyanins Induce Leukemia WEHI-3 Cells Apoptosis, Autophagy, Differentiation and Prolong Leukemic Mice Survival

Horng-Rong Chang¹,², Yun-Ching Chang³,⁴, Sz-Ya Shao³, Hui-Pei Huang³,⁵,⁶, *

Institute of Medicine, Chung Shan Medical University, Taichung 40201, Taiwan¹
Division of Nephrology, Department of Internal Medicine, Chung Shan Medical University Hospital, Taichung 40201, Taiwan²
Institute of Biochemistry and Biotechnology, Medical College, Chung Shan Medical University, Taichung, Taiwan³
Department of Medical Research, Chung Shan Medical University Hospital, Taichung, Taiwan⁴
Clinical Laboratory, Chung Shan Medical University Hospital, Taichung, Taiwan⁵
Department of Biochemistry, School of Medicine, Chung Shan Medical University, Taichung, Taiwan⁶
Corresponding author. Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung, Taiwan*

Accepted 2017-05-24; Published 2017-06-08

Abstract:
Recently, interest in dietary phytochemicals and Chinese herbs for potential cancer chemoprevention has increased substantially owing to their multiple capacities of a combination of several distinct intracellular events (such as cell proliferation, apoptosis, angiogenesis, and metastasis). In our previous studies, we showed that Mulberry anthocyanins (MACs), a natural polyphenol product extracted from Morus, have the antitumor effects on various cancers, such as gastric cancer, melanoma, and hepatoma. Acute myeloid leukemia (AML) patients usually suffer from the side effects of chemotherapy. We investigated effects of MACs on murine Leukemia WEHI-3 cells in vitro. Firstly, 50% of WEHI-3 cells were decreased after treatment with 1.76 mg/ml and 1.48 mg/ml for 24 and 48 hr by MTT assay, respectively. The results of flow cytometry assay showed that low does MACs caused the WEHI3 cells apoptosis when they were exposed to high dose MACs. In addition, caspase-9 was activated from pro-caspase to cleaved-caspase in response to treatment MACs with WEHI-3 cells. Immunofluorescence analysis and western blotting assay demonstrated that MACs caused WEHI3 cells death by triggering autophagic flux. By co-treatment with ATRA, MACs also increased CD11b and GM-CSF level, decreased M-CSF expression, which led to WEHI3 cells differentiation. Moreover, co-treated ATRA and MACs cells showed the suppression of MEK, ERK and STAT1 phosphorylation. Altogether, MACs can prevent the growth of WEHI-3 cells for potentially combination chemotherapy therapy to prevent side effects of acute myeloid leukemia.

Key words: Mulberry anthocyanins (MACs), acute myeloid leukemia (AML), apoptosis, autophagy, differentiation
Introduction:
Acute myeloid leukemia (AML) is a hematological malignancy representing chromosomal abnormalities and gene mutations as well as transcriptional, proteomic, metabolic and epigenetic modifications. The present chemotherapeutic approach for the disease is based on the concept of targeting leukemic cells specifically to eliminate them while minimally affecting normal cells. The induction therapy with cytosine arabinoside in combination with anthracycline antibiotics such as doxorubicin or idarubicin is a mainstream approach for AML treatment[1]. However, even though good responses to initial chemotherapy, the majority of AML patients have poor prognosis due to primary resistance and frequent relapse. Relapse of the disease is thought to be associated with the failure of chemotherapy to eradicate leukemia stem cells which utilizing the survival (NF-kB, STAT, PI3 kinase, PTEN, p53) and self-renewal (Wnt, Hedgehog, Notch) pathways [2-9]. Furthermore, the therapy is highly toxic and poorly tolerated, particularly by older patients. Therefore, the development of novel therapeutic agents and strategy is in an urgent need for improving outcomes in patients with AML.

