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Anti-tumor and Anti-inflammatory Properties of Ankaflavin and Monaphilone A from *Monascus purpureus* NTU 568

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ABSTRACT: An azaphilonidal derivative monaphilone A (MA) was recently isolated from the fermented products of *Monascus purpureus* NTU 568 by our laboratory. We report here the exploration of apoptosis-related and anti-inflammatory properties of MA and ankaflavin (AK) by some experiments about inducing death of human laryngeal carcinoma cell line HEp-2 and reducing inflammatory responses on murine macrophage RAW 264.7 cells. We employed a ssDNA enzyme-linked immunosorbent assay (ELISA) kit to investigate the nuclear changes of early apoptosis induced by AK and MA on HEp-2 cells and used a western blot and an enzyme activity assay to demonstrate the activation of caspase-3, caspase-8, and caspase-9 by MA and AK. Our studies revealed that AK and MA may decrease lipopolysaccharide (LPS)-induced inflammatory responses, including nitrite productions and expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) in RAW 264.7 cells. All evidence support that azaphilonidal derivatives from *M. purpureus* NTU 568, such as AK and MA, are suitable for the development of chemotherapy or chemopreventive agents.

KEYWORDS: Ankaflavin, monaphilone A, *Monascus purpureus*, lipopolysaccharide, nitric oxide synthase, cyclooxygenase 2

INTRODUCTION

Monascus species has traditionally been used to make food more colorful and easy to preserve in Asian countries for thousands of years. Using rice as a fermented substrate, its product, red mold rice (RMR), has been proven to possess various biological functions. For example, RMR exhibited hypolipidemic effects in a hyperlipidemia hamster model,[1] antifatigue activities to Wistar rats through a swimming exercise,[2] neuroprotective properties against Alzheimer's risk factors in Aβ40-infused rats,[3] preventive ability for obesity in a high-fat diet rat model,[4] etc.

Some bioactive components from *Monascus* species have been identified and characterized. A family of monacolins possessed 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitory activity,[5] and monacolin K was the marker monacolin.[6] Other bioactive secondary metabolites, including γ-amino butyric acid (GABA) and dimerumic acid, were considered to possess hypotensive[7,8] and antioxidant activities, respectively.[9] Furthermore, yellow pigments in *Monascus* sp. were demonstrated to possess cytotoxic and anti-inflammatory effects.[10,11] Additionally, several *Monascus* pigment derivatives produced by fermentation with various amino acids[12] showed different kinds of usages, including antimicrobial activity,[13] inhibition of lipase,[14] and hypolipidemic effects.[15]

Carcinogenesis was involving in complicated steps, possibly resulting from chronic inflammatory stimuli in the beginning and leading to uncontrolled growth of tumor cells in the end. It was hoped that some nature products could exert an anti-tumor effect by the apoptosis-related mechanism or reduction of inflammatory responses. Our group has found that fermented products from *Monascus purpureus* NTU 568 showed anti-tumor and anti-inflammatory effects. For example, RMR extracts from *M. purpureus* NTU 568 significantly reduced tumor progression of Lewis lung carcinoma bearing mice[16] and also mitigated oral carcinogenesis through anti-inflammatory responses in a hamster model.[17] Recently, three new azaphilone pigments were isolated from *M. purpureus* NTU 568 and reported to be cytotoxic to cancer cell lines,[18] and monaphilone A (MA) was the most cytotoxic compound. Here, we report the exploration of apoptosis-related and anti-inflammatory mechanisms of MA and ankaflavin (AK). To the purpose, we designed some experiments including the induction of apoptosis in human laryngeal carcinoma cell line HEp-2 and reduction of inflammatory responses on murine macrophage RAW 264.7 cells.

MATERIALS AND METHODS

General Experimental Procedures. Nuclear magnetic resonance (NMR) spectra were run on Bruker NMR (Unity Plus 400 and 600 MHz) (Bruker BioSpin, Rheinstetten, Germany) and Varian NMR spectrometers (Varian Gemini 200 MHz, Varian, Inc., Palo Alto, CA) using d_6-acetone as the solvent. High-performance liquid chromatography (HPLC) separations were performed on a Shimadzu LC-6AD series apparatus with a SPD-6AV ultraviolet (UV) detector, equipped with a 250 × 20 mm inner diameter preparative Cosmosil AR-II column (Nacalai Tesque, Inc., Kyoto, Japan).

