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Ankaflavin from *Monascus*-Fermented Red Rice Exhibits Selective Cytotoxic Effect and Induces Cell Death on Hep G2 Cells

NAN-WEI SU,* YII-LIH LIN, MIN-HSIUNG LEE, AND CHEN-YING HO

Graduate Institute of Agricultural Chemistry, National Taiwan University, Taipei 106, Taiwan

Monascus-fermented red rice has traditionally been used as a natural food colorant or food preservative of meat and fish for centuries. Recently, it has become a popular dietary supplement due to many of its bioactive constituents being discovered. Commercial *Monascus*-fermented red rice was used in this study. According to the cell-based cytotoxicity assay, a compound with selective cytotoxicity was found and identified as ankaflavin. Ankaflavin was found to be toxic to human cancer cell lines Hep G2 and A549 with a similar IC_{50} value of 15 μg/mL, while it posed no significant toxicity to normal MRC-5 and WI-38 cells at the same concentration. For elucidating the possible mode of cell death, Hep G2 cells were treated with ankaflavin for 48 h to examine the morphological change of the chromatin. Chromosomal condensation and fragmentation were found, and a significant sub-G1 peak was found by flow cytometry. Apoptosis was therefore suggested as the possible mechanism. Monascin, an analogue of ankaflavin, was also tested in this study. However, it showed no cytotoxicity and did not induce death of Hep G2 cells.

KEYWORDS: Angkak; *Monascus*; ankaflavin; monascin; cytotoxicity; apoptosis

INTRODUCTION

Monascus-fermented red rice (ang-kak) is a common red-colored product of cooked rice fermented by some *Monascus* genus molds. In Taiwan, *Monascus anka*, *Monascus ruber*, or *Monascus purpureus* are the common speices used to make *Monascus*-fermented red rice. These molds produce intense red pigments and some physiologically active metabolites when grown on cooked rice. Traditionally, ancient Chinese used *Monascus*-fermented red rice as a food colorant, preservative, or medicinal agent. Recently, *Monascus*-fermented red rice extracts have been recommended as a dietary supplement for reducing the cholesterol and lipoprotein level of human blood because it contains monacolin K. Monacolin K is a powerful drug for lowering cholesterol due to its competitive inhibitory effect on HMG-Co A reductase. Thus, almost all of recent studies on *Monascus* metabolites have investigated this effect (*1*−*5*). Organisms in the genus *Monascus* produce a mixture of six major pigments of polyketide origin (*6*−*8*). They are respectively the orange pigments monascorubrin and rubropunctatin, the red pigments monascorubramine and rubropunctamine, as well as the yellow pigments ankaflavin and monascin. There were few reports on the bioactive functions of these pigments. Yasukawa et al. (*9*, *10*) demostrated that monascorubrin suppressed the tumor promotion induced by 12-*O*-tetradecanoylphorbol-13-acetate and followed by initiation with 7,12-dimethylbenz[*a*]anthracene in mice.

In this study, we investigated the tumoricidal effect of ankaflavin. We employed a cell-based cytotoxicity assay to determine the differential cytotoxic activities of ankaflavin between human normal cell lines, MRC-5 and WI-38, and cancer cell lines, Hep G2 and A549, and we examined its effects on cell cycle distribution and its apoptotic effect on Hep G2 cells. Bioassay-guided fractionation enabled us to establish ankaflavin from *Monascus*-fermented red rice as a potent anticancer compound.

MATERIALS AND METHODS

Materials. Fetal bovine serum was obtained from Gibco BRL (Gaithersburg, MD). F12K, BME, MTT, DMSO, phenol, propidium iodide, and Hoechst 33258 were purchased from Sigma (St. Louis, MO). Trypan blue and MEM were purchased from Invitrogen (Carlsbad, CA). An in situ cell death detection kit was obtained from Roche (Mannheim, Germany). All chemicals and kits used in this study were purchased from their local agencies in Taiwan.

Cell Lines and Culture. Hep G2 (human hepatocellular carcinoma), A549 (human lung epithelial carcinoma), WI-38 (human lung fibroblast), and MRC-5 (human lung fibroblast) were obtained from Bioresources Collection and Research Center (Hsinchu, Taiwan). All cell lines in this study were maintained in the media according to their respective product sheets supplemented with 10% fetal bovine serum in a carbon dioxide incubator at 37 °C in a 5% CO_2−air mixture.