Natural products have played a major beneficial role in cancer chemotherapy for over 50 years [10]. When compared with synthesized chemical compounds, natural products show a favorable profile in terms of their absorption and metabolism in the body with low toxicity. Natural products are also shown to possess multi-faceted mechanism that can hit multiple pathways that are de-regulated in cancer cells to achieve greater therapeutic efficacy [10]. Mulberry belongs to the genus Morus of the family Moraceae and is rich in phenolic compounds, including flavonoids, anthocyanins, and carotenoids[11]. Mulberry has been reported to have not only anti-oxidative, anti-inflammatory, anti-tumor, and anti-diabetic effects, but also cardiovascular, hepato-, and neuro-protective properties[12]. Many studies in vitro and in vivo illustrated the protective role of Mulberry against different types of cancers. Mulberry extracts promote human glioma cell death in vitro through the reactive oxygen species (ROS) dependent mitochondrial pathway and glioma tumor growth in vivo via reduction of tumor cell proliferation and the induction of apoptosis[13]. Our studies also demonstrated that Mulberry anthocyanins (MACs) suppressed gastric cancer cell survival and tumorigenesis, and induced apoptotic death by targeting p38/p53 and the c-jun pathways in vitro and in vivo[14]. Besides, MACs could mediate B16-F1 cell metastasis by reduction of matrix metalloproteinase-2 and -9 activities involving the suppression of the Ras/PI3K signaling pathway[15]. The multiple bioactivity of Mulberry to affect the several chronic diseases prompted us to investigate the role of MACs on AML. We found that MACs could induce AML cells apoptosis via reducing of mitochondrial membrane potential. When co-treatment with ATRA, MACs promoted MEK/STAT1 signaling to regulate AML cells differentiation.

Materials and Methods:
Cell line and culture condition:
WEHI-3 cell line, Mouse myelomonocyte, was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Cells were grown in RPMI1640 medium supplemented with 10% FBS, penicillin (100U/ml), streptomycin (0.1 mg/ml), and glutamine (1%) at 37°C under a humidified 5% CO₂ atmosphere.

**Cell treatment and cytotoxicity assay:**

Cell cytotoxicity was determined by MTT assay. MACs at concentrations of 0, 1, 2, 3, 4 and 5 mg/ml was added to 1x10⁵ cells grown overnight in 24-well-plates. The treated cells were incubated for 24, and 48 hr. After incubation, the culture supernatant was replaced with 20 μL of MTT (5 mg/ml in PBS) (Sigma Aldrich, St.Louis, MO, USA) and incubated at 37°C for 3 hr. The color products were measured by spectrophotometer at 563 nm. The experiments were performed in triplicate.

**Annexin V/ propidium iodide staining:**

Briefly, 2 × 10⁵ cells were harvested, washed once with PBS and resuspended in 100 μl of Annexin V binding buffer (10 mM HEPES/NaOH, 140 mMNaCl, 2.5 mM CaCl₂, pH7.4). 5 μl of annexin V FITC and 1μl of PI (propidium iodide) were then added and cells were incubated at room temperature for 20 min in the dark. Then 400 μl of binding buffer was added and the stained cells were analyzed in flow cytometer (FACSCalibur; BD Biosciences, SanJose, CA, USA) equipped with Cell Quest software (BD Biosciences). For each analysis 10,000 events were recorded.

**Acridine orange and ethidium bromide (AO/EtBr) staining:**

Cells (2 × 10⁵) were collected by centrifugation and doubly stained with 14 μg/ml acridine orange and 14 μg/ml ethidium bromide[16]. Nuclear morphology of stained cells was examined by fluorescent microscopy (ZEISS Company, Jena, Germany) at a magnification of 400 x.

**Detection of mitochondrial membrane potential (MMP):**

MMP was assessed by flow cytometry after staining with JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical Company, USA). 1x10⁵/ml cells in 6-well plates were treated with various concentrations of MACs for 24 h. The cells were then washed with PBS and incubated with 0.5 ml JC-1 working solution (5 μM) for 30 min at 37°C in the dark. Cells were washed with PBS and resuspended in 500 μl PBS. The green signal of JC-1 was measured at 480 nm, whereas that of the red was measured at 590 nm. The stained cells were analyzed by flow cytometry to determine the change in the florescence from red to green.