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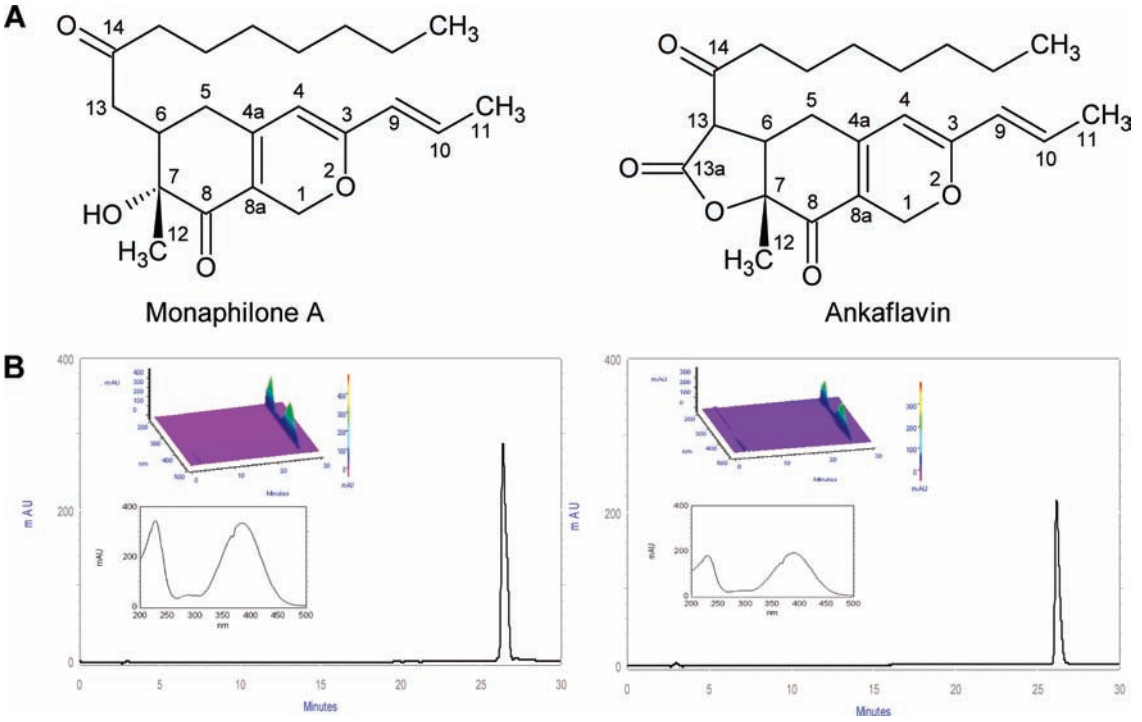

Figure 1. (A) Structures of MA and AK. (B) HPLC analysis and UV spectrum of purified MA and AK showed that their purities were more than 98%. Purified MA and AK were detected at 236 nm.

Reagents. Methanol, acetonitrile (HPLC grade), acetone, ethyl acetate, *n*-hexane, and methanol (analytical grade) were purchased from ECHO (Miaoli, Taiwan). Trifluoroacetic acid (TFA), anisaldehyde, and sulfuric acid were purchased from Merck. Fetal bovine serum (FBS), minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), and trypan blue were purchased from Biological Industries (Kibbutz Beit-Haemek, North District, Israel). Other chemicals, such as lipopolysaccharides (LPSs, from *Escherichia coli* O55:B5), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO).

Extraction and Purification. Preparations of AK and MA followed the experimental procedures from our previous publication.[18] Briefly described, the dried RMR was extracted 3 times with acetone. After filtration and concentration, the residue was chromatographed on a silica gel column and Sephadex (LH-20) gel column. Finally, the semi-pure fractions were purified twice by preparative HPLC to obtain AK and MA.

Cell Lines and Culture. Human laryngeal carcinoma cell line HEp-2 and murine macrophage cell line RAW 264.7 were obtained from Bioresources Collection and Research Center (Hsinchu, Taiwan). HEp-2 cells were maintained in MEM (5% FBS) in a humified incubator with 5% CO_2 at 37 °C, and RAW 264.7 cells were maintained in DMEM (5% FBS).

