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 ssDNA ELISA kit to investigate the nuclear changes of early apoptosis induced by AK and MA on HEp-2 cells, and used Western blot and enzyme activity assay to demonstrate the activation of caspase-3, -8 and -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 evidences support that azaphilonidal derivatives from *Monascus purpureus* NTU 568, such as AK and MA, are suitable for the development of chemotherapy or chemopreventive agents.

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 fermented substrate, its product, red mold rice (RMR), has been proved to possess various biological functions. For example, RMR exhibited hypolipidemic effects in a hyperlipidemia hamster model (*1*), antifatigue activities to Wistar rats through swimming exercise (*2*), neuroprotective properties against Alzheimer's risk factors in Aβ40-infused rats (*3*), and 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 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 anti-microbial activity (*13*), inhibition of lipase (*14*), and hypolipidemic effects (*15*).

Carcinogenesis was involving in complicated steps, possibly resulted 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 anti-tumor effect by 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 *Monascus 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 *Monascus 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 of 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. NMR spectra were run on a Brucker NMR (Unity Plus 400 and 600 MHz) (Brucker BioSpin, Rheinstetten, Germany) and Varian

NMR spectrometers (Varian Gemini 200 MHz, Varian Inc., Palo Alto, CA, USA) using d_6-acetone as solvent. HPLC separations were performed on a Shimadzu LC-6AD series apparatus with a SPD-6AV UV detector, equipped with a 250 x 20 mm i.d. preparative Cosmosil AR-II column (Nacalai Tesque, Inc., Kyoto, Japan).

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

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

high-performance liquid chromatography (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^{o}C, and RAW 264.7 cells were maintained in DMEM (5% FBS).

Cytotoxicity Assay. Cytotoxicity assay was modified from the reported methods (*19, 20*). HEp-2 cells (3 x 10^3 per well) were seeded with 180 μL of MEM in 96-well plates. After 4 hr, 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^{o}C incubator with 5% CO_2. Culturing for 24, 48 and 72 hr, 20 μL of MTT solution (2 mg/mL) was added to each well and incubated for 4 hr to make cellular conversion of a tetrazolium salt into a formazan product. Then the supernatant was removed and 200 μL of 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 x 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 x 10^5 per well) were seeded and maintained with 500 μL of DMEM in 24-well plates. After 12 hr, cells were treated with LPS (1 μg/mL) and test agents (10 μg/mL) dissolved in DMEM. After 24 hr of incubation, determination of the nitrite levels in supernatants was performed by Griess reagent kit (Promega, Madison, WI, USA), and adapted from the reported methods (*21*).

Early Detection of Apoptosis. This assay was designed for detection of early apoptosis by using ssDNA apoptosis ELISA kit (Millipore, Billerica, MA, USA), and adapted from the reported methods (*22*). HEp-2 cells (about 10000 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 hr. As positive control and negative control, 100 μL of ssDNA (0.3 μg/mL) and S1 nuclease were added separately. For all conditions, antibody mixture (recognized for ssDNA) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic 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 x 10^5 for HEp-2 and 8 x 10^6 for RAW 264.7) were seeded with 10 mL of media in a 75 cm^2 flask. After 12 hr, 10 mL of test agents dissolved in media were replaced. After 12 and 24 hr of incubation, the cells were

harvested and extracted by RIPA lysis buffer (Millipore, Bellerica, MA, USA) with 1% protease inhibitor (Sigma, St. Louis, MO, USA). The cell lysates were analyzed with primary antibodies, including of caspase-3 antybody (Novus biologicals, Littleton, CO, USA), β-actin antibody (Epitomics, Burlingame, CA, USA), inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) polyclonal antibody (Cayman Chemical, Ann Arbor, MI, USA). The anti-rabbit or anti-mouse secondary horseradish peroxidase antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were further added. Finally, the detection was performed using the Western lightning chemiluminescence reagent (PerkinElmer Life Sciences, Waltham, MA, USA).

Caspase Activity Assay. 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 hr, 10 mL of test agents dissolved in media were replaced. After 12 and 24 hr of incubation, the cells were harvested and tested for caspase-8 and caspase-9 activities respectively by using colorimetric assay kit (BioVision, Linda Vista Avenue, CA, USA). Caspase activity was determined according to the manufacturer's protocol.