**Immunofluorescence analysis:**

After treatment with MACs for 24 h, the cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.25% Triton X-100 for 10 min and blocked with PBS containing 10% FBS for 30 min at room temperature. Cells were then incubated with an anti-LC3B primary antibody (Cell Signaling Technology, USA) at 4°C overnight, washed three times with PBS and stained with fluorescent secondary antibody (1:10,000) (Donkey anti-Rabbit Alexa Fluor 488, Jackson ImmunoResearch, West Grove, PA, USA) for 2 h. Nuclei were stained with DAPI for visualization. Fluorescence images were taken on a spinning disk confocal microscope using a 100X oil-immersion objective.

20213

*International Journal of Contemporary Research and Review*, Vol. 8, Issue. 6, Page no: MS 20211-20224
doi: http://dx.doi.org/10.15520/ijcrr/2017/8/06/198
siRNA Treatment:

Atg5 RNA interference was accomplished by transfecting WEHI-3 cells with the Atg5-targeted siRNA and the Universal Control siRNA (Invitrogen; 100 pmol/well). Short oligo-RNAs were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 24 h transfection, cells were treated with MACs for an additional 24 or 48 h. Then cells were collected and cell lysates were subjected to immunoblotting of Atg5. Cells were also processed for cell viability analysis.

Western blotting:

WEHI-3 cells were treated with MACs at various concentrations for 24 hr. Proteins involved in apoptosis and differentiation in the treated cells were measured by Western blot analysis. The harvested cells were washed, centrifuged, and the cell pellets were lysed with RIPA buffer (25 mMTris-HCl pH 7.6, 150 mMNaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Merck, Frankfurt, Germany). 50 μg of cellular proteins from each treatment was separated by SDS polyacrylamide gel. After transferred to a nitrocellulose membrane (Millipore, Atlanta, GA, USA), the membrane was blocked with 5% skimmed milk in TBS (100 mMTris-base, 150 mMNaCl, 0.1% Tween20) for 1 hr followed by immunoblotting with primary antibodies at 4°C overnight. The membrane was washed and incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) for 2 h at room temperature. After washing three times with TBS for 2 min, the protein bands on the membrane were stained with ECL Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, United Kingdom) and analyzed using AlphaImager Series 2200 software (Alpha Innotech, San Leandro, CA, USA). The results were representative of at least three independent experiments.

Differentiation detection:

The induction of cell differentiation was determined by assessing morphologic changes and CD11b expression. The cell morphology was evaluated by Giemsa staining. The cytopsin preparations were fixed with methanol and air-dried. The slides were then stained with the Giemsa solution and examined with a Leica microscope. To measure the CD11b expression, the harvested cells (1 × 10⁶) were washed with PBS, blocked with 5% bovine serum albumin (BSA) in PBS for 30 minutes, and incubated with anti-human CD11b antibody (PE conjugated) for 45 minutes on ice. After incubation, the CD11b expression levels were analyzed with a FACS flow cytometer.

Enzyme-linked immunosorbent assay (ELISA):

After treatment ATRA and MACs for 24 hr, the concentration of GM-CSF in the supernatant of WEHI3 cell cultures was determined by ELISA (ELISA kits were purchased from R & D systems, Minneapolis, USA). The assays were conducted according to the manual instructions.

Animal model:

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the Chung Shan Medical University (IACUC, CSMC), Taichung, Taiwan. Male BALB/c wild-type mice (7 weeks old) were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) with a 12/12 h light/dark cycle at 18–23°C and ad libitum access to food and water at least 1 week before each study. In total, 38...
mice were randomly divided into five groups. Group 1, normal control (distilled water), Group 2, MACs alone (1%), Group 3, leukemic control (WEHI-3), Group 4, leukemia + MACs 0.5%, and Group 5, leukemia + MACs 1%. For the leukemia-implanted mouse acute myelomonocytic leukemia model, BALB/c mice were injected intravenously through their tail vein with WEHI-3 cells (1×10⁵ cells/animal) in 100 μl of PBS.

Organ weight and histopathological analysis:
Mice spleens and livers were harvested and weighed at the end of the experiment. Full-thickness biopsies were taken and fixed in 4% formaldehyde and embedded in paraffin. Tissues were sectioned (5 μm) and stained with Hematoxylin & Eosin, then examined under the light microscope (Nikon Instruments Inc., Melville, NY, USA).

Statistical analysis:
Results are reported as mean±standard deviation (SD) of three independent experiment. Statistical comparisons were evaluated by one-way analysis of variance (ANOVA). A P value less than 0.05 was considered to be statistically significant.

