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Apoptosis inducing activity of 4-substituted coumarins from *Calophyllum brasiliense* in human leukaemia HL-60 cells

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Abstract

With the objective of identifying anti-tumour-promoting agents, we carried out a primary screening of ten 4-substituted coumarins isolated from *Calophyllum brasiliense* Camb. (Guttiferae), to determine the ability of these compounds to inhibit proliferation of the human leukaemia cell line HL-60. Among the 4-substituted coumarins isolated, calophyllolide (**2**) and mammea B/BB (**3**) 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 calophyllolide (**2**) or mammea B/BB (**3**). In addition, the activity of caspase-9 and caspase-3 was also enhanced in a time-dependent manner upon treatment with the 4-substituted coumarins 2 and 3. Caspase-9 and caspase-3 inhibitors suppressed apoptosis induced by 4-substituted coumarins 2 and 3. These results suggest that calophyllolide (**2**) and mammea B/BB (**3**) induced apoptosis in HL-60 cells through activation of the caspase-9/caspase-3 pathway, which is triggered by mitochondrial dysfunction.

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Note: *Both authors contributed equally to this work.

Introduction

In 1992, a research group at the National Cancer Institute reported that (+)-calanolide A, one of the 4-propyldipyranocoumarins isolated from *Calophyllum* plants, showed strong activity against human immunodeficiency virus type 1 (HIV-1) (Kashman et al 1992). Since then, the chemical constituents of *Calophyllum* species have been actively studied (Patil et al 1993; Taylor et al 1994; Currens et al 1996; Zembower et al 1997; McKee et al 1998; Ishikawa 2000; Sekino et al 2004). Systematic studies of the chemical constituents of *Calophyllum* plants have been conducted by our group, and we showed that *C. brasiliense* Camb. (collected in Brazil) contained many kinds of 4-substituted coumarins and xanthones as major components (Ito et al 2002, 2003). In addition, in a primary screening test for novel cancer chemopreventive agents (inhibition of tumour promoters), we found that several 4-substituted coumarins and xanthones showed potent inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells (Itoigawa et al 2001; Ito et al 2002, 2003). Furthermore, we have demonstrated that some of the 4-substituted coumarins isolated from *C. brasiliense* display cytotoxicity against six human leukaemic cell lines (Kimura et al 2005). In particular, a novel anti-cancer drug, GUT-70 (5-methoxy-2,2-dimethyl-6-(2-methyl-1-oxo-2-butenyl)-10-propyl-2*H*,8*H*-benzo[1,2-*b*,3,4-*b*′]dipyran-8-one) induced caspase-mediated and p53 independent apoptosis to overcome multidrug resistance (Kimura et al 2005).

The aim of this study was to examine the cytotoxic potential of 4-substituted coumarins present in *C. brasiliense* as compared with GUT-70. Ten 4-substituted coumarins were tested for cytotoxicity against the human leukaemia cell line HL-60 in a primary screening.

Materials and Methods

Plant material and test products

Calophyllum brasiliense Camb. was collected from the garden of the Federal University of Santa Catarina, Brazil, in March 1998. Plant material was classified by Dr Ademir Reis. A voucher specimen has been deposited at Barbosa Rodrigues Herbarium under number VC Filho 007. The isolation and characterization of all the compounds tested in this study have been described in previous papers (Ito et al 2002, 2003). Their purity was corroborated by measurements of melting point, IR, UV and ^{1}H NMR spectra. The structures of the compounds tested are shown in Figure 1.

Cell culture and treatment

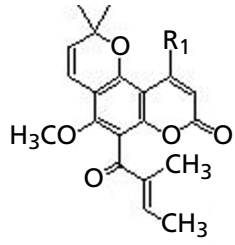
The human leukaemia cell line HL-60 was provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Miyagi, Japan). Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The test compounds were dissolved in dimethyl sulfoxide (DMSO) and were added to the 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 cell line tested (data not shown).

