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Induction of apoptosis in human leukaemia HL-60 cells by furanone-coumarins from *Murraya siamensis*

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Abstract

To identify potential anti-tumour agents, we screened five furanone-coumarins isolated from *Murraya siamensis* Craib (Rutaceae) for their ability to inhibit the growth of human leukaemia HL-60 cells. Among the furanone-coumarins tested, murrayacoumarin B (compound 2) showed significant cytotoxicity against HL-60 cells. Fluorescence microscopy with Hoechst 33342 staining revealed that the percentage of apoptotic cells with fragmented nuclei and condensed chromatin increased in a time-dependent manner after treatment with murrayacoumarin B. Interestingly, this furanone-coumarin induced the loss of the mitochondrial membrane potential. In addition, treatment with murrayacoumarin B stimulated the activities of caspase-9 and caspase-3, and caspase-9 and caspase-3 inhibitors suppressed the apoptosis induced by murrayacoumarin B. These results suggest that murrayacoumarin B induced apoptosis in HL-60 cells through activation of the caspase-9/caspase-3 pathway triggered by mitochondrial dysfunction.

Introduction

As a part of our systematic investigation of the chemical constituents of plants of the Rutaceae family, we identified various constituents in *Murraya* and *Clausena* species used in folk medicine in China and other Asian countries as analgesics, astringents, antidiarrhoeal agents or antipyretics (Kan 1972). We have previously reported the isolation and identification of several natural furanone-coumarins from *M. siamensis* Craib (Ito et al 2005) and *C. excavata* Burm. f. (Ito et al 2000). 3-Substituted γ-lactone rings (referred to as furanones in this paper) are a characteristic feature of furanone-coumarins. These furanone-coumarins act as a C_{10}-terpenoido side-chain bonded to the coumarin nucleus. In a primary screening of novel anti-tumour agents, we found that several furanone-coumarins were potent inhibitors of Epstein–Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells (Ito et al 2000, 2005).

Recent studies have suggested that anti-cancer drugs and cancer chemopreventive agents act by inducing apoptosis to inhibit tumour growth and cellular inflammatory responses other than necrosis (Nicholson 2000). Cells undergoing apoptosis go through a cascade of biochemical and morphological changes (Kaufmann & Earnshaw 2000). DNA fragmentation is a biochemical hallmark of apoptosis, and chromatin condensation is an early morphological change of apoptosis. These changes result from the proteolytic cleavage of various intracellular polypeptides, mostly catalysed by the capsases, a family of cysteine-dependent proteases. The sequential activation of caspases is a necessary part of the apoptosis signalling pathway. Caspase-3 is major downstream effector of apoptosis, and caspase-3-mediated proteolytic cleavage is a critical step leading to DNA fragmentation and chromatin condensation.

The aim of this study was to examine the cytotoxic potential of furanone-coumarins from *M. siamensis*. Five furanone-coumarins were tested for cytotoxicity against the human leukaemia HL-60 cell-line as a primary screen. We further evaluated the molecular mechanisms underlying the apoptotic effects induced by compound **2**, murrayacoumarin B.

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Materials and Methods

Plant material and test products

The plant material used in this study, *M. siamensis* Craib, was collected at the Sakaeraj Environmental Research Station, Nakorn-rachasima Province, Thailand, in March 1998. Authentication was achieved by comparison with the herbarium specimen at the Royal Forest Department, Ministry of Agriculture and Cooperative, Bangkok, Thailand. A voucher specimen is deposited at the Barbosa Rodrigues Herbarium under accession number NSR-092515.

The acetone extract of the dried leaves of *M. siamensis* was fractionated by silica-gel column chromatography and preparative thin-layer chromatography, as described in our previous papers (Ito et al 2000, 2005). Purity was corroborated by inspection of infrared, mass spectrometry and ^{1}H-NMR spectra. The structures of the five furanone-coumarins are shown in Figure 1.

Cell culture and treatment

The human leukaemia HL-60 cell-line was provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Miyagi, Japan). Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The test compounds were dissolved in DMSO and were added to culture medium to give a final DMSO concentration of 0.5% (v/v). This concentration of DMSO had no significant effect on the growth of the cells (data not shown).