Results:
MACs suppressed cell growth and induce cell apoptosis in WEHI3 cells:
First, we studied the effect of MACs on suppressing proliferation in WEHI3 cells in vitro. WEHI3 cells were treated with MACs at the various concentrations for 24 hr and 48 hr. Cell viability was assessed by MTT assay. MACs reduced cell viability in a dose-dependent manner (Fig. 1). IC50 value was calculated to be 1.76 mg/ml and 1.48 mg/ml for 24 and 48 hr treatment MACs with WEHI3 cells, respectively.

Fig. 1 Effects of MACs on cytotoxicity in WEHI-3 cells. Counted WEHI-3 BALB/c myelomonocytic leukemia cell were treated with 0.25, 0.5, 1, 2, 3 or 5 mg/mL of dry Mulberry MACs for 24 hr and 48 hr. Cell survival was measured by MTT assay. The data were shown as mean ± SD from three independent experiments, *P<0.01, **P<0.001 compare with the control.

Apoptosis is typically characterized by cell death-related biochemical and morphological changes including cell shrinkage, DNA fragmentation, chromatin condensation, and formation of apoptotic bodies [17]. To determine whether MACs induces cell death by apoptosis, morphological changes of MACs treated WEHI3 cells were observed using microscopy. The inhibitory effect of MACs on cell growth was accompanied by membrane blebbing, as observed using a phase contrast microscope (Fig. 2A), indicating that cell death was due to apoptosis death. Then, the apoptotic effect of MACs on WEHI3 cells was examined through Annexin V/PI double staining assay. Incubation of MACs (0.5, 1 and 2 mg/ml) significantly led to an increased apoptosis for 24 hr (0.71%, 3.45%, and 23.11%), respectively (Fig. 2B). We also studied the nuclear morphology.
of the cells using AO/EtBr staining and the fluorescence microscopy technique. The live cells showed green in color with unscathed nuclei, while the early apoptotic cells showed condensed nuclei and were in green color with bright green dots in their nuclei, and the late apoptotic cells appeared in orange color. In Fig. 2C, fluorescence microscopy analysis showed that in control cells, the nucleus was big and round without condensation or fragmentation, while the treated cells showed chromatin condensation and fragmentation, emphasizing the happening of apoptosis. However, MACs treated cells appeared the early apoptotic characteristic, especially at MACs concentration of 2 mg/ml representing the late apoptotic status. These results indicated that the apoptotic WEHI3 cells were induced by MACs in both dose- and time-dependent manners.

Fig. 2. MACs promoted WEHI3 cells apoptosis. WEHI-3 cells were treated with 0, 0.5, 1, 2 mg/ml of MACs for 24hr. The morphology of WEHI-3 cells was observed (Magnification 200X) (A). The apoptotic cells harvest and stained in Annexin V/ propidium iodide. The percentage population of apoptotic cells is indicated for total FL1. (FL1: FITC annexin V, FL2: PI) and H2O2-treated cells were as positive control (B). Acridine orange/ethidium bromide staining shows viable cells in green fluorescence and dead cells in red-orange fluorescence (C).

MACs reduced mitochondrial membrane potentials and induce cellular caspase activation in WEHI3 cells:

Reduction of mitochondrial membrane potential is one of the molecule events for early apoptosis. When the cell is in a normal state, MMP is high and JC-1 predominantly appears as red fluorescence. A change in the fluorescence from red to green indicates a decrease in the MMP. In our study, MACs treated cells showed loss of red fluorescence and production of obvious green fluorescence, suggesting reduction of mitochondrial membrane potentials. In comparison, control cells emitted mitochondrial red fluorescence with less green fluorescence (Fig. 3A), resulting in the cytosolic accumulation of monomeric JC-1, which is an indicator of apoptosis by activation of the intrinsic pathway[18].