Statistical Analysis. Data were presented as mean ± standard deviation (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. **Monaphilone A:** ESIMS m/z 383 [M + Na]+. ^{1}H NMR (d6-acetone, 400 MHz) : 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 (d6-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). **Ankaflavin:** ESIMS m/z 387 [M + H]+. ^{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 and resulting a breakage on the ester bond. The purity of MA and AK was confirmed by 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 utilized MTT assay for a three-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 as 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 human laryngeal carcinoma cell line was possibly resulted from the structural changes between C-12 and C-14. The results also suggested that MA and AK were suitable for apoptotic approach for the next step.

Induction of Early Apoptosis by MA and AK on HEp-2 Cells. Since MA possessed moderate cytotoxicity against HEp-2 cells, we have examined whether MA induced apoptosis or necrosis. After HEp-2 cells treating with CPT (0.2 μg/mL,

reference compound for cytotoxity), AK (50 μg/mL) and MA (50 μg/mL) for 12 hr, the occurrences of early apoptosis were analyzed by ssDNA apoptosis ELISA kit (**Figure 3**). Comparing with the negative control, both of the two azaphilones exhibited significant increment of ssDNA formation, which was regarded as early apoptosis. The results confirmed that it was apoptosis, not necrosis, induced by the treatment of MA and AK.

Caspase Activation of MA and AK on HEp-2 Cells. HEp-2 cells were treated with 25 and 50 μg/mL of MA and AK for 12 and 24 hr, and further analyzed for the cleaved caspase-3 by Western blot (**Figure 4**) and enzyme activity of caspase-8 and -9 by 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 resulted from apoptosis. As 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. Comparing with all conditions, only treatment of MA (50 μg/mL, 24 hr) exhibited significant increase of caspase-8. Thus, MA was demonstrated to induce apoptosis through both of caspase-8 and -9 activations, but AK was 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 utilized Griess reagent and 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 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 of LPS alone, or in combinations with 10 μg/mL of MA and AK for 24 hr. 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 with AK.

DISCUSSION

RMR was recognized to posses various biological functions, but in most cases, the mechanisms remained unclear. In order to explore the mechanisms for each biological function, the preparation of large-scale bioactive components were needed. [An exception was monacolin K (MK), which was structural 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 from neovascularization and intravasation of malignant cell by 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 cell (*16*). Similarly, treating on colon cancer cell line Caco-2, MK induced apoptosis (*24*) and exerted some proteomic changes (*25*). As to the study of *Monascus* pigments, treatment of pigment-rich fraction from RMR also induced apoptosis on colon cancer cells (*26*). Besides, a scarce but critical paper, reported about 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 utilized AK and MA purified by our group to explore their

apoptotic mechanisms, including caspase-9 mediated intrinsic pathway and caspase-8 mediated extrinsic pathway (*28, 29*). Our first results were that AK and MA induced caspase-3 activations, which were recognized a down-steam consequence of apoptosis (**Figure 4**). This outcome was consistent with a reported data, which showed AK increased the amounts of apoptotic cells with cycle cell analysis (*27*). Further study of up-stream events for caspase-3, we also demonstrated AK and MA induced casepase-9 activations through the intrinsic pathway. However, the situation of MA was much more complicated. We found 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 that of caspase-8 (**Figure 5**). This result suggested that apoptosis induced by MA was major through caspase-9 mediated intrinsic pathway, but minor through 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 ligand to death receptor.

Moreover, we utilized 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 describing that RMR extracts significantly decreased the NO production and pro-inflammatory cytokines (*17*). Further exploring the up-stream 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, not purified component (*17*). The evidences implied that the anti-inflammatory properties of AK, MA and RMR extracts were major resulted from inhibition of iNOS, but minor from that 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 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 DMBA-induced oral carcinogenesis in a hamster model (*17, 36*). Studies from other group 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 were 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 due to the fermented products of *M. purpureus* NTU 568 containing available amount of yellow pigments.