Preparation of Crude Extracts from *Monascus*-Fermented Red Rice. The dried *Monascus*-fermented red rice (13.8 kg), which had been collected at a local traditional market in Taipei, was extracted twice with 10 L of acetone at room temperature for 4 h. The extracts were combined and concentrated under reduced pressure. The residue

* To whom correspondence should be addressed. Tel: +886-2-23630231, ext. 4011. Fax: +886-2-23632714. E-mail: snw@ntu.edu.tw.

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was suspended in *n*-hexane, subjected to a silica gel (70−230 mesh) column in *n*-hexane, and eluted with increasing concentrations of ethyl acetate to increase polarities. The fractions eluted with the solvents of less than 50% ethyl acetate in *n*-hexane were collected and concentrated under reduced pressure to obtain the crude extract (ca. 1 kg).

Isolation of Ankaflavin from Crude Extracts. A part of the crude extract was coated on silica gel (1:5, sample/adsorbent, *w*/*w*) and subjected to dry flash chromatography. Sufficient *n*-hexane was passed through the column to remove the oily hydrophobic materials. Extensive gradient elution was then employed using 20%, 40%, 60%, and 80% ethyl acetate in *n*-hexane (2 L, each) to yield 16 fractions. Similar fractions were combined to give nine main fractions according to thin-layer chromatography (TLC), and the solvent was removed under reduced pressure. The fraction with most cytotoxic activity was further separated by rechromatography in a silica gel column to give fine 10-mL fractions. These resultant fractions were further analyzed by HPLC, and then fractions with a similar single peak profile were combined, respectively. Finally, the fraction with the desired compound was concentrated to dryness and then resuspended in hot ethyl alcohol. The supernatant of suspension was separated and incubated at room temperature overnight. The desired compounds, ankaflavin and monascin, were obtained respectively by means of crystallization from their respective fractions. The purity of the desired compounds was established by TLC and HPLC.

General Methods and Apparatus for Compound Purification. HPLC was performed according to the method of Teng and Feldheim (*8*) and carried out on an LDC analytical HPLC system (Thermo Separation Products) equipped with an L-7455 diode array detector (Hitachi, Tokyo, Japan). The analytical column was a 250 × 4.6 mm i.d., 5 μm, Polarity dC18 column (Waters Co.). The mobile phase was acetonitrile/water (80/20, *v*/*v*) pumped at a flow rate of 1.0 mL/min. Injection volume was 20 μL. TLC was carried out on 0.25 mm precoated Kiesel gel 60 F_{254} plates (Merck). The developing solvent system was 30% ethyl acetate in *n*-hexane. The developed spots were visualized directly or under UV light (254 and 360 nm) irradiation. Dry flash column chromatography was performed with 70−230 mesh silica gel (Merck). Mass spectrometric analyses were performed with a Fisons VG-Trio-2000 mass spectrometer system with a solid probe at 350 °C and an EI energy of 70 eV. ^{1}H and ^{13}C NMR spectra were measured with a Bruker 300 FT-NMR spectrometer in DMSO-d_6 operating at 300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR. The UV and IR spectra were taken with a Hitachi U-3210 spectrophotometer and a Nicolet 510P FT-IR spectrometer, respectively.

Assay for Cytotoxicity. Cell toxicity was assessed by MTT assay based on the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, the cells were subcultured into a 96-well plate with 2500−5000 cells per well in 100 μL of medium. After 24 h of incubation, the medium in the 96-well plate was discarded and replaced with 100 μL of medium which contained tested agents at final concentrations of 1, 10, 25, and 50 μg/mL and 0.4% DMSO as the control, each in triplicate. These plates were then incubated in a 37 °C humidified incubator with 5% CO_2 for 48 h. At the end of the incubation, the media in these plates were discarded, and the cells were washed once with PBS. Then 200 μL of MTT solution (100 μg/mL) in fresh medium was added directly to each well. The culture was then incubated for 4 h to convert MTT to formazan. Thereafter, the supernatant was aspirated and 200 μL of DMSO was added to dissolve the formazan. Plates were agitated on a plate shaker to ensure a homogeneous solution, and the optical densities (OD) were read at 570 nm. Data were expressed in terms of relative cell number % [(OD of treated cells/OD of control cells) × 100].