Cytotoxicity Assay. The cytotoxicity assay was modified from the reported methods.[19,20] HEp-2 cells (3×10^3 per well) were seeded with 180 μL of MEM in 96-well plates. After 4 h, 20 μL of test agents dissolved in PBS solution were added at final concentrations of 5, 10, 25, and 50 μg/mL and incubated in a 37 °C incubator with 5% CO_2. After culturing for 24, 48, and 72 h, 20 μL of MTT solution (2 mg/mL) was added to each well and incubated for 4 h to make cellular conversion of a tetrazolium salt into a formazan product. Then, the supernatant was removed, and 200 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan. Finally, the formazan can be detected by spectrophotometry in the absorbance at 570 nm and provided a relative estimate of cell viability. The same test agents were treated on RAW 264.7 cells with similar procedures. However, RAW 264.7 cells were seeded for 2×10^5 per well and maintained with 500 μL of DMEM in 24-well plates. Test agents were added at final concentrations of 5 and 10 μg/mL.

Nitrite Production Assay. RAW 264.7 cells (2×10^5 per well) were seeded and maintained with 500 μL of DMEM in 24-well plates. After 12 h, cells were treated with LPS (1 μg/mL) and test agents (10 μg/mL) dissolved in DMEM. After 24 h of incubation, determination of the nitrite levels in supernatants was performed by the Griess reagent kit (Promega, Madison, WI) and adapted from the reported methods.[21]

Early Detection of Apoptosis. This assay was designed for the detection of early apoptosis using a ssDNA apoptosis enzyme-linked immunosorbent assay (ELISA) kit (Millipore, Billerica, MA) and adapted from the reported methods.[22] HEp-2 cells (about 10 000 cells per well) were transferred into a 96-well microplate and treated with camptothecin (CPT, 0.2 μg/mL), AK (50 μg/mL), and MA (50 μg/mL) in 200 μL of MEM (5% FBS) for 12 h. As positive and negative controls, 100 μL of ssDNA (0.3 μg/mL) and S1 nuclease were added separately. For all conditions, the antibody mixture (recognized for ssDNA) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS solution) were added. Finally, we easily detect early apoptotic cells by measuring the absorbance at 405 nm in a standard microplated system.

Western Blot Analysis. Cells (about 5×10^5 for HEp-2 and 8×10^6 for RAW 264.7) were seeded with 10 mL of media in a 75 cm^2 flask. After 12 h, 10 mL of test agents dissolved in media were replaced. After 12 and 24 h of incubation, the cells were harvested and extracted by RIPA lysis buffer (Millipore, Bellerica, MA) with 1% protease inhibitor (Sigma, St. Louis, MO). The cell lysates were analyzed with primary antibodies, including the caspase-3 antibody (Novus Biologicals, Littleton, CO), β-actin antibody (Epitomics, Burlingame, CA), inducible nitric oxide synthase (iNOS), and cyclooxygenase 2 (COX-2) polyclonal antibody (Cayman Chemical, Ann Arbor, MI). The anti-rabbit or anti-mouse secondary horseradish peroxidase antibodies (Jackson ImmunoResearch, West Grove, PA) were further added. Finally, the detection was performed using the Western Lightning chemiluminescence reagent (PerkinElmer Life Sciences, Waltham, MA).

Caspase Activity Assay. Cells (about 5×10^5 for HEp-2) were seeded with 10 mL of media in a 75 cm^2 flask. After 12 h, 10 mL of test agents dissolved in media was replaced. After 12 and 24 h of incubation, the cells were harvested and tested for caspase-8 and caspase-9 activities, respectively, by using a colorimetric assay kit (BioVision, Mountain View, CA). Caspase activity was determined according to the protocol of the manufacturer.

Statistical Analysis. Data were presented as the mean ± standard deviation (SD) (n = 3). The statistical comparisons were performed by one-way analysis of variance (ANOVA) with Duncan's test. The significant differences were indicated as $p < 0.05$ or 0.01.

RESULTS

Identification of MA and AK from RMR. We purified these two compounds by preparative HPLC twice in the final step and identified them according to the following MS and NMR data.

MA. ESIMS m/z: $[M + Na]^+$ 383. ^{1}H NMR (d_6-acetone, 400 MHz) δ: 0.87 (3H, t, J = 6.8, H-21), 1.15 (3H, s, H-12), 1.27 (8H, m, H-17−20), 1.54 (2H, m, H-16), 1.82 (3H, d, J = 7.2, H-11), 2.21 (1H, m, H-5a), 2.47 (1H, m, H-13a), 2.49 (3H, m, H-5b, H-15), 2.54 (1H, m, H-6), 2.91 (1H, d, J = 15.2, H-13b), 4.71 (1H, d, J = 12.4, H-1a), 4.88 (1H, d, J = 12.4, H-1b), 5.41 (1H, s, H-4), 6.01 (1H, d, J = 15.2, H-9), 6.39 (1H, dq, J = 15.2, 7.2, H-10). ^{13}C NMR (d_6-acetone, 100 MHz) δ: 14.2 (C-21), 18.3 (C-11), 20.0 (C-12), 23.2 (C-20), 24.4 (C-16), 29.9 (C-17, C-19), 32.4 (C-18), 32.9 (C-5), 40.3 (C-6), 42.4 (C-13), 43.4 (C-15), 64.3 (C-1), 74.8 (C-7), 104.3 (C-4), 114.2 (C-8a), 125.7 (C-9), 134.1 (C-10), 152.4 (C-4a), 160.3 (C-3), 198.6 (C-8), 209.8 (C-14).

