Cudraflavanone A purified from *Cudrania tricuspidata* induces apoptotic cell death of human leukemia U937 cells, at least in part, through the inhibition of DNA topoisomerase I and protein kinase C activity

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A chloroform extract of the root bark of *Cudrania tricuspidata* showed an inhibitory effect on mammalian DNA topoisomerase I. The topoisomerase I inhibitory compound was purified and identified as 2S-2′,5,7-trihydroxy-4′,5′-(2,2-dimethylchromeno)-6-prenyl flavanone (cudraflavanone A). Cudraflavanone A was shown to inhibit the activity of topoisomerase I with approximately 0.4 mmol/l 50% inhibitory concentration. A concentration of 6 μmol/l cudraflavanone A caused a 50% growth inhibition of human cancer cell U937. Cudraflavanone A-induced cell death was characterized by the cleavage of poly(ADP-ribose) polymerase and pro-caspase-3. Furthermore, cudraflavanone A induced the fragmentation of DNA into multiples of 180 bp (an apoptotic DNA ladder), indicating that the inhibitor triggered apoptosis. This induction of apoptosis by cudraflavanone A was also confirmed using flow-cytometry analysis. In addition, this compound inhibited protein kinase C activity with approximately 150 μmol/l 50% inhibitory concentration. Taken together, these results suggest that cudraflavanone A may function by inhibiting oncogenic disease, at least in part, through the inhibition of protein kinase C and topoisomerase I activity. *Anti-Cancer Drugs* 18:1023–1028 © 2007 Lippincott Williams & Wilkins.

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Introduction

DNA topoisomerases are nuclear enzymes that alter the DNA topology required for the replication, transcription, recombination and segregation of daughter chromosomes [1–3]. Eukaryotic cells have two types of topoisomerases, topoisomerase I and II. Topoisomerase I catalyzes the passage of the DNA strand through a transient single-strand break in the absence of any high-energy cofactor, whereas topoisomerase II catalyzes the passage of DNA double strands through a transient double-strand break in the presence of ATP.

In recent years, topoisomerase I has been considered as an attractive target for antitumor agents [4]. The expression of topoisomerase I is enhanced in several types of leukemia, lymphoma and colon carcinoma cells [5,6]. Topoisomerase I-targeted drugs, such as the plant alkaloid camptothecin (CPT) and its derivatives including topotecan, 9-amino-CPT and CPT-11, are used in cancer chemotherapy [7,8]. Recently, some compounds such as β-lapachone, bulgarein and diospyrin have also been reported as topoisomerase I inhibitors [9–11]. These results have prompted us to screen topoisomerase I inhibitors derived from medicinal herbs.

Cudrania tricuspidata is a deciduous shrub or tree that is found throughout Korea, China, and Japan. Its roots have been used in the clinical treatment of digestive apparatus tumors and inflammation in East Asia [12,13]. The mechanisms by which the extract of the roots of *C. tricuspidata* exerts its anticarcinogenic effects, however, remain largely unknown. In this report, we first demonstrate that cudraflavanone A, purified from *C. tricuspidata*, inhibits not only topoisomerase I activity but also protein kinase C (PKC) activity. Cudraflavanone A killed human cancer cells by apoptosis. The current results suggest that cudraflavanone A is likely to function by inhibiting oncogenic disease; such an effect would be due, at least in part, to the inhibition of PKC and topoisomerase I activity.

Materials and methods

Plant materials, enzyme and antibodies

C. tricuspidata (Carr.) Bureau was collected in Hyoupchun (South Korea) and identified by Professor Jae-Hong Pak. A voucher specimen (Park, K. H. 110) of this raw material was deposited at the Herbarium of Kyungpook National University. Calf thymus topoisomerase I was purchased from Takara Shuzo (Otsu, Shiga, Japan).

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Specific antibodies against a cleaved form of poly(ADP-ribose) polymerase (PARP) and pro-caspase-3 were from Cell Signaling Technology (Beverley, Minnosota, USA) and Santa Cruz Biotechnology (Santa Cruz, California, USA), respectively.