Assay for Cell and Nuclear Morphology. Hep G2 cells were cultured in 6-well plates at a concentration of 4×10^5/well. After 48 h of incubation, cells were treated with 25 μg/mL ankaflavin or 25 μg/mL monascin for another 48 h and were observed in parallel with cell cultures in the absence of test agents to determine the morphological effects on cell growth. In the meantime, the treated cells were fixed with methanol/acetic acid (3/1, *v*/*v*) and were stained with 50 μg/mL Hoechst 33258 (molecular probes). The stained nuclei were viewed under a fluorescence microscope.

Assay for Cell Cycle Distribution. Hep G2 cells were treated with 30 μg/mL ankaflavin or 30 μg/mL monascin for 12, 24, 36, and 48 h. Cells were collected using trypsin−EDTA and then washed once with PBS. Cells were fixed with 75% ethanol at −20 °C overnight. Cell pellets were suspensed in 800 μL of PBS, followed by adding 100 μL of 1 mg/mL RNase A and the same volume of 0.4 mg/ mL propidium iodide and incubated in the dark at 37 °C for 30 min. The cell suspension was filtered through a 35-μm mesh nylon filter. Data acquisition and analysis were performed by a FACSCalibur flow cytometer system (BD Biosciences). Cell cycle analysis was performed with the CellQuest (BD Biosciences) software. For each experiment, at least 5000 events were counted.

Assay for DNA Fragmentation. For observation of DNA fragmentation on tested Hep G2 cells, a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay using an in situ cell death detection kit−fluorescein (Roche, Mannheim, Germany) was employed in this study. The procedure was conducted according to the manufacturer's guidelines. Briefly, cells were cultured on glass slides and analyzed 48 h after treatment. Cells growing on the slides were fixed with cold 4% formaldehyde, washed twice in PBS, and incubated in cold permeabilization solution (0.5% Tween 20) after inactivating endogenous peroxidase with 3% H_2O_2. The cells were washed with PBS again and incubated with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-labeling reaction mixture for 60 min at 37 °C. After washing with PBS, cells were stained with 0.5 μg/mL propidium iodide for 10 min. For the observation of DNA fragmentation of the tested Hep G2 cells, the resulted slides were mounted with 90% glycerol and viewed under an Eclipse E600 fluorescence microscope (Nikon).

Data Analysis. Experiments were carried out in six cultures and expressed as mean ± standard deviation. Statistical evaluation was done by Student's *t*-test.

RESULTS

Purification of Ankaflavin and Monascin from *Monascus*-Fermented Red Rice. The eluate from the third chromatographic silica gel column was collected in 10-mL fractions, and the contents of ankaflavin and monascin were analyzed by HPLC with a photodiode array detector. The collected eluate was then combined into three main fractions according to the results of HPLC detection. On the basis of the elution volume, they were respective ankaflavin concentrate, ankaflavin and monascin mixture, and monascin concentrate in that order. Both the ankaflavin and monascin concentrates were collected respectively and further purified by recrystallizing in ethanol. The purity of recrystallized ankaflavin and monascin are confirmed by HPLC chromatogram (**Figure 1**). Because commercial standards of ankaflavin and monascin were not available, these two isolated compounds were identified according to the following UV, MS, and NMR data. Ankaflavin: UV λ_{max}: 230, 291 (shorter), 390. MS (70 eV) *m*/*z*: 386 (M^+), 162. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 0.85 (3H, t, J = 5.80, H-8′), 1.23 (8H, m, H-4′-7′), 1.36 (3H, s, H-16), 1.50 (2H, m, H-3′), 1.82 (3H, d, J = 6.95, H-15), 4.43 (1H, d, H-11), 4.65 (1H, d, H-1), 4.87 (1H, d, H-1), 5.57 (1H, s, H-4), 6.06 (1H, d, H-13), 6.35 (1H, M, H-14). ^{13}C NMR (DMSO-d_6, 300 MHz) δ: 14.1 (C-8′), 17.5 (C-16), 18.3 (C-15), 22.1 (C-7′), 22.6 (C-3′), 28.5 (C-4′), 28.6 (C-6′), 28.9 (C-5), 31.3 (C-5′), 42.5 (C-2′), 43.5 (C-6), 54.5 (C-11), 63.3 (C-1), 83.4 (C-7), 104.3 (C-4), 113.9 (C-9), 124.8 (C-13), 134.3 (C-14), 151.5 (C-10), 159.0 (C-3), 171.0 (C-12), 190.2 (C-8), 203.7 (C-1′). Monascin: UV λ_{max}: 230, 288 (shorter), 388. MS (70 eV) *m*/*z*: 358 (M^+), 162. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 0.85 (3H, t, J = 5.8, H-6′), 1.23 (4H, m, H-4′, H-5′), 1.36 (3H, s, H-16), 1.50 (2H, m, H-3′), 1.82 (3H, d, J = 6.95, H-15), 2.57 (2H, d, J = 9, H-5), 2.66 (1H, t, J = 6.9, H-2′), 2.77 (1H, t, J = 7.2, H-2′), 2.78 (1H, m, H-6), 4.44 (1H, d, J = 10.2, H-11), 4.65 (1H, d, J = 12.6, H-1), 4.87