AK. ESIMS m/z: $[M + H]^+$ 387. ^{1}H NMR (d_6-acetone, 400 MHz) δ: 0.87 (3H, t, J = 6.8, H-21), 1.29 (8H, m, H-17−20), 1.45 (3H, s, H-12), 1.60 (2H, m, H-16), 1.82 (3H, d, J = 7.2, H-11), 2.66 (2H, m, H-5), 2.70 (1H, m, H-15a), 2.92 (1H, m, H-15b), 3.15 (1H, m, H-6), 4.27 (1H, d, J = 13.2, H-13), 4.68 (1H, d, J = 12.4, H-1a), 4.90 (1H, d, J = 12.4, H-1b), 5.50 (1H, s, H-4), 6.01 (1H, d, J = 15.6, H-9), 6.41 (1H, dt, J = 15.6, 7.2, H-10). ^{13}C NMR (d_6-acetone, 100 MHz) δ: 14.2 (C-21), 17.7 (C-12), 18.3 (C-11), 23.2 (C-20), 23.6 (C-16), 29.2 (C-5), 29.9 (C-17, C-19), 32.3 (C-18), 43.4 (C-15), 44.4 (C-6), 55.3 (C-13), 64.2 (C-1), 84.0 (C-7), 104.6 (C-4), 115.1 (C-8a), 125.5 (C-9), 134.6 (C-10), 151.6 (C-4a), 160.2 (C-3), 171.3 (C-13a), 190.6 (C-8), 203.6 (C-14). Both of them were yellow pigments and structurally similar to each other. They were azaphilones containing the same alkyl group, but MA was decarboxylated, resulting in a breakage on the ester bond. The purity of MA and AK was confirmed by a HPLC chromatogram (Figure 1).

Cytotoxicity of MA and AK on HEp-2 Cells. To study the inhibitory effects on cell viability of HEp-2 (human laryngeal carcinoma) cell lines by MA and AK, we used the MTT assay for a 3 day course (Figure 2). Both of the two azaphilone derivatives possessed dose-dependent and moderated cytotoxic activity against HEp-2 cells. The structure of MA was similar to AK, whereas it had better cytotoxic activity (IC_{50} = 20.97 ± 2.27 μg/mL) against HEp-2 cells than that of AK (IC_{50} = 31.62 ± 2.51 μg/mL). The increase of cytotoxicity against the human laryngeal carcinoma cell line possibly resulted from the structural changes between C-12 and C-14. The results also suggested that MA and AK were suitable for the apoptotic approach for the next step.

Induction of Early Apoptosis by MA and AK on HEp-2 Cells. Because MA possessed moderate cytotoxicity against HEp-2 cells, we have examined whether MA induced apoptosis or necrosis. After