Assay of cell viability/cell growth

Cells were seeded into 96-well plates at a density of 1×10^5 cells per well, and maintained for 24 h at 37°C. Each compound to be tested (30 μM) was then added to the culture medium. Cell viability was determined using a CellTiter 96 aqueous assay kit (Promega, Madison, WI, USA), which is based on the metabolic conversion by living cells of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS tetrazolium) compound to a coloured formazan product. The absorbance of the formazan product is directly proportional to the number of viable cells in the culture. MTS solution was added to the 96-well plates at 0, 6, 12, 18 and 24 h after addition of the test compound, and the cells were incubated for 1 h at 37°C. The absorbance was measured at a wavelength of 490 nm using a Wallac 1420 ARVOsx microplate counter (Applied Biosystems, Foster City, CA, USA).

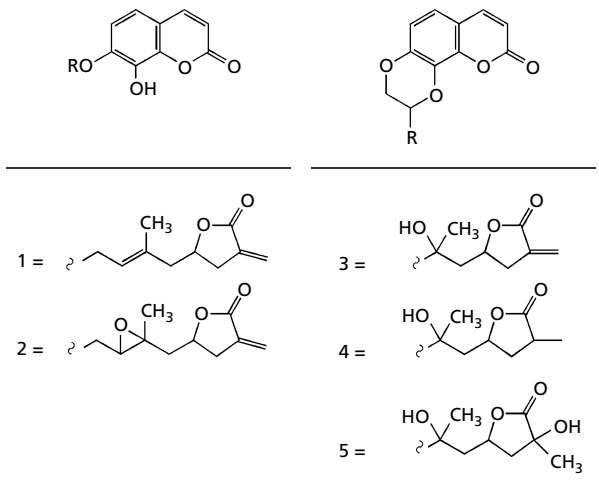

Figure 1 Structures of furanone-coumarins isolated from *Murraya siamensis*.

Assessment of apoptosis

To detect apoptotic cells, we stained the cells with the DNA-binding dye Hoechst 33342 (Dojindo, Kumamoto, Japan). After the cells had been exposed to the test compound for the allotted time periods, they were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at 4°C, and then washed with PBS. For nuclear staining, the cells were incubated for 20 min with 20 μg mL^{-1} Hoechst 33342. Cells were then washed with PBS, and observed under a fluorescence microscope (excitation 352 nm, emission 461 nm; Zeiss Axiophoto 2, Carl Zeiss, Jena, Germany). Cells that exhibited condensed chromatin and fragmented nuclei were scored as apoptotic cells. At least 200 cells from each sample were scored.

Measurement of caspase-9 and -3 activities

The enzyme activities of caspase-3 and -9 were measured using a commercial fluorometric assay kit (R&D Systems Inc., Minneapolis, MN, USA). Cells were seeded into 24-well plates at a density of 3×10^6 cells per well. After exposing the cells to the test compound for the allotted time periods, the cells were washed three times with PBS, and then lysed in a lysis buffer for 10 min on ice. The protein content of the cell lysates was measured using Micro BCA reagent (Pierce, Rockford, IL, USA). Cell lysates containing 50 μg of protein were incubated with a caspase-9 fluorogenic substrate (LEHD-AFC) or a caspase-3 fluorogenic substrate (DEVD-AFC) for 1 h at 37°C. The enzyme activity was measured fluorometrically using a Wallac 1420 ARVOsx microplate counter (excitation 400 nm, emission 505 nm; Applied Biosystems, Foster City, CA, USA).

Caspase inhibition assay

The caspase-9-specific inhibitor Z-LEHD-FMK and the caspase-3-specific inhibitor Z-DEVD-FMK (both from R&D Systems Inc., Minneapolis, MN, USA) were dissolved in DMSO at a concentration of 50 μM. Cells were pretreated with medium containing either DMSO or caspase inhibitor in DMSO for 2 h. Control medium or medium containing the test compound was then added to achieve a final concentration of 30 μM. The number of apoptotic cells was determined as described above.

Measurement of mitochondrial membrane potential

Changes in the mitochondrial membrane potential of HL-60 cells were detected using a fluorescence-based ApoAlert

mitochondrial membrane sensor kit (Clontech Laboratories, Palo Alto, CA, USA). The cells (10^6 mL^{-1}) were treated with 30 μM test compound for the desired period. After treatment, the cells were pelleted, washed with fresh culture medium and then resuspended in 0.5 mL culture medium containing the mitosensor reagent (5 μg mL^{-1}). After incubation at 37°C for 20 min, the cells were washed with 1 mL culture medium and then pelleted. The final cell pellet was resuspended in 30 μL culture medium and then analysed by confocal laser-scanning microscopy (Zeiss LSM510 META, Carl Zeiss MicroImaging, Inc., Jena, Germany). The mitosensor reagent, which is cationic by nature, is taken up by normal mitochondria, where it forms aggregates that emits red fluorescence. A loss in mitochondrial membrane potential inhibits this uptake and subsequent aggregation, and the mitosensor remains in the cytoplasm as a monomer where it emits green fluorescence.