Extraction and isolation

The dried root barks (3 kg) were chopped and extracted with $CHCl_3$ at room temperature. The combined extracts were concentrated, and the dark residue (86 g) was partitioned between H_2O and $CHCl_3$. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and then concentrated to give a dark-brown residue (41 g). The residue was placed onto a silica gel column (7×60 cm, 230–400 mesh) eluted initially with $CHCl_3$, and then with $CHCl_3$/MeOH mixtures of increasing polarity ($30:1 \rightarrow 1:1$), yielding 11 fractions (F1–F11). The fraction F6 (1.4 g) was submitted to a flash silica gel column chromatography (25 g, 230–400 mesh) and eluted with a hexane/EtOAc gradient ($20:1 \rightarrow 1:1$), resulting in 80 subfractions. Subfractions 31–36 were evaporated to yield 32 mg of topoisomerase I inhibitory compound.

DNA topoisomerase I assay

The topoisomerase I activity was monitored by a DNA relaxation assay. The assay was carried out in 20 µl of the reaction mixture, which contained 35 mmol/l Tris–HCl (pH 8.0), 72 mmol/l KCl, 5 mmol/l $MgCl_2$, 5 mmol/l dithiothreitol, 5 mmol/l spermidine, 0.01% bovine serum albumin, supercoiled pUC118 (0.4 µg) and topoisomerase I (1 unit). One unit of topoisomerase I activity was defined as the minimum amount of enzyme required for the complete relaxation of 0.4 µg of supercoiled DNA. In some experiments, the reaction mixtures were supplemented with cudraflavanone A (1 µl in dimethylsulfoxide). After incubation for 15 min at 37°C, the reactions were terminated by 5 µl of a stop buffer containing 5% sodium dodecyl sulfate (SDS), 50 mmol/l ethylenediaminetetraacetic acid (EDTA), 20% Ficoll, 0.1 mg/ml bromophenol blue and 0.1 mg/ml of xylene cyanol; then the DNA samples were electrophoresed in a 0.7% agarose gel. The gels were stained with ethidium bromide (5 µg/ml) and photographed.

Cytotoxicity assay

Cytotoxicity was determined with the MTT dye reduction assay as described by Mosmann [14] with minor modifications. Human U937 cells were maintained in RPMI-1640 medium (Sigma-Aldrich Company; Poole, Dorset, UK) with a 10% heat-inactivated bovine calf serum (FBS), 20 mmol/l *N*-2-hydroxyl piperazine-*N*′-2-ethane sulfonic acid (pH 7.0), 100 µg/ml gentamycin and 5×10^{-5} mol/l β-mercaptoethanol. The growing cells were plated in a 96-well plate at 5×10^4 cells per well with 100 µl RPMI-1640. Subsequently, a serial dilution of cudraflavanone A, concentration ranging from 0 to 50 µmol/l, was added. The cells were incubated for 48 h at 37°C in an atmosphere containing 5% (v/v) CO_2; then 50 µl of an MTT solution (1.1 mg/ml; Sigma) was added to the wells for 4 h. The cells were harvested by centrifugation and dissolved in 150 µl dimethysulfoxide. The optical density was measured at 540 nm using an enzyme-linked immunosorbent assay reader (Bio-Rad, Hercules, California, USA).

Western blotting analysis

Cudraflavanone A-treated or untreated cells were washed with ice-cold phosphate buffered saline, collected by centrifugation and lysed in 100 µl of ice-cold radioimmunoprecipitation assay buffer (50 mmol/l Tris–HCl, pH 8.0, 150 mmol/l NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mmol/l phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/ml pepstatin). The particulate debris was removed by centrifugation at 12 000*g*. The protein concentration in the supernatants was determined using Bradford protein dye reagent (Bio-Rad) and the volumes of the supernatants were adjusted for equal protein concentration. Protein samples were separated on 10% polyacrylamide gel in the presence of SDS and then transferred electrophoretically to nitrocellulose membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline Tween-20, (20 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl and 0.05% Tween 20) for 2 h and then incubated with specific antibodies against a cleaved PARP form or pro-caspase-3 in 1% skim milk for 1 h. The membrane was washed three times in Tris-buffered saline Tween-20 and then treated with ECL system (Biosciences, Little Chalfont, Buckinghamshire, UK).