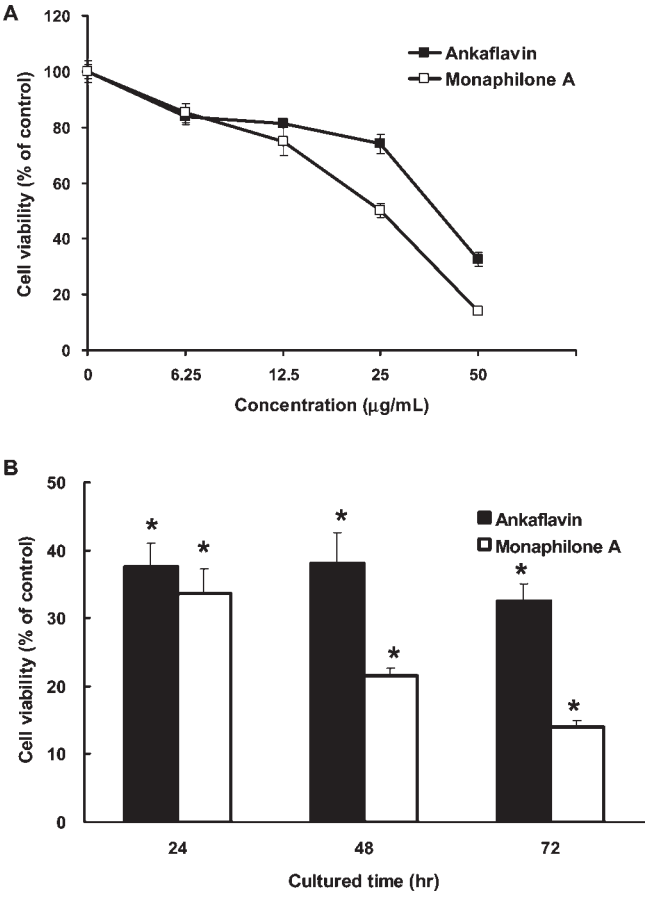

Figure 2. Inhibition of HEp-2 cell viability by AK and MA. (A) HEp-2 cells were treated with test agents for 72 h. (B) HEp-2 cells were treated with 50 μg/mL test agents for 24, 48, and 72 h. Data were expressed as the mean ± SD (n = 3). (*) Significantly different ($p < 0.05$) versus the negative control (without any treatment).

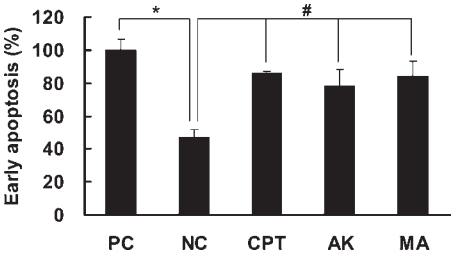

Figure 3. Detection of early apoptosis of HEp-2 cells treated with AK and MA by a ssDNA ELISA kit. HEp-2 cells were treated with CPT (0.2 μg/mL, reference compound for cytotoxity), AK (50 μg/mL), and MA (50 μg/mL) for 12 h. PC, positive control (0.3 μg/mL ssDNA); NC, negative control; CPT, camptothecin. Data were expressed as the mean ± SD (n = 3). (*) Significantly different ($p < 0.05$) versus the positive control. (#) Significantly different ($p < 0.05$) versus the negative control.

HEp-2 cells were treated with CPT (0.2 μg/mL, reference compound for cytotoxity), AK (50 μg/mL), and MA (50 μg/mL) for 12 h, the occurrences of early apoptosis were analyzed by a ssDNA apoptosis ELISA kit (Figure 3). In comparison to the negative control, both of the two azaphilones exhibited a significant increment of ssDNA formation, which was regarded as early apoptosis. The results confirmed that it was apoptosis and not necrosis, induced by the treatment of MA and AK.

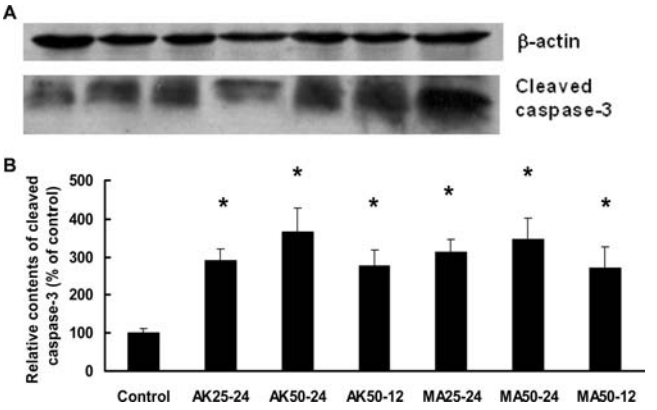

Figure 4. Effects of MA and AK on caspase-3 activation in HEp-2 cells. Cleaved caspase-3 and β-actin were detected by Western blot. HEp-2 cells were treated with 25 or 50 μg/mL test agents for 12 or 24 h. (A) From the left side: lane 1, control; lane 2, 25 μg/mL AK for 24 h; lane 3, 50 μg/mL AK for 24 h; lane 4, 50 μg/mL AK for 12 h; lane 5, 25 μg/mL MA for 24 h; lane 6, 50 μg/mL MA for 24 h; and lane 7, 50 μg/mL MA for 12 h. (B) Quantification of cleaved caspase-3 presented above. Data were expressed as the mean ± SD (n = 3). (*) Significantly different (p < 0.01) versus the control (without any treatment).