Assay of cell viability/cell growth

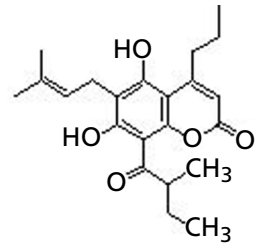
Cell viability was determined using a CellTiter 96 Aqueous assay kit (Promega, Madison, WI, USA). Cells were seeded in 96-well plates at a density of 1×10^5 cells per well. The cells were maintained for 24 h at 37°C and then each compound to be tested (30 μM) was added to the culture medium. MTS solution was added to the 96-well plates at the time points indicated in the figures, and the cells were incubated for 1 h at 37°C. The absorbance was measured at a wavelength of 490 nm with a Wallac 1420 ARVOsx microplate counter (Applied Biosystems, Foster City, CA, USA).

Assessment of the percentage of apoptotic cells

To detect apoptosis, cells were stained with the DNA-binding dye Hoechst 33342 (Dojindo, Kumamoto, Japan). After exposure of the cells to the test



	R_1
1	*n*-Pr
2	Ph



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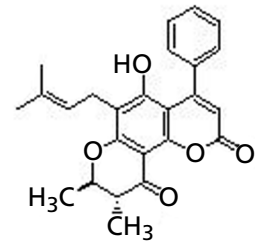

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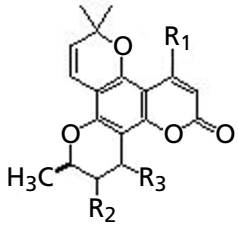
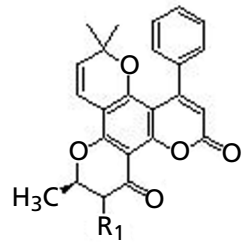
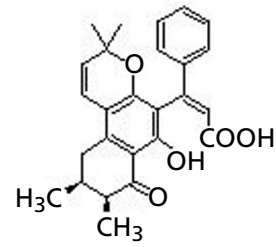


	R_1	R_2	R_3
5	Ph	β-CH_3	α-OH
6	Ph	β-CH_3	β-OH
7	*n*-Pr	α-CH_3	β-OH



	R_1
8	β-CH_3
9	α-CH_3



10

Figure 1 Chemical structures of 4-substituted coumarins isolated from *Calophyllum brasiliense*.

compounds 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. To stain the nuclei, cells were incubated for 20 min with 20 μg mL^{-1} Hoechst 33342. After washing with PBS, the cells were observed under a fluorescence microscope (excitation at 352 nm, emission at 461 nm; Zeiss Axiophoto 2, Carl Zeiss, Germany). Cells exhibiting condensed chromatin and fragmented nuclei were scored as apoptotic cells. Results from the time-course experiments are expressed as the percentage of apoptotic cells relative to the total number of counted cells (200 cells/time point).

Measurement of enzyme activity of caspase-3 and -9

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

Caspase inhibition assay

The caspase-9-specific inhibitor Z-LEHD-FMK (50 μM), the caspase-3-specific inhibitor Z-DEVD-FMK (50 μM) and the pan-caspase inhibitor Z-VAD-FMK (50 μM (all from R&D Systems Inc., Minneapolis, MN, USA) were dissolved in DMSO. Cells were pretreated with either medium containing DMSO or inhibitor for 2 h. Medium alone or medium containing each test compound at a final concentration of 30 μM was then added. The number of apoptotic cells was determined by counting cells that exhibited nuclear condensation and fragmentation, as confirmed by Hoechst 33342 staining. A minimum of 200 cells was scored from each sample.

Statistical analysis

Statistical analysis was performed with SPSS version 10.0J for Windows (SPSS Inc., Chicago, IL), using the Kruskal–Wallis test; individual differences were examined by the Mann–Whitney *U*-test (Figure 2, Table 1 and 2). Differences between the two groups (Table 3) were statistically examined using the Mann–Whitney *U*-test. $P < 0.05$ denoted significance in all cases.