DNA fragmentation assay

U937 cells treated with or without cudraflavanone A for 48 h were resuspended in 1 ml of lysis buffer (200 mmol/l NaCl, 0.2% SDS, 5 mmol/l EDTA and 100 mmol/l Tris–HCl, pH 7.4) for 45 min on ice and centrifuged at 16 000*g* for 10 min. Genomic DNA was extracted twice using phenol/chloroform (1 : 1), precipitated with isopropanol and resuspended in Tris–EDTA buffer (10 mmol/l Tris–HCl, pH 8.0, and 1 mmol/l EDTA). The DNA was treated with RNase A, analyzed by electrophoresis on 1% agarose gel and then stained with a solution containing 0.5 µg/ml of ethidium bromide.

Fluorescence-activated cell sorting analysis

U937 cells (2×10^5) were seeded in 100-mm dishes containing RPMI-1640 medium with 10% FBS and treated with cudraflavanone A for 48 h. The cells were collected in phosphate-buffered saline containing 2% FBS, fixed in 70% ethanol for 16 h at −20°C, and then incubated in 50 µg/ml DNase-free RNase A (Sigma), 25 µg/ml propidium iodide (Sigma) and 0.6% sodium citrate for 30 min at 37°C. Flow-cytometric determination of cellular DNA content was performed on a Coulter Elite ESP Cell Sorter (Beckman Coulter, Fullerton, California,

USA). The forward-scatter and side-scatter gates were set to exclude any dead cells from the analysis; 10 000 events within this gate were acquired per sample.

Assays for casein kinase II and protein kinase C activity

The standard assay for the phosphotransferase activity of casein kinase II (CKII) was conducted in a reaction mixture containing 20 mmol/l Tris–HCl, pH 7.5, 120 mmol/l KCl, 10 mmol/l $MgCl_2$ and 100 µmol/l [γ-^{32}P]ATP in the presence of 1 mmol/l synthetic peptide substrate (RRREEETEEE) in a total volume of 30 µl at 30°C. The reactions were started by the addition of cell lysates and incubated for 15 min. For PKC activity assay, cell lysates were incubated with a kinase buffer [10 mmol/l $MgCl_2$, 20 mmol/l Tris–HCl, pH 7.5, 0.25 mmol/l ethylene glycol-bis(*b*-aminoethyl ether), 0.4 mmol/l $CaCl_2$, 100 µg/ml phosphatidyl serine] containing 50 µmol/l PKC substrate peptide (AAKIQASFRGHMARKK) and [γ-^{32}P]ATP at 30°C for 5 min. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 10%. It was then centrifuged and 10 µl of supernatant applied to P-81 paper. The paper was washed in 100 mmol/l phosphoric acid and the radioactivity was measured by scintillation counting.

Other experimental procedures

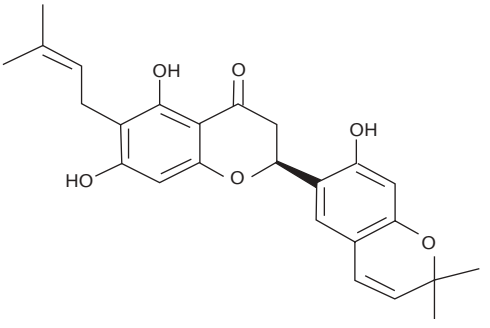
Melting points were measured on a Thomas Scientific Capillary Melting Point Apparatus (Thomas Scientific, Swedesboro, New Jersey, USA). Infrared spectra were recorded on a Bruker IFS66 (BRUKER, Silberstreifen, Rheinstetten/Karlsruhe, Germany) infrared Fourier transform spectrophotometer (KBr). Ultraviolet spectra were measured on a Beckman DU650 spectrophotometer. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) along with two-dimensional (2D)-NMR data were obtained on a Bruker AM 500 (^{1}H-NMR at 500 MHz, ^{13}C-NMR at 125 MHz) spectrometer in $CDCl_3$; EIMS and HREIMS data were collected on a Jeol JMS-700 spectrometer (JEOL; Tokyo, Japan).