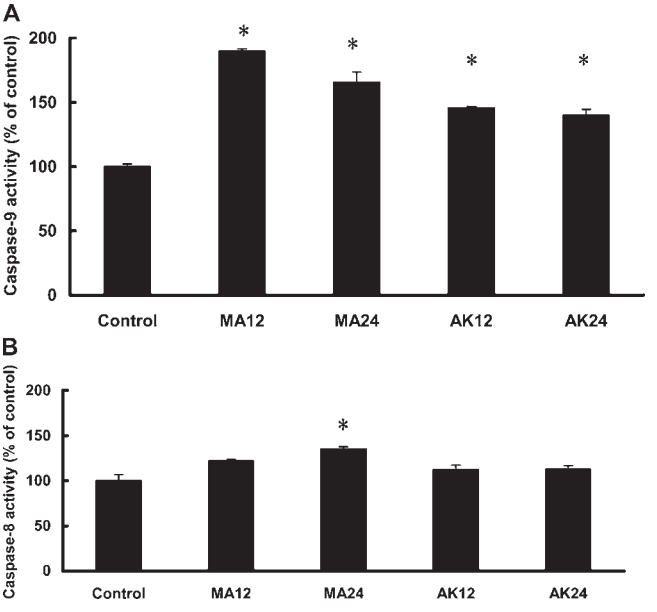

Figure 5. Effects of MA and AK on caspase-9 and caspase-8 activities. HEp-2 cells were treated with 50 μg/mL test agents for 12 or 24 h. From the left side: control; MA, 12 h; MA, 24 h; AK, 12 h; and AK, 24 h. Data were expressed as the mean ± SD (n = 3). (*) Significantly different (p < 0.01) versus the control (without any treatment).

Caspase Activation of MA and AK on HEp-2 Cells. HEp-2 cells were treated with 25 and 50 μg/mL MA and AK for 12 and 24 h and further analyzed for the cleaved caspase-3 by western blot (Figure 4) and enzyme activity of caspase-8 and caspase-9 by the colorimetric assay kit (Figure 5). Treatment of MA and AK (25 and 50 μg/mL) exhibited remarkable contents of cleaved caspase-3, which were regarded as caspase-3 activation resulting from apoptosis. With regard to caspase-9 activation, treatment of MA (50 μg/mL) and AK (50 μg/mL) exhibited significant increases of caspase-9 activity. However, caspase-8 activation was quite different between MA and AK. In comparison to all

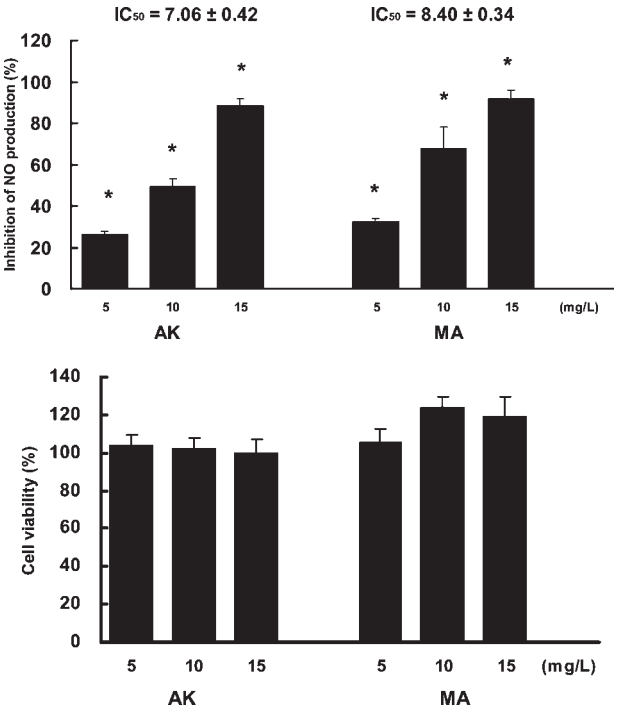

Figure 6. AK and MA suppressed LPS-induced NO production on RAW 264.7 cells. Cells were treated with LPS (1 μg/mL) and in combinations with AK (10 μg/mL) or MA (10 μg/mL) for 24 h. Data were expressed as the mean ± SD (n = 3). (*) Significantly different (p < 0.05) versus the negative control (treatment only with LPS).

conditions, only treatment of MA (50 μg/mL, 24 h) exhibited a significant increase of caspase-8. Thus, MA was demonstrated to induce apoptosis through both caspase-8 and caspase-9 activations, but AK was demonstrated to induce apoptosis merely through caspase-9 activation.