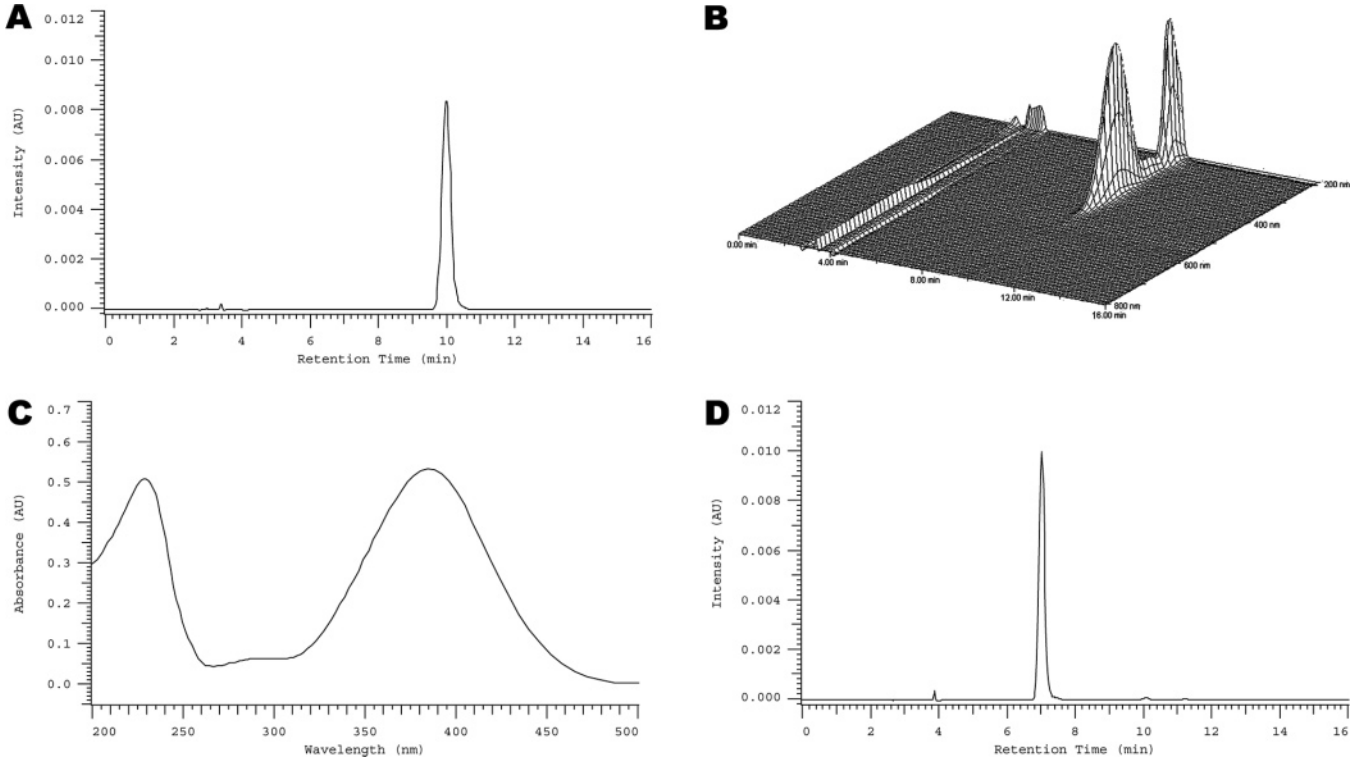

Figure 1. Chromatograms of purified ankaflavin and monascin of this study. Purified ankaflavin detected at 233 nm (**A**); detected under full wavelength (**B**); UV spectrum of purified ankaflavin (**C**); and purified monascin detected at 233 nm (**D**).