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Figure legends

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

Figure 2. Inhibition of HEp-2 cell viability by AK and MA. (A) HEp-2 cells were treated with test agents for 72 hr. (B) HEp-2 cells were treated with 50 μg/mL of test agents for 24, 48 and 72 hr. Data were expressed as means ± 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 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 hr. (PC, positive control (0.3 μg/mL of ssDNA); NC, negative control; CPT, camptothecin) Data were expressed as means ± SD (n = 3). *, significantly different ($p < 0.05$) versus the positive control. #, significantly different ($p < 0.05$) versus the negative control.

Figure 4. The effects of MA and AK on caspase-3 activation in HEp-2 cells. Cleaved

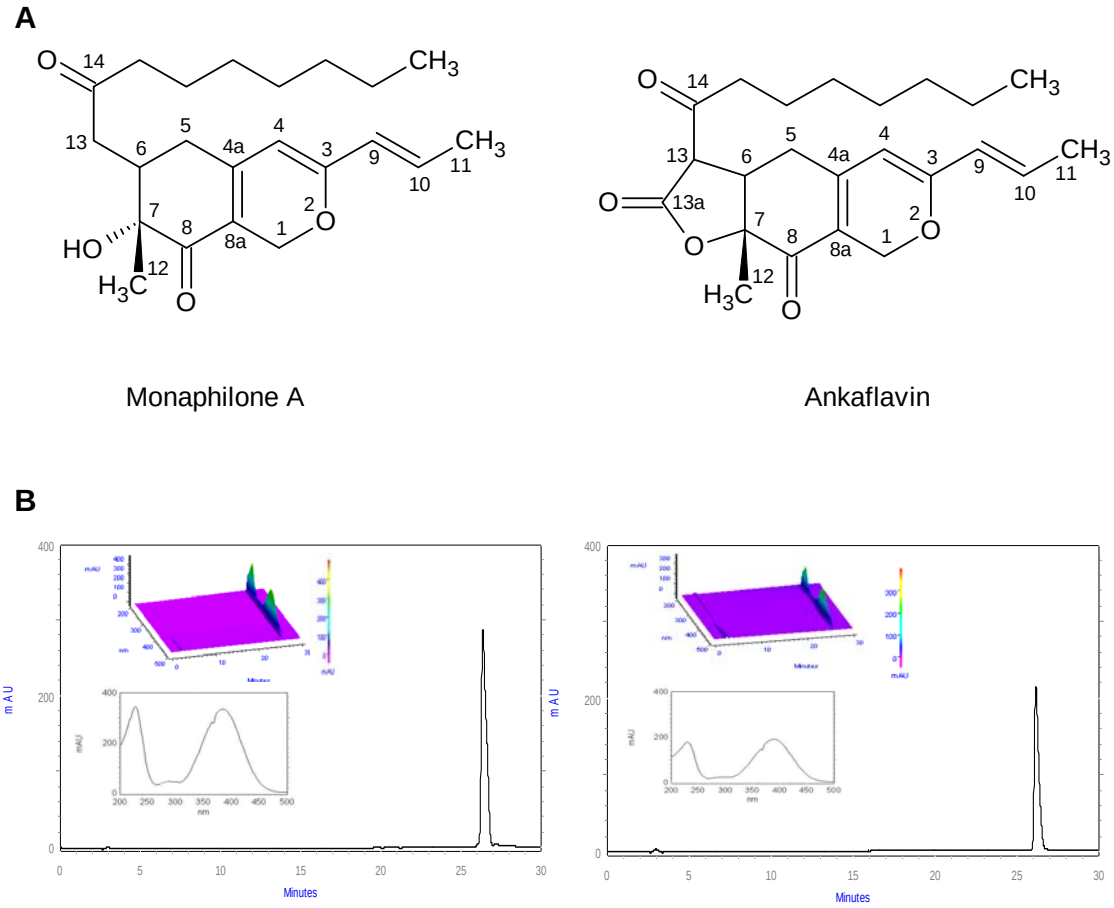
caspase-3 and β-actin were detected by Western blot. HEp-2 cells were treated with 25 or 50 μg/mL of test agents for 12 or 24 hours. (A) From the left side: Lane 1, control; lane 2, AK 25 μg/mL for 24 hr; lane 3, AK 50 μg/mL for 24 hr; lane 4, AK 50 μg/mL for 12 hr; lane 5, MA 25 μg/mL for 24 hr; lane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

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

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 hr. Data were expressed as means ± SD (n = 3). *, significantly different ($p < 0.05$) versus the negative control (treatment only with LPS).