Inhibitory Effect of MA and AK on LPS-Induced NO Production in RAW 264.7 Cells. To study the anti-inflammatory effects of MA and AK on LPS-stimulated RAW 264.7 cells, we first used Griess reagent and the MTT assay to estimate the NO production and cell viability of RAW 264.7 cells (Figure 6). Both of the two azaphilone derivatives reduced NO production in a dose-dependent manner but did not alternate cell viability obviously. The anti-NO activity of MA (IC_{50} = 7.06 ± 0.42 μg/mL) was slightly better than that of AK (IC_{50} = 8.40 ± 0.34 μg/mL). The results suggested that MA and AK were good anti-inflammatory agents, which was suitable for further anti-inflammatory studies at a dosage of 10 μg/mL.

Suppression of LPS-Induced iNOS/COX-2 Expression by MA and AK. RAW 264.7 cells were treated with 1 μg/mL LPS alone or in combinations with 10 μg/mL MA and AK for 24 h. The alternation of iNOS and COX-2 expression were detected by western blot (Figure 7). Treatment of MA and AK exhibited a remarkable decrease of iNOS expression, which was regarded as a key enzyme directly responsible for NO production. As mention to COX-2, treatment of MA or AK showed only a slight decrease of protein expression. Nevertheless, MA inhibited iNOS and COX-2 expressions obviously compared to AK.

DISCUSSION

RMR was recognized to possess various biological functions, but in most cases, the mechanisms remained unclear. To explore

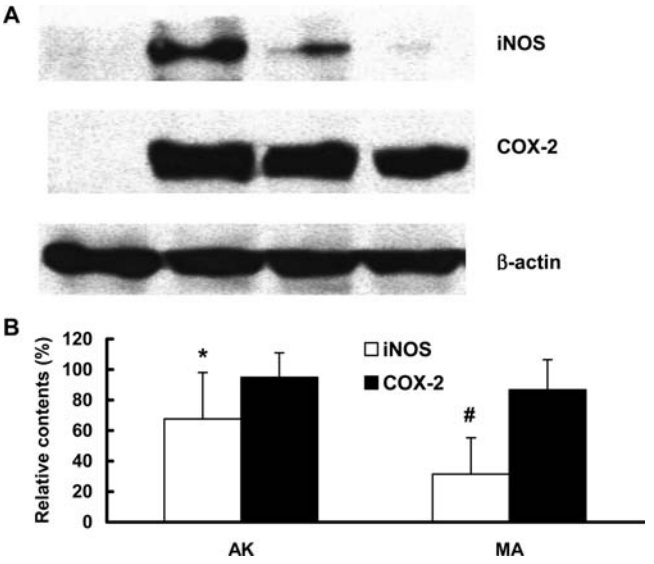

Figure 7. AK and MA suppressed LPS-induced inflammatory iNOS/COX-2 expression on RAW 264.7 cells. Cells were treated with test agents for 24 h and detected for iNOS/COX-2 by Western blot. (A) From the left side: lane 1, control; lane 2, LPS (1 μg/mL); lane 3, LPS (1 μg/mL) and AK (10 μg/mL); and lane 4, LPS (1 μg/mL) and MA (10 μg/mL). (B) Quantification of iNOS/COX-2 expression presented above. Data were expressed as the mean ± SD ($n = 3$). (*) Significantly different ($p < 0.05$). (#) Significantly different ($p < 0.01$).

the mechanisms for each biological function, the preparation of large-scale bioactive components were needed [An exception was monacolin K (MK), which was structurally identical and commercially available as lovastatin]. Generally speaking, the study of anti-tumor properties of RMR were started from the extracts and then narrowed down to its major components. For example, treatments of RMR extract not only mitigated oral carcinogenesis in hamster[17] but also prevented neovascularization and intravasation of malignant cells by the chicken embryo model.[23] One of the major anti-tumor components from RMR was MK, which inhibited metastasis and tumor progression of Lewis lung carcinoma cells.[16] Similarly, upon treatment of colon cancer cell line Caco-2, MK induced apoptosis[24] and exerted some proteomic changes.[25] With regard to the study of *Monascus* pigments, treatment of the pigment-rich fraction from RMR also induced apoptosis on colon cancer cells.[26] Besides, a scarce but critical paper reported the purification and treatment of AK to induce apoptosis on human liver cancer cell line Hep G2.[27] In our recent research, we have successfully developed the procedures of purifying a series of yellow pigment derivatives from the fermented products of *M. purpureus* NTU 568 and further proved their cytotoxicity to cancer cells but not to normal cells.[18] The above experiences are useful for us to execute large-scale preparation of related known or novel derivatives and investigate the interesting biological mechanisms, such as apoptosis-related and anti-inflammatory properties.