Apoptosome complex formation starts with the release of cytochrome c (Cyt-c); it is a crucial step in mitochondria mediated apoptosis. Western blot analysis showed that MACs induced the release of Cyt-c from the mitochondria to the cytosol. The reduced anti-apoptotic Bcl-2 level and increased apoptotic proteins such as Bax and tBid were detected (Fig. 3B). Caspases are cytosolic proteins that exist normally as inactive precursors with higher molecular weights (55, 46, and 32 kDa). They are cleaved proteolytically into low molecular weights.
Hui-Pei Huang et al. Mulberry Anthocyanins Induce Leukemia WEHI-3 Cells Apoptosis, Autophagy, Differentiation and Prolong Leukemic Mice Survival

(20–23 kDa) when cell undergoes apoptosis [19]. In this study, we observed that the bands of inactive caspase (procaspase) diminished as the cleaved fragments increased in the MACs-treated WEHI3 cells. Thus, MACs reduced mitochondrial membrane potentials and induced WEHI3 cells apoptosis by activation of caspase pathway.

Fig. 3. MACs reduced mitochondrial membrane potentials and induce cellular caspase activation in WEHI3 cells. (A) The mitochondrial membrane potentials were measured by JC-1 staining and showed by fluorescent micrographs. (B) The most representative images of western blot for apoptosis related protein were assayed by Western blotting.

MACs induced autophagy in the WEHI3 cells: Fig. 1 and Fig. 2B indicated that MACs might induce the cancer cell death induced by other cell death mechanism. Many common anticancer drugs induce not only apoptosis but also autophagy in cancer cells. Therefore, we investigated whether MACs could induce autophagy on AML cells. To confirm MACs-induced autophagy, we measured autophagy induction with the fluorescence microscopy and using anti-LC3B that can stain autophagosomes in the WEHI3 leukemia cells, emitting green fluorescence and forming punctate structures.

Fig. 4. MACs induced autophagy in WEHI3 cells. (A) The WEHI3 leukemia cells were treated with MACs for 24 h. The cells were then loaded with anti-LC3B primary antibody and DAPI, then imaged by confocal microscopy. (B) The cells were treated with 0~2 mg/ml MACs for 24 h and immunoblotting was used to determine protein levels of LC1, LC3-BII, AFG5, and Beclin-1. β-actin was used as a loading control. After transfection of siTAG5 and confirmed by western blotting (C), the siTAG5-WEHI3 cells were treated with 2 mg/mL of MACs. Cell viability was measured by MTT assay (D). The data were shown as mean ± SD from three independent experiments, **P<0.001 compared with the control cells. ##p<0.001 compared with
siTAG5 cell. $p<0.001$ siTAG5 cell compared with siTAG5+MACs cell.

In Fig. 4A, it clearly showed that there was no fluorescence was detected in the no-treated MACs group. The increase in green fluorescence could be observed in 1 and 2 mg/ml MACs treated cells. The autophagic flux in the MACs-treated cells was further assayed by western blotting analysis. The assay showed decrease LC1 protein level and increase LC3-BII/ATG5/Beclin-1 proteins levels in the MACs-treated WEHI3 cells (Fig. 4B). After siATG5 transfection (Fig. 4C), cell viability inhibited by MACs was restored (Fig. 4D), suggesting that MACs caused autophagy in the WEHI3 cells.

**Combined treatment of WEHI3 cells with ATRA and MACs enhanced cell differentiation**

*In vitro* and *in vivo* studies, the successful clinical application of differentiation therapy is ATRA-based therapy of acute promyelocytic leukemia (APL), but ATRA does not work for other AMLs. Indeed, in Fig. 5A, ATRA and 2 mg/ml MACs had slight effect to change WEHI3 cells morphology. Interestingly, morphology of the WEHI3 cells changed from circular, suspended to spindle and adhesion after treatment ATRA and MACs. On the other hand, cell differentiation was then measured by determining the expression level of CD11b (identified by mean fluorescence intensity). The CD11b$^+$ cells increased when WEHI3 cells were co-cultured with MACs and ATRA dose dependently (Fig. 5B). Single treatment with 0.5 μM ATRA or 2 mg/ml MACs increased the differentiated cells approximately 8% or 4%, respectively.

**Fig. 5** MACs enhanced the differentiation-inducing effect of ATRA in WEHI3 cells. WEHI3 cells were incubated with indicated concentration MACs and/or 0.5 μM ATRA for 24 hr. Micrographs of WEHI3 cells were shown in (A). CD11b-positive cells were counted by flow cytometry (B). GM-CSF secreted in conditional medium were detected by ELISA kit test (C). The data were shown as mean ± SD from three independent experiments, *$P<0.01$, **$P<0.001$ compare with the control.