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 hr 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); lane 4, LPS (1 μg/mL) and MA (10 μg/mL). (B) Quantification of iNOS/COX-2 expression presented above. Data were expressed as means ± SD (n = 3). *, significantly different ($p < 0.05$); #, significantly different ($p < 0.01$).

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



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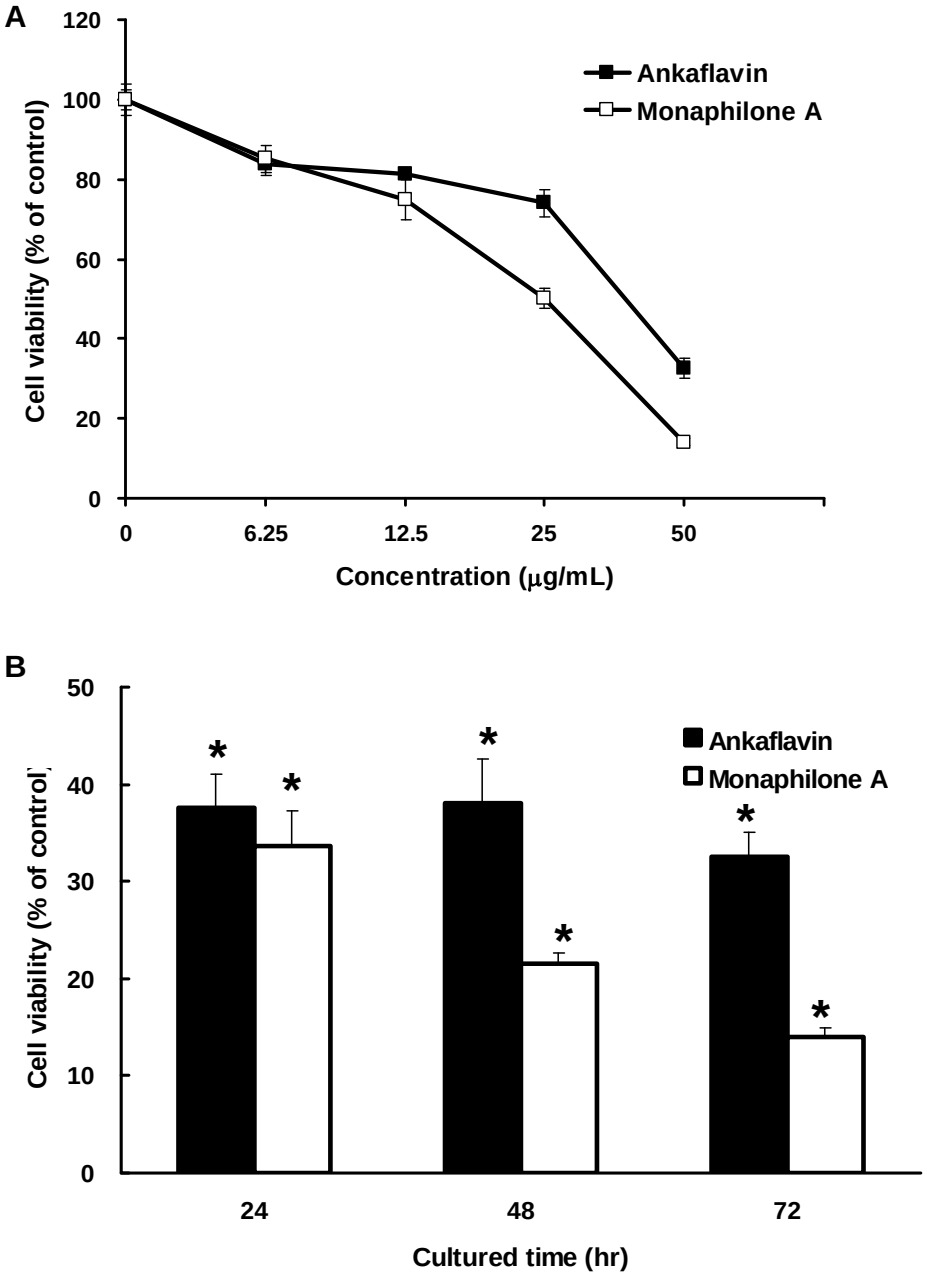