Statistical analysis

Statistical analysis was performed using SPSS version 10.0J for Windows (SPSS Inc., Chicago, IL, USA), using the Kruskal–Wallis test. Individual differences were examined by the Mann–Whitney *U*-test (Figures 2 and 3 and Table 1). Differences between the two groups (Figure 4) were examined statistically using the Mann–Whitney *U*-test. *P* values below 0.05 were considered to be significant.

Results

Inhibition of cell growth by furanone-coumarins

First we examined the effect of five furanone-coumarins (Figure 1), isolated from the acetone extract of leaves of *M. siamensis*, on the growth of human leukaemia HL-60 cells.

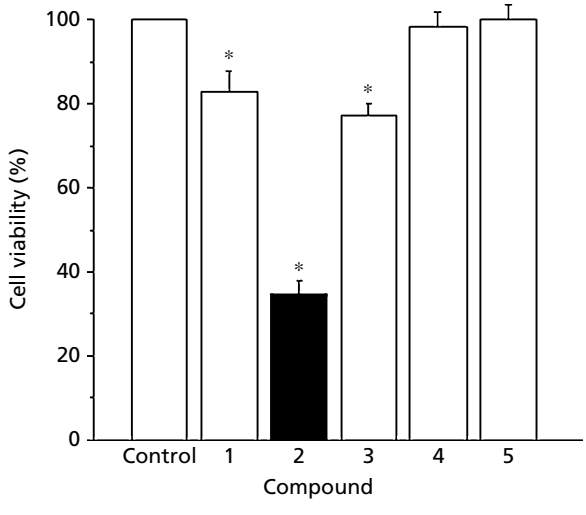

Figure 2 Effect of the five isolated furanone-coumarins (30 μM) on the viability of HL-60 cells. Cell viability after 24 h' incubation was estimated by MTS assay. Values are mean ± s.e. of three independent experiments performed in triplicate. $^*P < 0.05$ vs control value at the same time point.

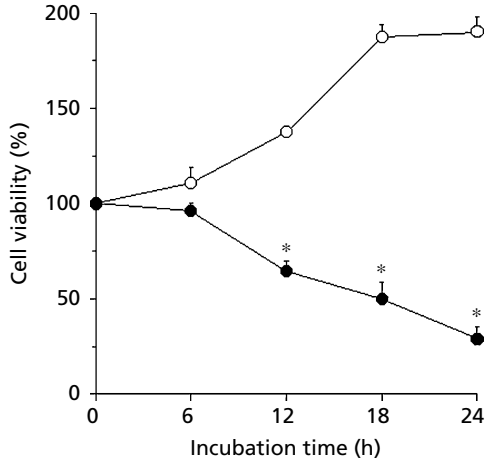

Figure 3 Effect of murrayacoumarin B (30 μM) on the growth of HL-60 cells. HL-60 cells were treated in the presence (●) and absence (○) of murrayacoumarin B for the indicated time periods. Cell viability was estimated using the MTS assay. Cell growth is expressed as a percentage of the cell viability level at time 0. Values are mean ± s.e. for three independent experiments performed in triplicate. $^*P < 0.05$ vs control values at the same time.

We used the MTS assay to estimate the number of viable cells in the culture as a measure of cell growth. HL-60 cells were exposed to each of the furanone-coumarins (30 μM) for 24 h. As shown in Figure 2, murrayacoumarin B (compound **2**) significantly decreased cell viability (35% compared with control). Compounds **1** and **3** slightly reduced cell viability. The other compounds did not affect cell viability.

Induction of apoptosis by murrayacoumarin B

We focused on murrayacoumarin B (compound **2**) and examined the time course of its cytotoxic effect. As shown in Figure 3, this compound inhibited HL-60 cell growth in a time-dependent fashion.

To determine whether the loss of cell viability occurred as a consequence of apoptosis, we examined the nuclear morphology of HL-60 cells treated with murrayacoumarin B (30 μM) using Hoechst 33342 staining. As shown in Table 1, the percentage of apoptotic cells increased significantly with time in cells exposed to this compound. No apoptotic nuclei were observed in the untreated (control) cells (Table 1). These results indicate that murrayacoumarin B induced apoptotic cell death in HL-60 cells.

Activation of caspase-9 and -3

To examine whether the apoptotic pathway was induced by murrayacoumarin B, we measured the activities of caspase-9 and caspase-3. As shown in Table 1, the activities of both caspase-3 and caspase-9 activity increased with time in cells treated with murrayacoumarin B.