Emerging evidence also suggests that granulocyte-macrophage colony-stimulating factor (GM-CSF) is a multi-lineage hemopoietic growth factor that stimulates proliferation, differentiation, and survival of progenitor cells[20]. Incubation with ATRA or MACs alone led to a slight increase GM-CSF level, whereas co-treatment ATRA and MACs significantly promoted GM-CSF expression (Fig.
5C), indicating the synergistic effect of ATRA and MACs on cell differentiation.

**WEHI3 cells differentiation is dependent on the phosphorylation of the MEK-ERK/STAT1 cascade:**
Raf/MEK/ERK, PI3K/PTEN/ Akt/mTOR and Jak/STAT pathways are often activated by mutations in leukemia [21]. Numerous reports demonstrate the critical role of STAT1 in ATRA-induced myeloid differentiation [22]. To observes the regulation of ATRA and MACs on differentiation in WEHI3 cells, related signaling proteins were detected by immunoblotting assay. The results showed in Fig. 6 revealed that MACs and ATRA could activate MEK, ERK and STAT1 phosphorylation respectively. Co-treatment with MACs and ATRA distinguished that protein activation, suggesting the synergistic effect of MACs and ATRA on AML.

**Fig. 6.** WEHI3 cells differentiation is dependent on the phosphorylation of the MEK-ERK/STAT1 cascade. Cells were collected after treatment with MACs and ATRA for 24 hr.

**MEK signaling related proteins were measured by Western blotting.**

**Effects of MACs on mice leukemia model:**

*In vivo*, we determined the antitumor efficacy of MACs in Balb/c mice via injecting intravenously through their tail vein with WEHI-3 cells (1×10^5 cells/animal) in 100 µl of PBS. The percent survival of each group was calculated and is presented in Fig. 7A. The MACs-treated leukemic mice (WEHI-3/MACs 0.5% and WEHI-3/MACs 1%) exhibited a concentration-related higher survival rate compared to the untreated-leukemic mice. Leukemia-induced mice spleen and liver weighed at autopsy showed splenomegaly and hepatomegaly, whereas MACs treatment significantly reduced the spleen and liver sizes compared to the leukemic control (WEHI3 along) group (Fig. 7B and C). Histopathological examination was also presented in Fig. 7C. In WEHI3 along group, soft tissue tumor nodules were found within the enlarged spleen and liver. In spleen, decreased white pulp and infiltrated with aggressive leukemic cells in red pulp were found in leukemic control group. However, clear distribution of white pulp and red pulp were showed in MACs-treated leukemic groups. MACs also improved tumor cell infiltration of liver in leukemic control mice. Taken together, MACs supplementation could ameliorate or abolish in leukemic mice disorder.
Discussion:

Unbalanced control of proliferation, apoptosis, autophagy and differentiation are the main characteristics of cancer cells. Polyphenols are a group of metabolites that are widely distributed in the plants and found in abundance in a variety of fruits and vegetables [23]. The biological activities of polyphenols depend on their structure and the mechanism of anticancer activities of dietary polyphenols has been previously reviewed[24]. The major cellular signaling pathways and mechanisms of action of polyphenols on leukemia cells are through induction of apoptosis, inhibition of proliferation, eliminating the oxidative stress, modulation of multidrug resistance, and promoting of cell differentiation [25]. Our investigation evaluated the anticancer effect of MACs in AML cells. Exposure leukemia cell line to MACs increased apoptosis in dose- and time-dependent manners. The apoptotic effects of MACs on WEHI3 cells are exerted by reducing the mitochondrial membrane potential, the release of cytochrome C, and inducing intrinsic apoptotic signaling. In addition, MACs could promote autophagic flux by increasing Beclin-1, ATG5, and LC3BII levels those involved in autophagosome formation. Another important result of the present investigation is that MACs can synergistically induce differentiation in ATRA-treated WEHI3 cells. Although both MACs and ATRA have slight differentiation efficacy on WEHI3 cells, combination of MACs and ATRA treatment can significantly induce WEHI3 cells differentiation via activating MEK/STAT1 related proteins. These data support that MACs induce apoptosis, autophagy and differentiation will be good candidates as cancer chemo preventive and/or chemotherapeutic agents. Future studies will evaluate the antitumor activity of MACs in animal models.