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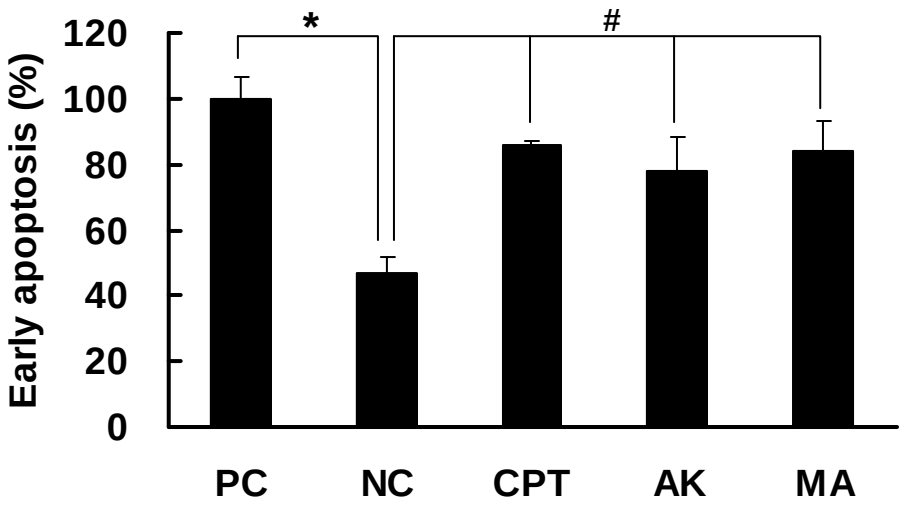

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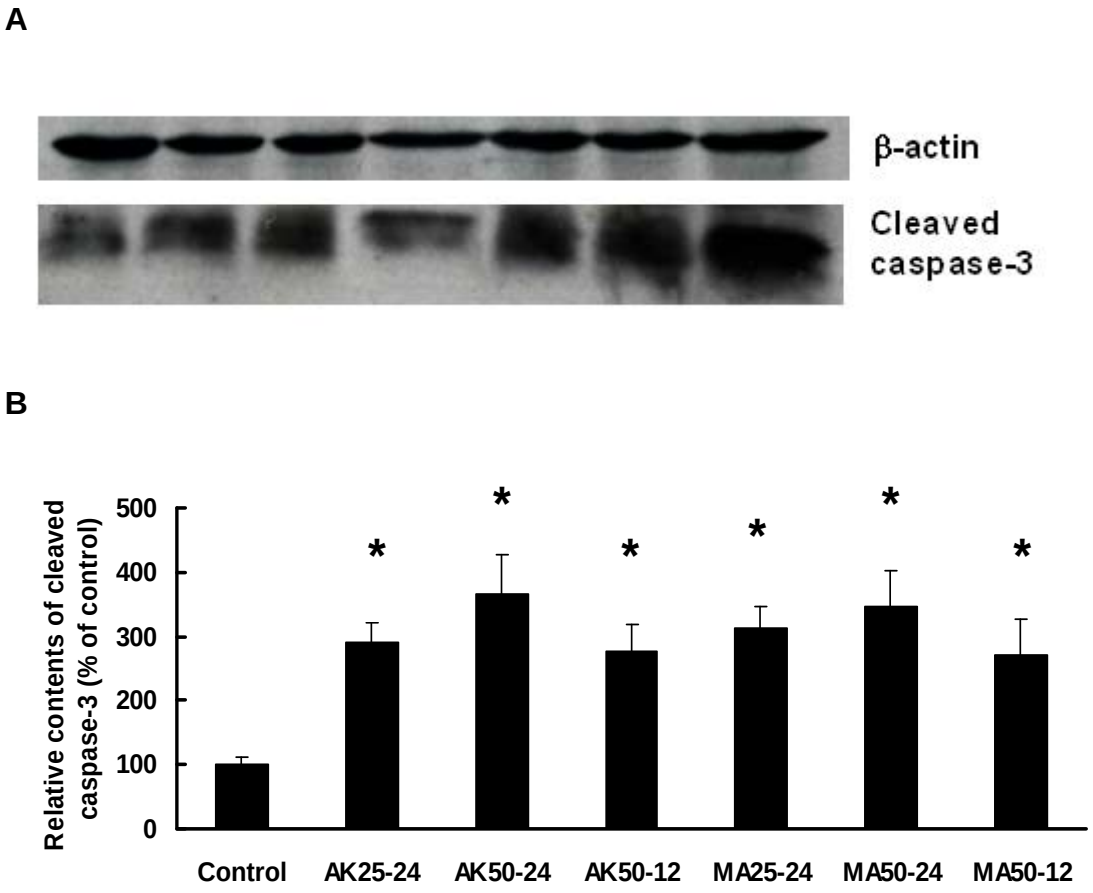