Table 1 Time course of the appearance of apoptotic nuclei and caspase activation in HL-60 cells incubated without and with 30 μM murrayacoumarin B (MCB) for the indicated periods. Apoptosis was determined by staining nuclear chromatin with Hoechst 33342. Results are expressed as the percentage of the cells showing characteristic nuclear morphological features (nuclear condensation and fragmentation) of apoptosis relative to the total number of counted cells (200 cells per time point). The activities of caspases-9 and -3, measured using fluorogenic substrates (see text), are expressed as arbitrary units of fluorescence (AUF)

	0 h	5 h	10 h	15 h
Apoptotic cells (%)				
Control	1.0 ± 0.0	1.3 ± 0.6	1.3 ± 0.6	1.3 ± 0.6
MCB-treated cells	1.0 ± 0.0	34.3 ± 3.8*	53.3 ± 4.8*	63.0 ± 1.7*
Caspase-9-activity (AUF)				
Control	1134 ± 240	1495 ± 150	1660 ± 167	1817 ± 123
MCB-treated cells	1134 ± 240	5540 ± 100*	12 528 ± 659*	15 632 ± 1867*
Caspase-3 activity (AUF)				
Control	8255 ± 592	12 341 ± 2597	12 467 ± 420	15 006 ± 346
MCB-treated cells	8255 ± 592	90 383 ± 3326*	184 326 ± 8284*	204 315 ± 9351*

Values are the mean ± s.e. for three independent experiments performed in triplicate. *$P < 0.05$ vs control values at the same time point.

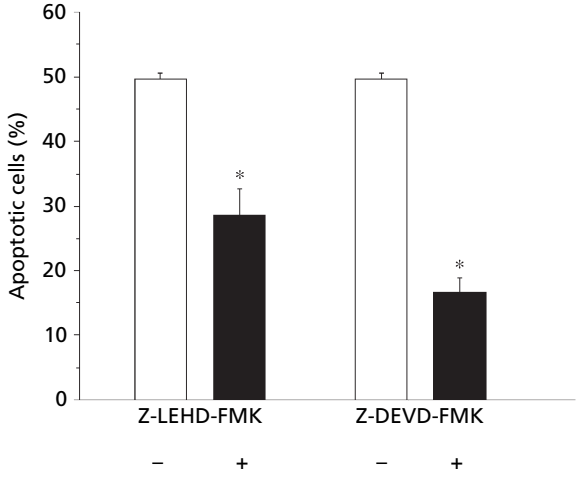

Figure 4 Effects of the caspase-9 inhibitor Z-LEHD-FMK (50 μM) and the caspase-3 inhibitor Z-DEVD-FMK (50 μM) on apoptosis induced by murrayacoumarin B. HL-60 cells were pretreated or not with the inhibitor for 2 h, and then treated with 30 μM murrayacoumarin B for 10 h. Nuclear condensation and fragmentation were analysed by staining with Hoechst 33342. Results are expressed as the percentage of apoptotic cells relative to the total number of counted cells (200 cells per time point). Values are mean ± s.e. for three independent experiments performed in triplicate. *$P < 0.05$ vs control values (no inhibitor pretreatment).

Effect of caspase-3 and -9 inhibitors on induction of apoptosis

To determine whether the activation of intracellular caspase-9 and caspase-3 is required for the induction of apoptosis by murrayacoumarin B, we examined the ability of specific caspase-9 and -3 inhibitors to prevent the induction of apotosis. As shown in Figure 4, both the caspase-9 inhibitor Z-LEHD-FMK and the caspase-3 inhibitor Z-DEVD-FMK significantly protected the HL-60 cells from apoptosis induced by murrayacoumarin B, indicating that the induction of apoptosis by this compound is mediated via activation of caspase-9 and caspase-3.

Alteration of mitochondrial membrane potential

We assessed the effect of murrayacoumarin B on the mitochondrial membrane potential using confocal microscopy and a fluorescent mitosensor reagent. Control cells loaded with the mitosensor dye exhibited a consistent red fluorescence (indicative of normal functioning mitochondria) when incubated for up to 10 h (Figure 5). However, treatment with murrayacoumarin B led to a massive loss of mitochondrial membrane potential, as shown by the complete conversion to green fluorescence throughout the cytoplasm (Figure 5). These results suggest that murrayacoumarin B induced mitochondrial dysfunction based on the loss of this membrane potential.