Results and discussion

A chloroform extract of the root bark of *C. tricuspidata* showed an inhibitory effect on calf thymus topoisomerase I (data not shown). The topoisomerase inhibitory compound was purified as described in Materials and methods. This compound (molecular formula of $C_{25}H_{26}O_6$) was obtained as a pale yellow needle and 13 degrees of unsaturation, as deduced from its high resolution electron ionization mass (HREIMS) data. Ultraviolet and infrared spectra showed that the compound had flavanone skeleton. The exact structure of CTR-103 was inferred from a detailed analysis of ^{1}H- and ^{13}C-NMR data, together with 2D-NMR experiments. The ^{1}H- and ^{13}C-NMR data with the distortionless enhancement by polarization transfer experiment showed the presence of 25 carbon atoms as one carbonyl group, four methyls, two sp^3 methylenes, seven methins and 11 quaternary carbons. The ^{13}C-NMR spectral data enabled one carbonyl and eight double bonds to be characterized, and these account for nine of the total 13 degrees of unsaturation. Hence, extra degrees of unsaturation were presumed to be due to a tetracyclic ring included in the flavanone ring system. The ^{1}H-NMR data showed δ_H 5.56 (1H, dd, J = 12.6, 2.9 Hz), 2.87 (1H, dd, J = 17.3, 3.0 Hz) and 3.13 (1H, dd, J = 17.3, 12.7 Hz), attributed to the C-ring protons (H-2, H-3) of the flavanone. It also indicated the presence of three aromatic protons [δ_H 6.01, 6.63 and 6.88 (each 1H, s)]. The prenyl group was determined on the basis of successive connectivities from C-1″ to C-5″ in the ^{1}H–^{1}H COSY spectrum. The HMBC correlation of H-1″ with C-5, C-6 and C-7, and of OH-5 (δ_H 12.37) with C-4a, C-5 and C-6 allowed the prenyl group to site at C-6 on the A-ring. The 2,2-dimethylpyran ring was easily deduced from the connectivity of two olefinic protons [H-1‴ and H-2‴ (δ_H 5.49 and 6.25)] in ^{1}H–^{1}H COSY spectrum, and also the correlation between C-3‴ and H-2‴,5‴ in the HMBC experiment. This 2,2-dimethylpuran ring was fused at C-4′ and C-5′ on the B-ring because of the HMBC correlation of H-3́ with C-5, H-1‴ with C-4 and C-6, and H-2‴ with C-5. The absolute configuration was determined as 2*S*-flavanone by the circular dichroism spectra. The positive Cotton effect at 339 nm and the negative Cotton effect at 295 nm allowed the assignment of the *S*-configuration at C-2. Consequently, the compound was identified as 2*S*-2′,5,7-trihydroxy-4′,5′-(2,2-dimethylchromeno)-6-prenyl flavanone (cudraflavanone A; Fig. 1).