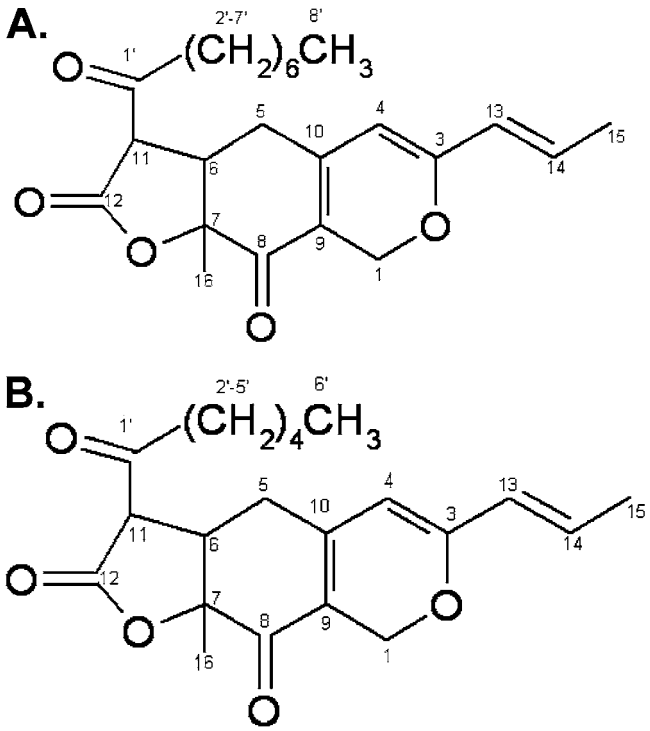

Figure 2. Structures of ankaflavin (**A**) and monascin (**B**).

(1H, d, J = 12.6, H-1), 5.57 (1H, s, H-4), 6.08 (1H, d, J = 15.6, H-13), 6.47 (1H, m, H-14). ^{13}C NMR (DMSO-d_6, 300 MHz) δ: 14.0 (C-6′), 17.5 (C-16), 18.3 (C-15), 22.1 (C-5′), 22.3 (C-3′), 28.9 (C-5), 31.1 (C-4′), 42.4 (C-2′), 43.5 (C-6), 55.0 (C-11), 63.3 (C-1), 83.4 (C-7), 104.3 (C-4), 113.9 (C9), 124.8 (C-13), 134.3 (C-14), 151.5 (C-10), 171.0 (C-12), 190.2 (C-8), 203.7 (C-1′). The structures are shown in **Figure 2**. These data are compatible with those previously reported by Martinkova et al. (*11*) and Teng (*12*).

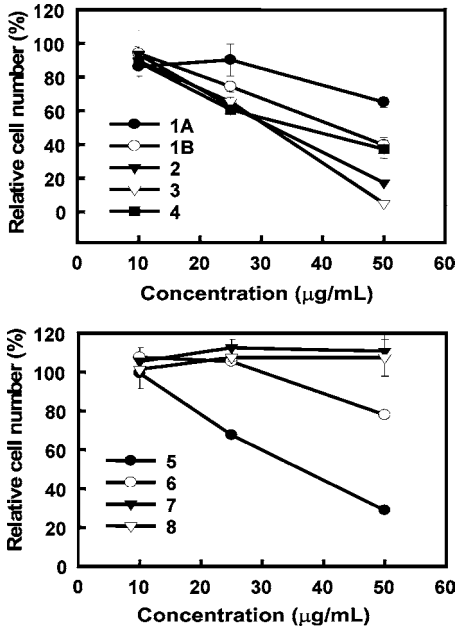

Figure 3. Cytotoxicity of fractions of acetone extract from *Monascus*-fermented red rice to A549 cells.