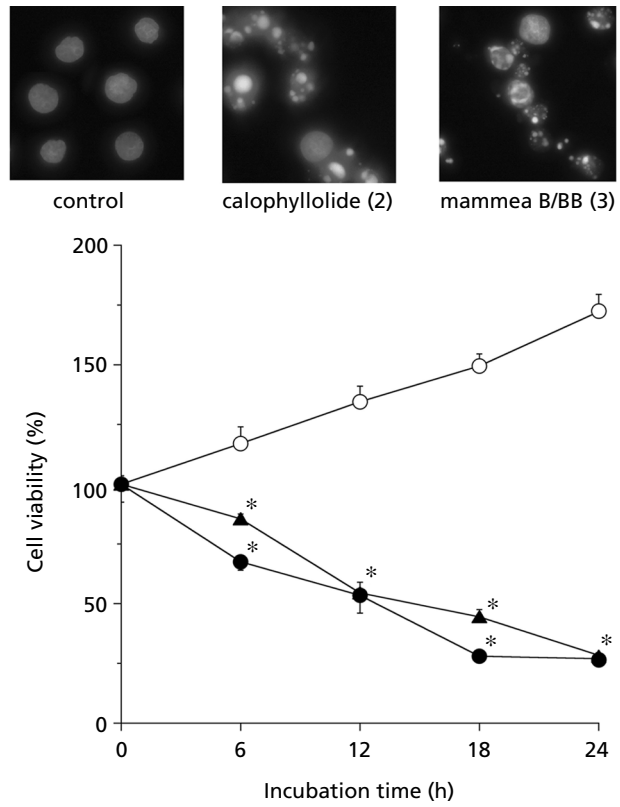


Figure 2 Effect of calophyllolide (**2**) and mammea B/BB (**3**) on the growth of HL-60 cells. HL-60 cells were left untreated (open circles) or treated with 4-substituted coumarins (**2** (closed circles) or **3** (triangles), concentration 30 μM), for the indicated time periods and cell growth was then estimated by metabolic conversion of the MTS tetrazolium compound to a colored formazan product. Cell growth at the indicated time points is expressed as a percentage of the MTS level at time 0. Values are mean ± s.e. for three independent experiments, in which each measurement was made in triplicate. *$P < 0.05$ compared with controls. The nuclear condensation and fragmentation was analysed by staining with Hoechst 33342. The fluorescence images indicate the typical apoptotic nuclear morphology in each treatment (incubation time 12 h).

Results and Discussion

We examined the effect of ten 4-substituted coumarins, isolated from the acetone extract of *C. brasiliense*, on the growth of human leukaemia HL-60 cells. To assess cell growth, we used the MTS assay to estimate the number of viable cells in culture. As shown in Table 1, when HL-60 cells were exposed to each of the ten isolated 4-substituted coumarins (30 μM) for 24 h, calophyllolide (**2**) and mammea B/BB (**3**) induced a more significant decrease in cell viability (**2** and **3** treated: cell viability decreased to 24 and 27%, respectively, compared with controls) than GUT-70 (**1**) (cell viability decreased to 58%, which represented the most potent

Table 1 Effect of the ten isolated 4-substituted coumarins on the cell viability of HL-60 cells

Compound	30 μM	IC50
Control	100.0 ± 0.0	
GUT-70 (**1**)	58.0 ± 3.3*	35.4 ± 3.2
Calophyllolide (**2**)	24.0 ± 4.4*	8.7 ± 1.2*
Mammea B/BB (**3**)	26.7 ± 3.5*	10.8 ± 2.2*
Brasimarin-C (**4**)	102.0 ± 3.3	
Inophyllum-D (**5**)	90.7 ± 5.1*	
Inophyllum-A (**6**)	81.7 ± 5.3*	
Calanolide-A (**7**)	76.7 ± 3.2*	
Inophyllum-E (**8**)	83.7 ± 5.4*	
Inophyllum-C (**9**)	98.0 ± 5.6	
Isocalophyllic acid (**10**)	102.7 ± 2.9	

IC50, 50% inhibitory concentrations (μM) on the cell viability. HL-60 cells (1×10^5 cells/well in a 96-well plate) were incubated with each of the ten 4-substituted coumarins (30 μM) for 24 h and then cell viability was determined using an MTS assay kit. Values are mean ± s.e. for three independent experiments, in which each measurement was made in triplicate. *$P < 0.05$ compared with controls.