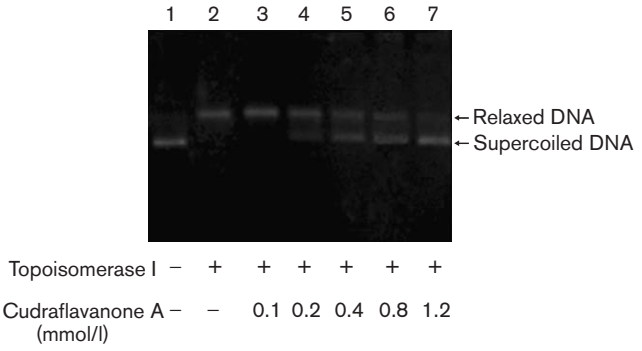
Next, we investigated the effect of cudraflavanone A on the catalytic activity of topoisomerase I in the presence of pUC118 as a substrate. As shown in Fig. 2, cudraflavanone A inhibited topoisomerase I activity in a dose-dependent manner. Little topoisomerase I inhibitory activity of cudraflavanone A was observed at a concentration of 0.1 mmol/l. At a concentration 0.2 mmol/l, however, it could inhibit the topoisomerase I activity. To obtain

Fig. 1



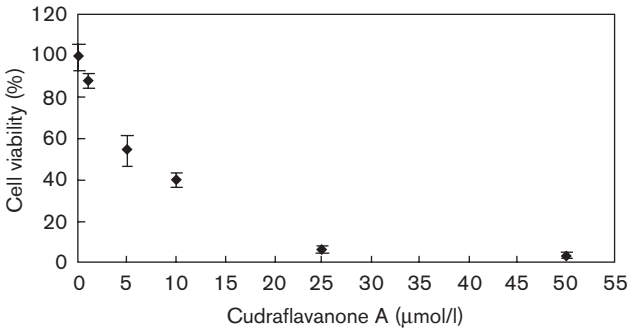
Structure of cudraflavanone A.

Fig. 2



Effect of increasing concentrations of cudraflavanone A on topoisomerase I activity. About 0.4 μg of supercoiled DNA was incubated with 1 unit of topoisomerase I in the presence of cudraflavanone A (lanes 3–7) and then analyzed by agarose gel electrophoresis. The control reactions contained an equivalent volume of dimethysulfoxide in the absence (lane 1) or presence (lane 2) of the enzyme.

Fig. 3



Cytotoxicity of cudraflavanone A against human cancer cells. U937 cells were incubated with various concentrations of cudraflavanone A for 48 h. The cytotoxicity was measured by a standard MTT assay as described in Materials and methods. Control cells were assigned 100% viability, having been incubated in a medium without cudraflavanone A. Each point represents the average ± SD of triplicate determinations.

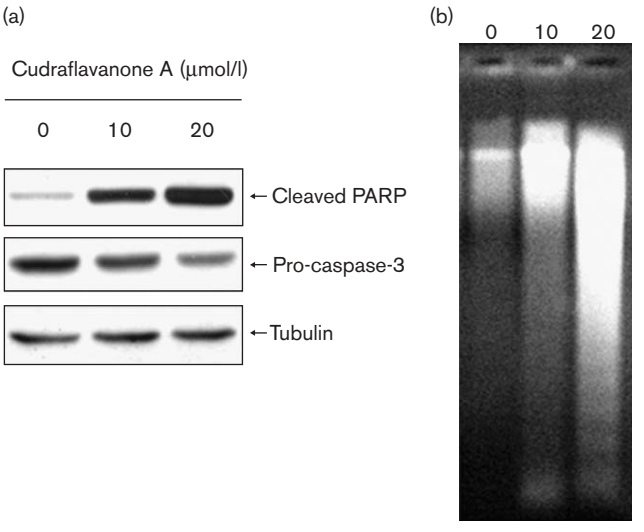
quantitative data, the amount of relaxed DNA was measured by scanning the photograph with an image analyzer (B. I. System, Ann Arbor, Michigan, USA). The 50% inhibitory concentration (IC_{50}) value of cudraflavanone A was approximately 0.4 mmol/l.