Discussion

Carcinogens usually cause genomic damage in cells exposed to them. As a consequence, rather than undergoing apoptosis, the damaged cells may be triggered to proliferate with genomic damage, leading to the formation of cancerous cells that usually exhibit cell-cycle abnormalities and are more susceptible to various apoptosis-inducing agents (Steller 1995). Therefore, identifying active plant-derived compounds with apoptosis-inducing activity against cancer cell-lines is considered to be important in the search for chemopreventive agents. Our results demonstrate for the first time that a furanone-coumarin inhibited the proliferation of human leukaemia cells by triggering apoptosis.

Apoptosis is distinguished from necrosis by various characteristic morphological changes, namely chromatin

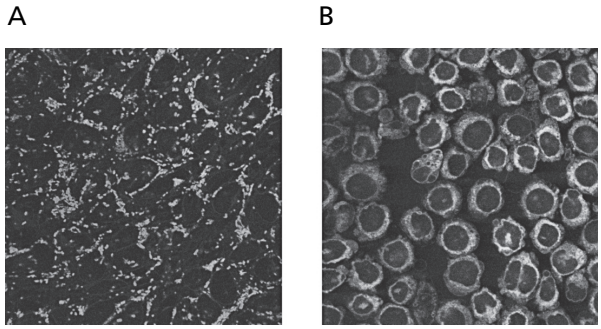

Figure 5 Change in mitochondrial membrane potential in HL-60 cells incubated without and with 30 μM murrayacoumarin B for 10 h, followed by 30 min' incubation with mitosensor, a mitochondrial-sensitive vital dye. Images were then collected by confocal microscopy. In control cells (A), the aggregates of red fluorescence indicates a high mitochondrial membrane potential. In cells treated with murrayacoumarin B (B), the mitosensor remains in the cytoplasm and emits green fluorescence, indicating a loss of membrane potential.

condensation, nuclear fragmentation, cell shrinkage, plasma membrane blebbing and the presence of apoptotic bodies (Kaufmann & Hengartner 2001). Apoptotic nuclei can be evaluated easily by fluorescence microscopy. HL-60 cells grown in the presence of murrayacoumarin B (compound **2**) showed marked nuclear fragmentation, indicating that this compound triggered apoptosis rather than necrosis.

The capsases are a family of aspartate-specific cysteine proteases that play pivotal roles in apoptosis (Shi 2002). We evaluated caspase-9 because it represents the apical caspase in the mitochondrial intrinsic pathway (Li et al 1997), and we analysed caspase-3 because it has been shown to be one of the important cell executioners of apoptosis (Shi 2002). The activities of caspase-9 and caspase-3 were stimulated by treatment with murrayacoumarin B in a time-dependent manner (Table 1). Furthermore, the caspase-9 and -3 inhibitors significantly protected HL-60 cells against the increase in apoptosis induced by murrayacoumarin B (Figure 4). These results indicate that caspases are indeed involved in the apoptotic response induced by this coumarin.

In addition, murrayacoumarin B induced mitochondrial dysfunction, as evidenced by the loss of membrane potential (Figure 5). In mitochondrial dysfunction, one of the early events that initiates apoptosis is the release of cytochrome *c* from the mitochondria into the cytosol (Green & Reed 1998). Once released into the cytosol, cytochrome *c* binds to Apaf-1 and pro-caspase-9 in the presence of deoxy-ATP to form the apoptosome (Li et al 1997). This complex then activates caspase-9, which in turn cleaves and thereby activates caspase-3 (Li et al 1997; Shi 2002). In cells treated with murrayacoumarin B, mitochondrial dysfunction was followed by activation of caspase-9 and caspase-3 (Table 1 and Figure 4). Our data show clearly that murrayacoumarin B induced the activation of caspase-9 and caspase-3 associated with mitochondrial dysfunction, which preceded the onset of apoptosis.

The α-methylene-γ-lactone moiety is known to play an important role in the cytotoxic activity of sesquiterpene lactones (Lee et al 1977; Hall et al 1977). The results of the present study show that the α-methylene-γ-lactone moiety of furanone-coumarins is important in the cytotoxic activity, as compounds **1, 2** and **3,** which have this moiety on the side-chain, were cytotoxic. In addition, the presence of an oxirane ring on the side-chain of murrayacoumarin B might improve cytotoxic activity. Further experiments on the structure–activity relationship are planned.

Conclusion

Murrayacoumarin B, a furanone-coumarin isolated from the leaves of *M. siamensis*, was able to induce apoptosis in HL-60 cells. The coumarin induced the loss of mitochondrial membrane potential and the subsequent activation of caspase-9/caspase-3. These findings suggest that murrayacoumarin B is a possible candidate for an anti-tumour agent.

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