Table 2 Time course of the appearance of apoptotic nuclei and caspase activation in calophyllolide (**2**)- and mammea B/BB (**3**)-treated HL-60 cells

	0 h	4 h	8 h	12 h
Apoptotic cells (%)				
Control	1.0 ± 0.0	1.3 ± 0.6	1.0 ± 0.0	1.3 ± 0.6
Calophyllolide (**2**)	1.0 ± 0.0	23.7 ± 4.0*	53.3 ± 3.2*	74.0 ± 1.4*
Mammea B/BB (**3**)	1.0 ± 0.0	17.3 ± 3.5*	56.3 ± 4.0*	71.0 ± 4.0*
Caspase-9 activity				
Control	2112 ± 207	2700 ± 398	2556 ± 221	3254 ± 139
Calophyllolide (**2**)	2112 ± 207	6198 ± 575*	20775 ± 4028*	29796 ± 3650*
Control	1449 ± 55	2243 ± 381	2664 ± 499	3154 ± 312
Mammea B/BB (**3**)	1449 ± 55	13734 ± 3112*	31331 ± 1931*	36054 ± 1968*
Caspase-3 activity				
Control	13903 ± 772	18280 ± 4203	19470 ± 1717	28136 ± 1832
Calophyllolide (**2**)	13903 ± 772	66428 ± 6668*	201968 ± 25041*	247856 ± 11287*
Control	11650 ± 366	20233 ± 2497	28998 ± 4648	35136 ± 3489
Mammea B/BB (**3**)	11650 ± 366	148743 ± 21950*	272318 ± 16725*	375747 ± 44642*

HL-60 cells were left untreated or treated with 4-substituted coumarins (**2** or **3**, concentration: 30 μM) for the indicated time periods. The number of apoptotic cells was determined by counting cells that exhibited nuclear condensation and fragmentation as confirmed by Hoechst 33342 staining. The activity of caspase-9 and caspase-3 was assayed using fluorogenic substrates, LEHD-AFC for caspase-9 and DEVD-AFC for caspase-3. Values are mean ± s.e. for three independent experiments, in which each measurement was made in triplicate. *$P < 0.05$ compared with controls.

inhibitory effect on proliferation of the cell line BV173, as previously reported (Kimura et al 2005)). The compounds showed potent dose-dependent inhibitory effects between 0.3 and 100 μM on the cell viability of HL-60 cells (data not shown). The IC50 values (50% inhibitory concentration) of calophyllolide (**2**; 8.7 μM) and mammea B/BB (**3**; 10.8 μM) were lower than that of GUT-70 (35.4 μM) (Table 1). The other compounds did not affect the cell viability.

We focused on two 4-substituted coumarins (calophyllolide (**2**) and mammea B/BB (**3**)), which showed especially strong cytotoxicity (cell viability decreased to < 30%), and examined the cytotoxic effect of these two compounds in detail. As shown in Figure 2, calophyllolide (**2**) and mammea B/BB (**3**) inhibited HL-60 cell growth within 24 h in a time-dependent fashion. To determine whether the loss of cell viability caused by calophyllolide (**2**) and mammea B/BB (**3**) occurred by apoptosis, we monitored the nuclear morphology of HL-60 cells treated with the two coumarins using Hoechst staining (Figure 2). As shown in Table 2, the percentage of apoptotic cells was significantly increased in cells exposed to 30 μM calophyllolide

Table 3 Effect of caspase inhibitors on calophyllolide (**2**)- and mammea B/BB (**3**)-induced apoptosis

		Control	Z-LEHD-FMK (50 μM)	Z-DEVD-FMK (50 μM)	Z-VAD-FMK (50 μM)
Calophyllolide (**2**)	8 h	68.7 ± 2.1	45.0 ± 1.7*	33.0 ± 1.0*	7.7 ± 0.8*
Mammea B/BB (**3**)	12 h	71.5 ± 0.7	46.7 ± 4.2*	39.5 ± 0.7*	16.8 ± 1.5*

