6´-Benzyloxy-4-bromo-2´-hydroxychalcone is cytotoxic against human leukaemia cells and induces caspase-8- and reactive oxygen species-dependent apoptosis

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Abbreviations: CHAL, 6´-benzyloxy-4-bromo-2´-hydroxychalcone; H_2-DCF-DA, 2´,7´-dichlorodihydrofluorescein diacetate; IC_{50}, 50% inhibition of cell growth; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; ROS, reactive oxygen species.

Keywords: Apoptosis; Caspases; Cytotoxicity; Chalcone

Abstract

In this study, we investigated the effects of synthetic 6′-benzyloxy-4-bromo-2′-hydroxychalcone on viabilities of seven human leukaemia cells. It was cytotoxic against U-937, HL-60, K-562, NALM-6, MOLT-3 cells, and also against Bcl-2-overexpressing U-937/Bcl-2 cells and P-glycoprotein-overexpressing K-562/ADR, but had no significant cytotoxic effects against quiescent or proliferating human peripheral blood mononuclear cells. This chalcone is a potent apoptotic inducer in human leukaemia U-937 cells. Cell death was (i) mediated by the activation and the cleavage of initiator and executioner caspases and poly(ADP-ribose) polymerase; (ii) prevented by the pan-caspase inhibitor z-VAD-fmk, and by the selective caspase-3/7, -6 and -8 inhibitors, and by a cathepsins B/L inhibitor; (iii) associated with the release of mitochondrial proteins, including cytochrome *c* and Smac/DIABLO; (iv) accompanied by dissipation of the mitochondrial membrane potential, (v) partially blocked by the inhibition of $p38^{MAPK}$ and (vi) mostly abrogated by catalase. In conclusion, the synthetic chalcone is cytotoxic against several types of human leukaemia cell with apoptosis being induced by activation of the extrinsic pathway and the generation of reactive oxygen species.

Introduction

Flavonoids are natural products that exhibit a wide array of pharmacological properties, including chemoprotective and chemotherapeutic activities [1]. Chalcones (1,3-diphenyl-2-propen-1-ones) are biosynthetic precursors of flavonoids and can be considered as flavonoids with an open structure. These compounds are commonly found in fruits and vegetables, and some of them are potential anticancer agents [2]. Two structural characteristics are important in determining their anti-proliferative effects on cancer cells: (i) the presence of hydroxy group/s in the chalcone skeleton and (ii) the conjugated enone system. Hydroxy derivatives of chalcone display more potent antiproliferative properties compared to the other chalcone derivatives and the presence of the central double bond plays a crucial role [3-4]. Naturally occurring and synthetic chalcones have been identified for modulating key pathways or molecular targets in cancer including apoptotic pathways [5]. Apoptosis induction is recognized as an efficient mechanism for destroying malignant tumour cells using chemotherapy [6]. This kind of cell death can be executed by aspartate-specific cysteine proteases, the caspases, which are activated by two main pathways [7,8]. The extrinsic pathway is initiated with the activation of the tumor necrosis factor receptor superfamily (TNF) or Fas receptor, involved in the recruitment and activation of the initiator caspase-8 or -10, which activate the effector caspases (-3, -6 and -7) [9]. The intrinsic pathway involves permeabilization of the mitochondrial outer membrane by the activation of the pro-apoptotic proteins Bax and Bak of the Bcl-2 family proteins [10]. This induces cytochrome *c* release in the cytosol which associates with Apaf-1 (apoptotic protease-activating factor 1) in a multimeric complex called the apoptosome. Initiator caspase-9 is then activated which in turn activates the effector caspases [11].

Although chalcone derivatives exhibit cytotoxicity against different tumour cell lines, little is known about the mechanism of action of these compounds. It has recently been reported that flavonoids with halogen substituents in the B ring show improved cytotoxicity against cancer cells relative to methoxylated, methylated or hydroxylated analogues [12]. In a previous report

we described a synthetic flavonol containing a bromine atom at position 4′ of the B ring that showed cytotoxic properties against human myeloid HL-60 tumor cells [13]. In addition, we have recently described how the introduction of two benzyloxy groups at positions 3′and 4′on the B ring of the flavonol skeleton enhances the cytotoxicity of the polyphenolic structure [14]. Moreover, Bu and coworkers have shown how the *ortho*-aryl substitution in the A ring is likely to be very important [15]. Since the presence of a bromine atom as well as a benzyloxy group may play an important role in the cytotoxic properties of chalcones we decided to synthesize new derivatives and explore the impact on human myeloid U-937 tumor cells.

In this study we synthesized 6′-benzyloxy-4-bromo-2′-hydroxychalcone (CHAL) and studied i) its potential cytotoxic effects against a panel of human leukaemia cells and ii) the signal transduction pathways involved in the mechanism of cell death.