The cytotoxic effect of cudraflavanone A on human leukemia U937 cells was investigated. The cytotoxicity was determined by an MTT assay, as described in Materials and methods. The inhibition of cell growth by cudraflavanone A appeared to be concentration-dependent in the cell line tested. At 10 μmol/l cudraflavanone A concentration, the viability of U937 cells was reduced to 40% after 2 days of incubation (Fig. 3). It has been reported that cudraflavanone C exerts cytotoxicity effect against four kinds of human digestive-apparatus tumor cell lines (HCT-116, SMMC-7721, SGC-7901 and BGC-823) with IC_{50} values of 24.37–65.86 μmol/l, whereas another flavonoid, kaempferol, is inactive [15].

The cleavage of PARP and caspase-3 is a typical feature of apoptosis [16]. We examined whether cudraflavanone A induced apoptotic cell death. As shown in Fig. 4a, in U937 cells treated with cudraflavanone A for 48 h, the 89-kDa cleaved fragment of PARP was observed by Western blotting. In untreated cells, however, there was little cleavage of PARP. To assess the activation of caspase-3, we assayed for the cleavage of the proenzyme of caspase-3 to its active form by Western blotting. Compared with the untreated control cells, pro-caspase-3 was apparently cleaved in cells treated with cudraflavanone A. The results suggest that cudraflavanone A kills human cancer cells by apoptosis. To confirm apoptotic cell death by cudraflavanone A, DNA fragmentation, another hallmark of apoptosis, was observed by agarose gel electrophoresis. Internucleosomal DNA fragmentation was observed with 10 μmol/l and increased further with 20 μmol/l cudraflavanone A (Fig. 4b).

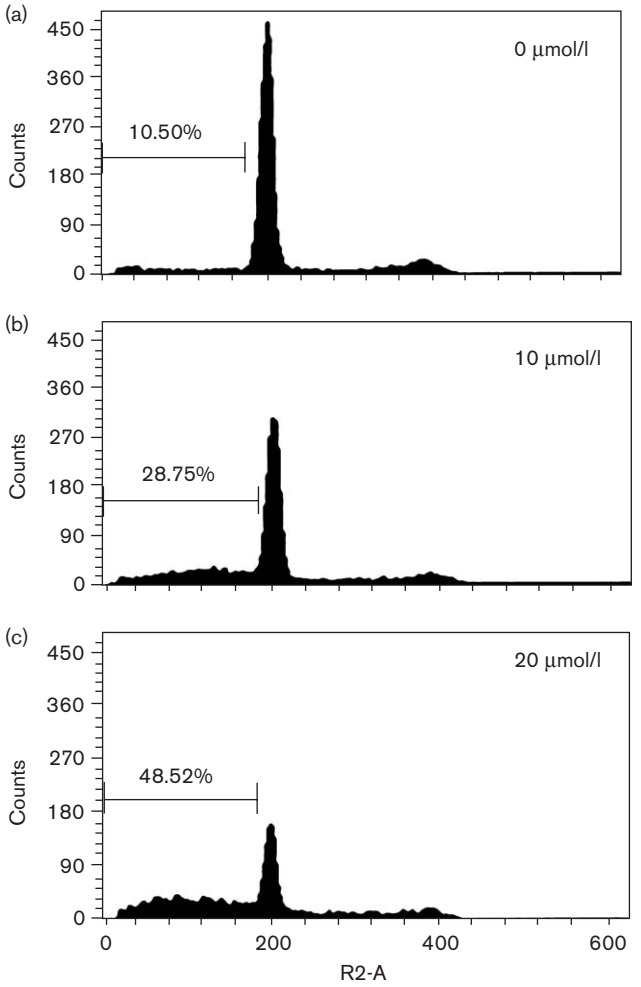
The apoptosis rate of U937 cells was monitored by flow cytometry. The cells increased the sub-G_1 DNA content