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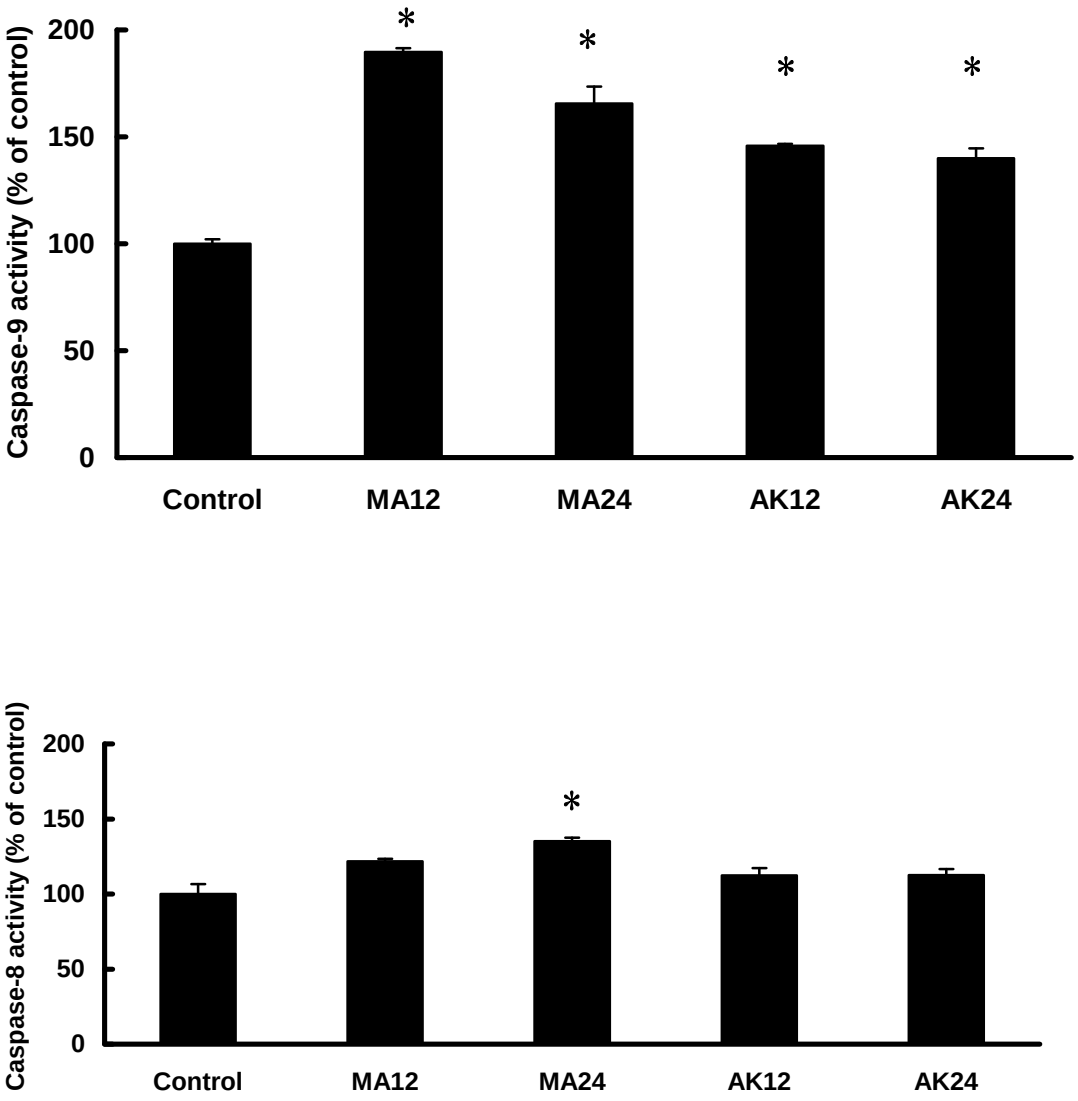