Cytotoxicity of *Monascus*-Fermented Red Rice Extracts, Ankaflavin, and Monascin. To study the cytotoxic effect of each fraction of *Monascus*-fermented red rice extract obtained from the silica gel fractionation, the tested cells were cocultured with various amounts of these fractions. The cytotoxic effects were determined by MTT assay. The cytotoxic effects of the nine main fractions from *Monascus*-fermented red rice extract are shown in **Figure 3**. The results indicate that the notable cytotoxic effects on A549 are found in fractions 2, 3, and 5. The effects were concentration-dependent. At 50 μg/mL, compared with the control, the relative cell number of A549

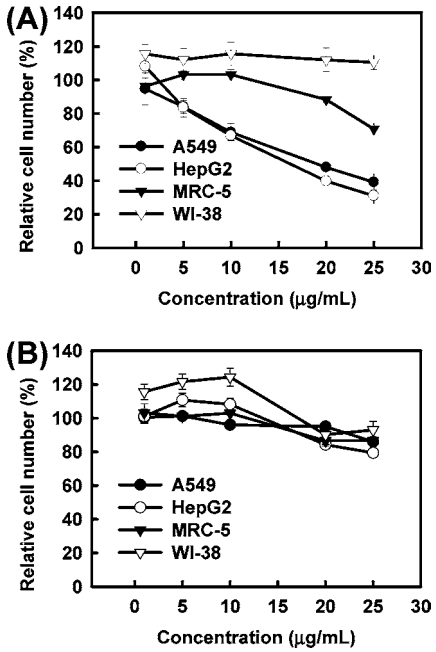

Figure 4. Cytotoxicity of ankaflavin (**A**) and monascin (**B**). The data shown are the mean ± SD of three independent experiments each with triplicate wells.

cells was 18% (fraction 2), 12% (fraction 3), and 27% (fraction 5). Furthermore, the cytotoxic effects of the purified ankaflavin and monascin, which were obtained from fraction 3 by rechromatography and crystallization, on the four tested cells are shown in **Figure 4**. Only ankaflavin exhibited cytotoxicity for the four tested cell lines. Additionally, the results of ankaflavin indicate selective tumoricidal effects on the human lung carcinoma A549 and liver cancer cell Hep G2 at the concentrations of 5−25 μg/mL with no significant toxicity toward normal fibroblasts (MRC-5 and WI-38) at the same concentrations of 5−20 μg/mL. The concentrations of 50% percent inhibition (IC_{50}) of ankaflavin in the two tumor cell lines (Hep G2 and A549), compared with the control, were found to be around 15 μg/mL. Moreover, the results of monascin did not show any cytotoxic effects on the four tested cells.

Cell Cycle Distribution on the Treated Hep G2 Cells. The effects of ankaflavin and monascin on the cell cycle distribution of Hep G2 was determinated by flow cytometric analysis after treating with different doses for different intervals. As shown in **Figure 5**, the results indicate that, compared with the control, ankaflavin induces a distinct sub-G1 peak in Hep G2 cells. The percentage of cells with a sub-G1 peak increased from 2.5% to 28% when treated with ankaflavin. **Figure 6** shows that an increase in sub-G1 peak is induced in a dose-dependent manner in ankaflavin-treated Hep G2 cells. The appearance of cells with low DNA stainability forms a sub-G_1 peak, which was considered to be the marker of cell death by apoptosis (*13*). The results suggest that apoptosis is a candidate mode of the cell death by means of ankaflavin treatment. In contrast, the results of cells treated with monascin for 48 h indicated that, compared with the control, there is no significant sub-G1 peak forming, and the number of cells in G_0/G_1, S, and G_2/M phases of the cell cycle distribution are not of any statistically significant difference.

Ankaflavin-Induced Apoptosis on Hep G2 Cells. Apoptosis is an active process of cell death. It has been recognized that apoptotic cells have reduced DNA stainability with a variety of fluorochromes (*14*, *15*). Thus, the observations of the