In this study, we used AK and MA purified by our group to explore their apoptotic mechanisms, including the caspase-9- and caspase-8-mediated extrinsic pathways.[28,29] Our first results were that AK and MA induced caspase-3 activations, which were recognized as a downsteam consequence of apoptosis (Figure 4). This outcome was consistent with reported data, which showed that AK increased the amount of apoptotic cells with cycle cell analysis.[27] As a further study of upstream events for caspase-3, we

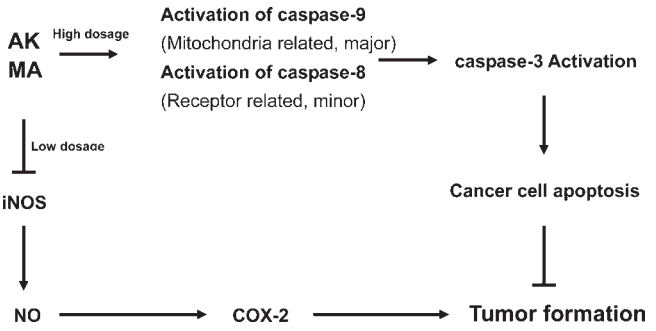

Figure 8. Correlations of AK and MA inhibited tumor formation via apoptosis of cancer cells or via suppression of inflammations.

also demonstrated that AK and MA induced casepase-9 activations through the intrinsic pathway. However, the situation of MA was much more complicated. We found that MA activated not only caspase-9 but also caspase-8. Generally, caspase-8 was indicated to activate caspase-9 through t-Bid,[30,31] but our data showed that MA-induced activations of caspase-9 were earlier and stronger than those of caspase-8 (Figure 5). This result suggested that apoptosis induced by MA was major through the caspase-9-mediated intrinsic pathway but minor through the caspase-8-mediated extrinsic pathway. In this case, the activations of caspase-9 were not amplified by caspase-8. Thus, the activations of caspase-8 were possibly triggered by clustering of death receptors[32,33] but not a direct binding of the ligand to a death receptor.

Moreover, we used the isolated AK and MA to explore their anti-inflammatory properties, such as inhibition of pro-inflammatory factors or enzymes. As shown in Figure 6, the LPS-induced NO production on RAW 264.7 was inhibited by AK and MA. This result was consistent with our previous published paper, which described that RMR extracts significantly decreased the NO production and pro-inflammatory cytokines.[17] Further exploring the upstream events for NO production, we analyzed the protein expressions of iNOS and COX-2. Therefore, we found that the expression of iNOS was severely decreased with the treatment of AK and MA, whereas the expression of COX-2 was just slightly decreased with the same treatment. This result was consistent with our previous report, however, in which experiments were performed by treating with RMR extracts and not a purified component.[17] The evidence implied that the anti-inflammatory properties of AK, MA, and RMR extracts were major resulting from the inhibition of iNOS but minor resulting from the inhibition of COX-2. It was possible that these yellow pigment derivatives inhibited iNOS and then reduced some NO-mediated responses, such as NO-stimulated COX-2 expression via a p38-dependent pathway[34] or blockage of the self-deactivation of COX-2.[35]

Previous studies in our group showed that RMR extracts or red mold dioscorea (RMD) extracts fermented from *M. purpureus* NTU 568 might prevent the 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced oral carcinogenesis in a hamster model.[17,36] Studies from other groups also showed that monascin possessed anti-tumor-initiating effects on mice skin.[11] These studies have indicated that the anti-tumor properties of fermented products from *Monascus* species resulted from the anti-inflammatory effects of yellow pigments. We have also investigated caspase activations and iNOS/COX-2 inhibitions with treatments of yellow pigment derivates. In a conclusion, we purposed that yellow pigment derivates, such as AK and MA, might inhibit tumor

formation by inducing apoptosis of cancer cells or through anti-inflammatory effects (Figure 8). These results strongly implied that RMR or RMD fermented from *M. purpureus* NTU 568 were potential candidates for tumor prevention because of the fermented products of *M. purpureus* NTU 568 containing an available amount of yellow pigments.

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