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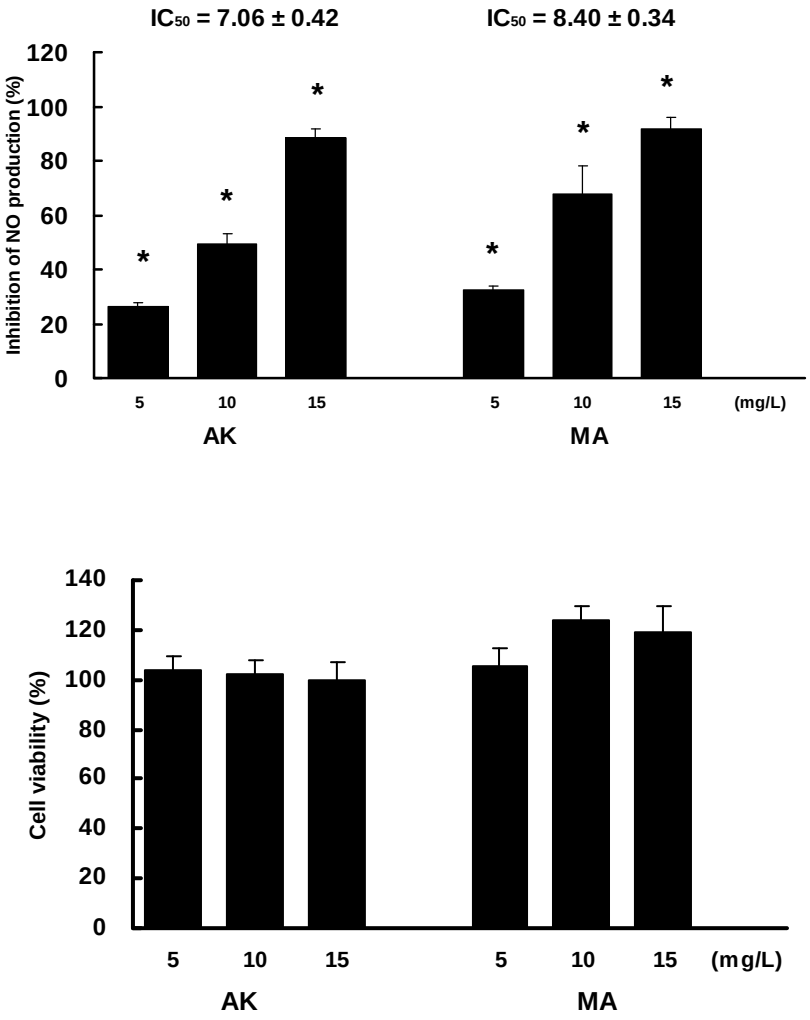