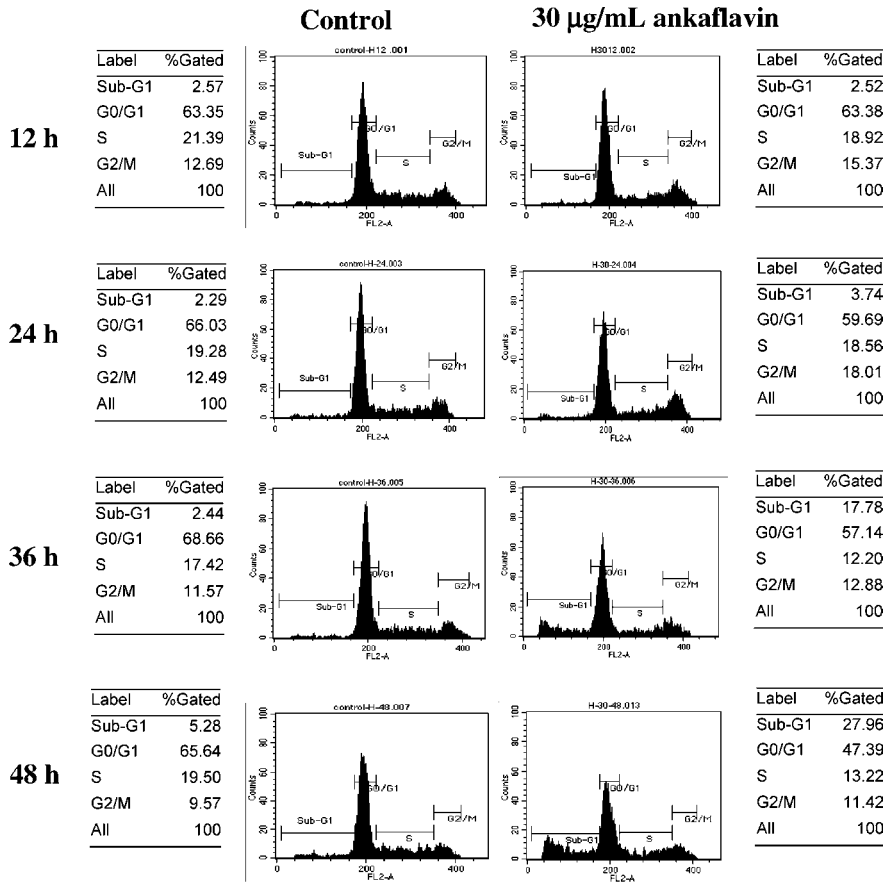

Figure 5. Cell cycle analysis of HepG2 cells after treating with 30 μg/mL ankaflavin for 12, 24, 36, and 48 h.

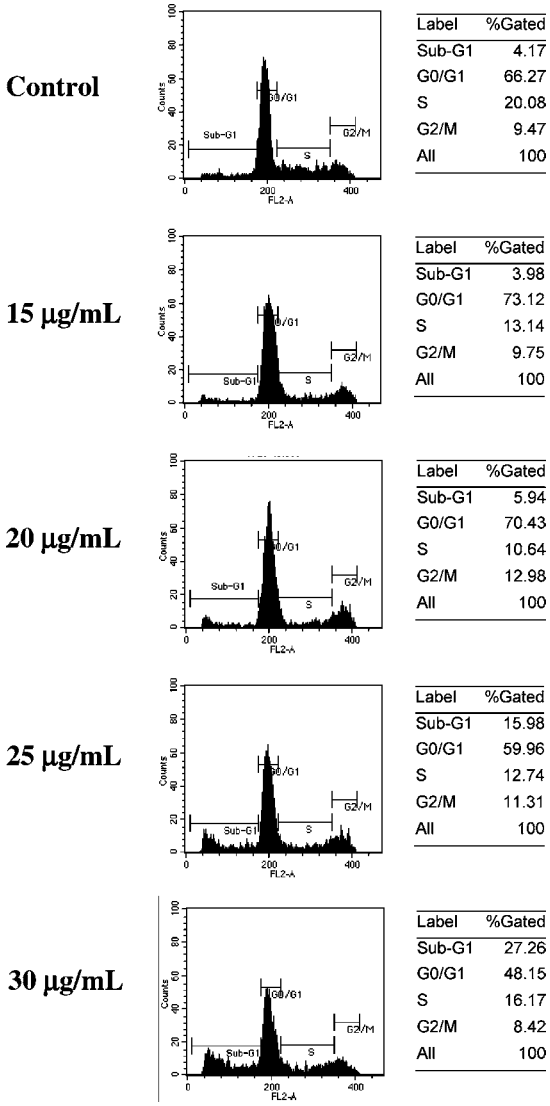

Figure 6. Cell cycle analysis of HepG2 cells after treating with 15, 20, 25, and 30 μg/mL ankaflavin for 48 h.

morphological changes of the nuclei in treated cells by Hoechst 33258 staining and TUNEL assay can precisely detect apoptotic response. According to our results (data not shown), Hep G2 cells treated with 25 μg/mL ankaflavin for 48 h exhibit significant chromatin condensation (fluorescent spot) through Hoechst staining. This phenomenon indicates many apoptotic bodies are formed after ankaflavin treatment. Furthermore, many TUNEL-positive cells are observed under a fluorescence microscopy and appeared fluorescent green in color. This result also suggests ankaflavin exerting its cytotoxicity to Hep G2 cells through apoptosis, which are characterized by DNA fragmentation.