Fig. 4



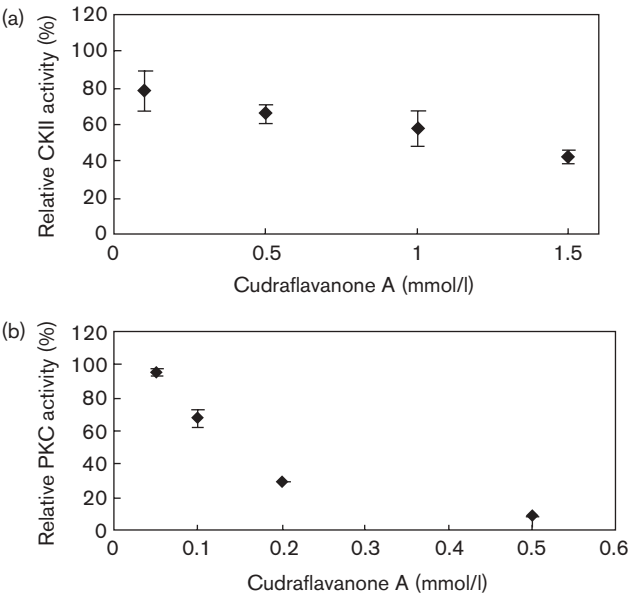
(a) Cudraflavanone A-induced poly(ADP ribose) polymerase (PARP) and caspase-3 cleavage in human cancer cells. U937 cells were incubated with 0–20 μmol/l cudraflavanone A for 48 h, lysed, electrophoresed on a 10% (w/v) sodium dodecylsulfate–polyacrylamide gel and visualized by Western blotting with specific antibodies against a cleaved form of PARP or pro-caspase-3. (b) DNA fragmentation in control or after treatment with cudraflavanone A. Genomic DNA was purified from U937 cells untreated or treated with cudraflavanone A for 48 h, analyzed by electrophoresis on 1% (w/v) agarose gel and then stained with a solution containing 0.5 μg/ml of ethidium bromide. Representative of two experiments.

Fig. 5



Analysis of apoptosis by flow cytometry. The cells (2×10^4) were treated with 0–20 μmol/l cudraflavanone A for 48 h, fixed with 70% ethanol and stained with propidium iodide. Propidium iodide signal was measured by flow cytometry. Apoptotic cells with a sub-G_1 ($<2N$ ploidy) were assayed by the computer program CellQuest (Beckton Dickinson, San Jose, California, USA). Data represent one of three independent experiments.

Fig. 6



Effect of cudraflavanone A on the activity of casein kinase II (CKII) (a) and protein kinase C (PKC) (b). The specific CKII substrate peptide (a) or PKC substrate peptide (b) were incubated with [γ-^{32}P]ATP and purified CKII or PKC, respectively, in the presence of various concentrations of cudraflavanone A under standard assay conditions as described in Materials and methods. The ^{32}P incorporation in the substrate peptides was measured by scintillation counting. Each point represents the average ± SD of triplicate determinations.

that characterized apoptosis at 48 h of cudraflavanone A exposure (Fig. 5). The apoptosis rates of the cudraflavanone A-treated cells (28.75 and 48.52%) were significantly higher than those in control cells (10.5%).

The inhibition of topoisomerase I at high cudraflavanone A concentration is unlikely to be the only event that could lead to apoptotic cell death occurring at a much lower concentration. It has been reported that several inhibitors of protein kinase CKII or PKC can induce apoptotic cell death [17,18]. Thus, we investigated the effect of cudraflavanone A on the catalytic activity of purified CKII and PKC, in the presence of ATP and specific peptide substrates. As shown in Fig. 6a, cudraflavanone A showed little effect toward CKII activity. Cudraflavanone A, however, inhibited strongly PKC activity with approximately 150 μmol/l of IC_{50}.

We first demonstrated here that cudraflavanone A, purified from *C. tricuspidata*, inhibited mammalian topoisomerase I and PKC activity, and induced the apoptotic cell death of human cancer cells. As roots of *C. tricuspidata* have been applied clinically to the treatment of tumors [13] and as topoisomerase I is strongly associated with oncogenesis [4–7], our current results suggest that cudraflavanone A is likely to function by inhibiting oncogenic disease, at least in part, through the inhibition of PKC and topoisomerase I activity.

Acknowledgements

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