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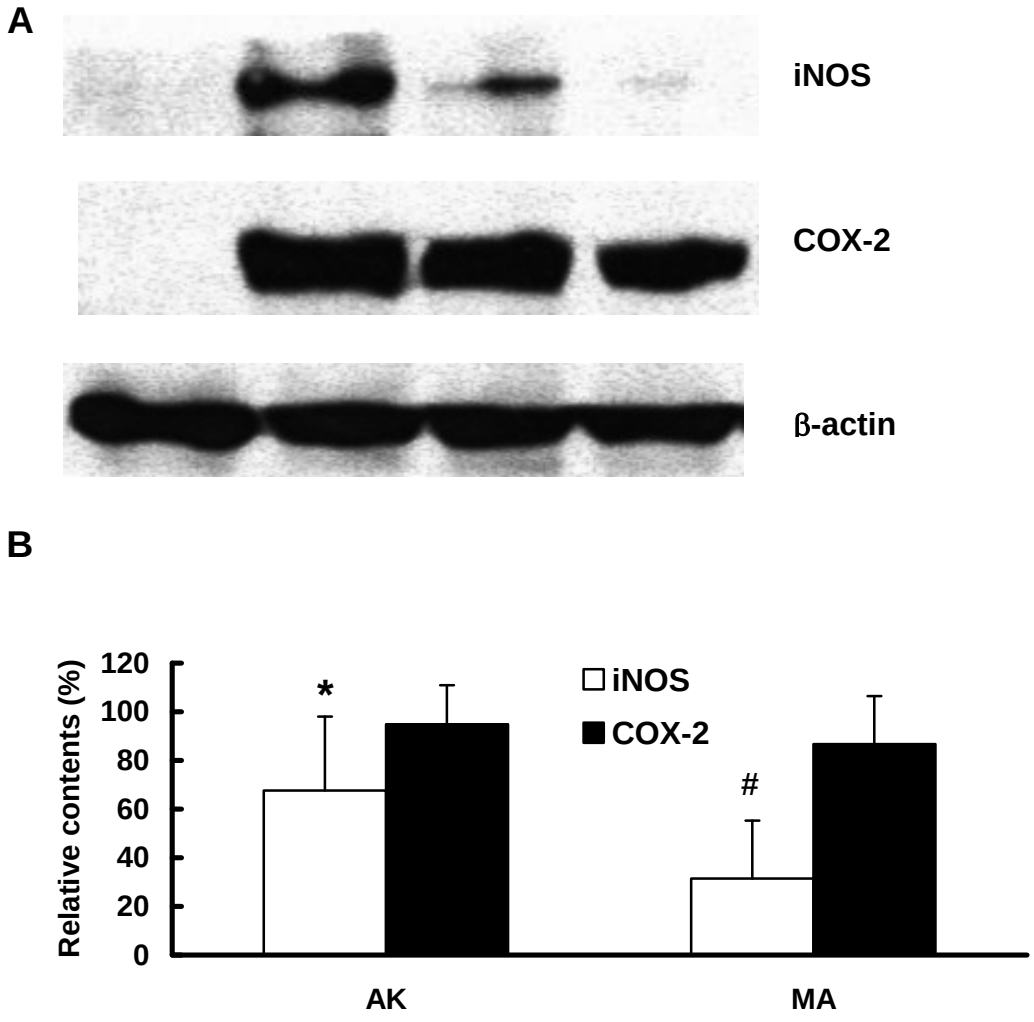

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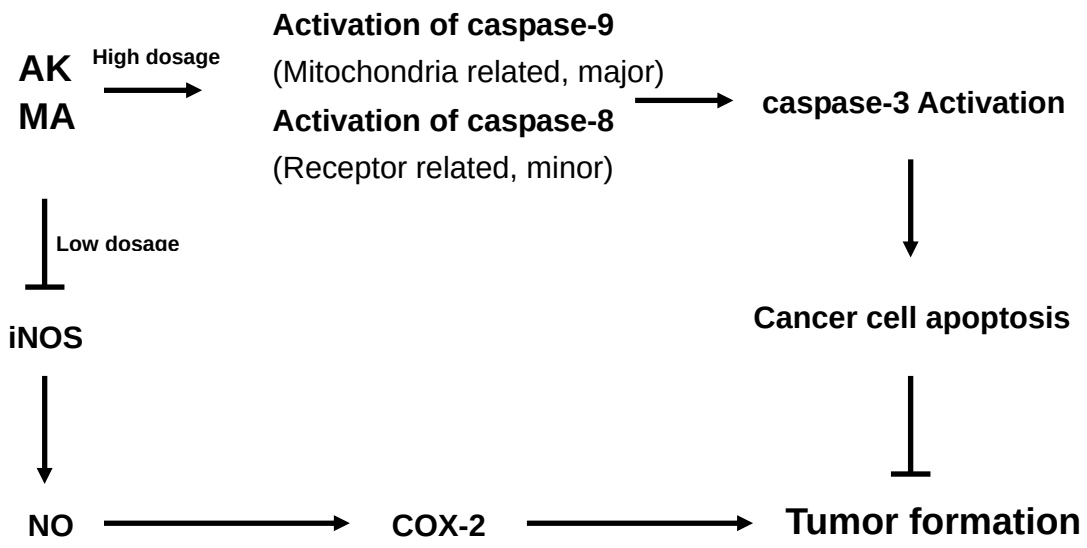

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