DISCUSSION

It is well-known that *Monascus* can produce the bioactive secondary metabolites such as monacolins, which can lower the plasma cholesterol levels (*1*−*5*), and γ-aminobutyric acid (GABA), which has several physiological functions, such as neurotransmitting, hypotensive, and diuretic effects (*16*−*19*). Moreover, mycelial extracts from *Monascus* were reported to be antimutagenic (*20*), antibiotic (*21*−*23*), embryotoxic, and teratogenic (*21*). However, there have been very few reports focusing on the antitumor effects of *Monascus* metabolites. In general, a compound that shows direct cytotoxic effects on tumor cells, and/or immunomodulatory activity may possess potency for antitumor application. In this study, a cell-assay-guided fractionation was employed to isolate the cytotoxic chemicals from *Monascus*-fermented red rice. On the basis of our preliminary results, the most polar fractions of the acetone extract of *Monascus*-fermented red rice did not show any detectable cytotoxic effects on A549 cells under our screening assay concentration (100 μg/mL) when the cells were analyzed by MTT assay. Therefore, we directly collected the eluate that was of less than 50% ethyl acetate in *n*-hexane as the crude extract during silica gel column fractionation. Moreover, the crude extract was further fractionated with a second chromatographic silica gel column by gradient elution through increasing the concentration of ethyl acetate in *n*-hexane to increase the polarities. The cytotoxic effects of these different fractions on A549 cells are shown in **Figure 3**. Those polar fractions that reveal no significant cytotoxicity are confirmed as the red pigments. However, the relatively weak polar fractions show significant cytotoxic effects on the tested tumor cells. We further obtained two *Monascus* metabolites, ankaflavin and monascin, from one of the relatively weak polar fractions by rechromatography and crystallization and showed that ankaflavin exhibited tumoricidal effects on human lung carcinoma A549 and hepatoma cell Hep G2 (**Figure 4**). However, monascin, an analogue of ankaflavin, shows no significant change on the cell cycle histograms, and no significant cell death was observed after 48-h treatment. Furthermore, according to the results of the TUNEL assay, Hoechst staining, and sub-G1/PI analysis, we suggest rationally that ankaflavin might mediate its cytotoxicity through apoptosis. In addition, the fact that ankaflavin shows no cytotoxic effects on the nontumorigenic WI-38 and MRC-5 cells at the same assayed concentrations indicates that it possesses a selective cytotoxicity to the tumor cells. Furthermore, we found a very interesting result in the structure−cytotoxic relationships. Both monascin and ankaflavin contain the same basic chromophoric structure and differ from each other only in the length of the saturated side chain (C_5 in monascin and C_7 in ankaflavin) on the ketonic carbonyl group (**Figure 2**). However, monascin was not cytotoxic toward the four tested cells and could not induce apoptosis or arrest the cell cycle progression in the treated Hep G2 cells as judged by the observations of DNA histograms and DNA condensation. These results indicate that the length of the saturated side chain on the ketonic carbonyl group of ankaflavin is an important factor toward the cytotoxicity. Recently, studies concerning the structure−activity relationship were reported, such as the antimelanoma effects of resveratrol and related substances (*24*), and the antibacterial action of the different alk(en)yl chain length or the number of double bonds in the side chain of anacardic acids (*25*). Therefore, our further work is to investigate the effects of the side chain group on the cytotoxic effects of the ankaflavin analogues. Our preliminary experiments have recently revealed the activation of caspase-3 at the concentration of 15 μg/mL of ankaflavin, which is one of the typical downstream molecular markers of apoptosis. The present study is the first report of ankaflavin from *Monascus* metabolites exerting the cytotoxicity through apoptosis. Therefore, further experiments are still needed to elucidate its exact mechanism of molecular action and its immunomodulatory effects.

ABBREVIATIONS USED

F12K, nutrient mixture F-12 Ham (Kaighn's modification); BME, basal medium Eagle; MEM, minimum essential medium

Eagle; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling.

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