Flavonoids have been reported to induce apoptotic cell death in various cancer cell lines including a variety of leukemic cell lines, but sparing the normal cells. They achieve this by several way including intrinsic, extrinsic and caspase cascade. Anthocyanins also can induce apoptosis in different cancer cells via a mitochondrial pathway and human leukemia U937 cells via activation of caspase 3, 8 and 9[26]. We previously demonstrated the effect of...
Mulberry extracts on inhibiting cancer development through inducing apoptosis via p53/Bcl-2/caspase and Fas/FasL signaling and inhibiting migration via Ras and p38 pathways [12]. Here, we again prove MACs induce leukemia cells apoptosis by regulation of signaling pathways, release of cytochrome c leading to activation of caspases 9, decreased expression of Bcl-2 but overexpression of Bax and t-Bid. However, extrinsic apoptosis signaling was not activated by MACs on WEHI3 cells, meaning the different mechanisms of MACs on various cancer cells.

Age and status of chromosomal and molecular aberrations remain the most important tools for outcome prediction in AML. An aggressive malignancy with poor outcomes, AML is most common in the elderly, and most elderly are thought to be unfit for intensive treatment because of the risk of fatal toxicity [27]. 32% of the older patients treated with standard therapy either died or could not receive additional therapy because of severe morbidity [28]. Despite decades of research, therapy has remained largely unchanged, with chemotherapy and bone marrow transplantation being the major treatments for AML. However, the high toxicity and serious complications caused by these conventional treatments has kept the survival rate for AML patients low. These problems have prompted us to search for novel agents with anticancer activity for AML treatment. New treatments need to be searched to replace the standard intensive treatment and not simply given to patients stated to be unfit for standard treatment. Recently, some natural products are viewed their ability of inducing differentiation of AML cells. Securinine is a plant-derived alkaloid that has previously been used clinically as a therapeutic for primarily neurological related diseases. It induces monocytic differentiation of a wide range of myeloid leukemia cell lines as well as primary leukemic patient samples and can synergize with ATRA to induce differentiation [29]. Pharicin B, a novel natural ent-kaurenediterpenoid derived from Isodonpharicus leaves, can rapidly stabilize RAR-α protein and presents a synergistic differentiation-enhancing effect when used in combination with ATRA in various AML cell lines [30]. In this work, we present the first demonstration that MACs were applied to ATRA-treated leukemic cells and induced differentiation through the activation of MEK/STAT1 related proteins. According to our previously studies, MACs might be a promising new differentiation inducing agent for AML for non-related disorders.

**Conclusion:**

We reveal two actions of MACs to affect AML. One is to promote AML cells death by reducing cell mitochondrial membrane potential to activate intrinsic apoptosis and triggering autophagy. The other is to induce AML cells differentiation combination with ATRA treatment through MEK/STAT1 pathway. However, to design more investigations such as further molecular mechanism and animal model to clarify the role of MACs on AML is our next task.

**Conflicts of interest:**

The authors declare that they have no competing interests.

**Acknowledgments:**

This investigation was supported by the Chung Shan Medical University and Chung Shan Medical University Hospital (CSH-2013 - C-028).

**Reference:**

Hui-Pei Huang et al. Mulberry Anthocyanins Induce Leukemia WEHI-3 Cells Apoptosis, Autophagy, Differentiation and Prolong Leukemic Mice Survival


16. Meshkini, A., Yazdanparast, R., Haidari, M.


29. Gupta, K., Chakrabarti, A., Rana, S., Ramdeo, R., Roth, B.L., Agarwal, M.L., Tse, W., Agarwal, M.K., Wald, D.N. Securinine, a myeloid differentiation agent with therapeutic potential for AML. *PLoS*
Hui-Pei Huang et al. Mulberry Anthocyanins Induce Leukemia WEHI-3 Cells Apoptosis, Autophagy, Differentiation and Prolong Leukemic Mice Survival