HL-60 cells were left untreated or pretreated with caspase-9 inhibitor (Z-LEHD-FMK 50 μM), caspase-3 inhibitor (Z-DEVD-FMK 50 μM) or pan-caspase inhibitor (Z-VAD-FMK 50 μM) for 2 h, and then treated with 4-substituted coumarins (**2** or **3**, concentration: 30 μM) for the indicated time periods. Nuclear condensation and fragmentation was analysed by staining with Hoechst 33342. Results from the time course experiments are expressed as the percentage of apoptotic cells relative to the total number of counted cells (200 cells/time point). Values are mean ± s.e. for three independent experiments, in which each measurement was made in triplicate. *$P < 0.05$ compared with controls.

(**2**) and mammea B/BB (**3**) in a time-dependent manner. No apoptotic nuclei were observed in untreated cells in each control (Table 2). These results indicate that the two compounds under study induce apoptotic cell death in HL-60 cells.

It is thought that the activation of caspases plays a central role in the execution stage of apoptosis (Patel et al 1996). The intrinsic apoptotic pathway is initiated by the release of cytochrome *c* from mitochondria. The released cytochrome *c* binds to, and activates, the adaptor protein Apaf-1, which in turn activates caspase-9, leading to the formation of an apoptosome and subsequent activation of downstream caspases, such as caspase-3 (Wang 2001). To examine whether the apoptotic pathway was induced by the coumarins **2** and **3**, the proteolytic activity of caspase-9 was measured in terms of its ability to cleave LEHD-AFC, a fluorescent substrate specific for caspase-9. As shown in Table 2, a time-dependent increase in caspase-9 activity was observed in calophyllolide (**2**)- and mammea B/BB (**3**)-treated cells. The activity of caspase-3, which is activated downstream of caspase-9, was then fluorometrically assayed using the fluorogenic substrate DEVD-AFC after treatment with calophyllolide (**2**) and mammea B/BB (**3**) (Table 2). In each case, caspase-3 was activated in a time-dependent manner in calophyllolide (**2**)- and mammea B/BB (**3**)-treated cells, as seen for caspase-9 activation (Table 2).

To determine whether the activation of intracellular caspase-9 and caspase-3 is required for the induction of apoptosis by calophyllolide (**2**) and mammea B/BB (**3**), we examined the ability of specific caspase-9 and -3 inhibitors to prevent coumarin-induced apoptosis. As shown in Table 3, Z-LEHD-FMK (a caspase-9 inhibitor), Z-DEVD-FMK (a caspase-3 inhibitor) and Z-VAD-FMK (a pan-caspase inhibitor) significantly protected HL-60 cells from calophyllolide (**2**)- and mammea B/BB (**3**)-induced apoptosis. These results suggest that calophyllolide (**2**) and mammea B/BB (**3**) induce apoptosis in HL-60 cells via caspase-9/caspase-3 activation. Caspase-9 and caspase-3 are activated by mitochondrial dysfunction (Yin 2000), hence it is possible that the apoptotic effect of these coumarins is mediated through this mechanism. It has been reported that mitochondrial-dysfunction-mediated apoptosis occurs through various intracellular processes such as death receptor signalling (Patel et al 1996) and DNA damage (Gross et al 1999). A study on the intracellular target site of coumarins **2** and **3** is currently in progress.

Conclusions

In conclusion, it was found that calophyllolide (**2**) and mammea B/BB (**3**), isolated from the leaves of *C. brasiliense*, are able to induce apoptosis in HL-60 cells through the activation of caspase-9/caspase-3. These findings suggest that these coumarins might be valuable as anti-tumour agents. Calophyllolide (**2**) and mammea B/BB (**3**) exhibited significant growth suppression due to apoptosis mediated by the activation of the caspase-9/caspase-3 pathway.

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