2. Materials and methods

2.1. Reagents

Compounds used as starting material and reagents were obtained from Aldrich Chemical Co. or other chemical companies and used without further purification. Nuclear Magnetic Resonance (NMR): ^{1}H and ^{13}C NMR spectra were obtained on a Bruker model AMX-400 spectrometer with standard pulse sequences operating at 500 in ^{1}H and 125 MHz in ^{13}C NMR. $CDCl_3$ was used as solvent. Chemical shifts (δ) are given in ppm relative to the residual solvent signals, and coupling constants (*J*) are reported in hertz. High resolution ESI mass spectra were obtained from a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer, an RF-only hexapole ion guide, and an external electrospray ion source. IR spectra were recorded using a Bruker model IFS-55 spectrophotometer. Melting points were determined on a Büchi B-540 apparatus and are uncorrected. Analytical thin layer chromatography (TLC) was performed using silica gel 60 (230−400 mesh) aluminum sheets. All commercially available chemicals were used without further purification.

2′-Hydroxychalcone, 4-bromo-2′-hydroxychalcone and 6′-benzyloxy-2′-hydroxychalcone were obtained by synthesis following established protocols and have been previously described. The

spectroscopic data were compared with those described in the literature in order to confirm the structures [12, 16, 17]. The inhibitors benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone (z-VAD-fmk), benzyloxycarbonyl-Val-Asp(OMe)-Val-Ala-Asp(OMe) fluoromethyl ketone (z-VDVAD-fmk), benzyloxycarbonyl-Asp(OMe)-Glu(O-Me)-Val-Asp(O-Me) fluoromethyl ketone (z-DEVD-fmk), benzyloxycarbonyl-Ile-Glu-Thr-Asp(OMe) fluoromethyl ketone (z-IETD-fmk), benzyloxycarbonyl-Leu-Glu-His-Asp(OMe) fluoromethyl ketone (z-LEHD-fmk), Ac-LEVD-CHO (*N*-acetyl-Leu-Glu-Val-Asp-CHO), PD98059, U0126, SP600125, SB203580, MeOSuc-Phe-Homo-Phe-fluoromethyl ketone (Mu-Phe-HPh-fmk; cathepsins B and L inhibitor) and pepstatin A were purchased from Sigma (Saint Louis, MO, USA). The caspase inhibitor Ac-VEID-CHO (*N*-acetyl-AAVALLPAVLLALLAPVEID-CHO) was from Calbiochem (Darmstadt, Germany). Acrylamide, bisacrylamide, ammonium persulfate and *N*,*N*,*N′*,*N′*-tetramethylethylenediamine were from Bio-Rad (Hercules, CA, USA). Antibodies for caspase-3, caspase-7, caspase-8 and caspase-9 were purchased from Stressgen-ENZO (Victoria, British Columbia, Canada). Anti-caspase-6 and anti-caspase-4 monoclonal antibodies were from Medical & Biological Laboratories (Nagoya, Japan). Monoclonal anti-β-Actin (clone AC-74) was purchased from Sigma (Saint Louis, MO, USA). Monoclonal anti-human Bcl-2 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal anti-human Bax and Bid antibodies and monoclonal anti-cytochrome *c* and poly(ADP-ribose) polymerase (PARP) antibodies were from BD Pharmingen (San Diego, CA, USA). Monoclonal antibody for Smac/DIABLO was from BD Transduction Laboratories. Secondary antibodies were from GE Healthcare Bio-Sciences AB (Little Chalfont, UK). PVDF membranes were from Millipore (Temecula, CA, USA). All other chemicals were obtained from Sigma (Saint Louis, MO, USA).

*2.1.1. General procedure for the synthesis of 6′-benzyloxy-4-bromo-2′-hydroxychalcone (**3**):* A mixture of the acetophenone **1** (5-10 mmol, 1 equiv.) and the corresponding aldehyde **2** (1 equiv.) in EtOH (20-40 ml) was stirred at room temperature. Then, a 50% aqueous solution of NaOH (5-8 ml) was added. The mixture was stirred at room temperature until the aldehyde had completely reacted. After that, HCl (10%) was added until neutrality. The solid was filtered and

crystallized from MeOH. The chalcone **3** was isolated as an orange solid (80%), mp 146-147 ºC. IR (KBr, cm^{-1}) ν_{max}: 3441, 3034, 1632, 1579, 1555, 1485, 1475, 1450, 1401, 1338, 1327, 1235, 1203, 1177, 1073, 1028, 1008, 972, 987, 816. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 13.39 (s, 1H); 7.73 (d, *J* = 15.6 Hz, 1H); 7.53 (d, *J* = 15.6 Hz, 1H); 7.41-7.38 (m, 2H); 7.38-7.34 (m, 1H); 7.33-7.27 (m, 3H); 7.21 (d, *J* = 8.4 Hz, 2H); 6.80 (d, *J* = 8.7 Hz, 2H); 6.56 (dd, *J* = 8.4, 1.0 Hz, 1H); 6.45 (dd, *J* = 8.3, 1.0 Hz, 1H); 5.01 (s, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ = 194.2, 165.6, 160.3, 141.7, 136.2, 135.5, 134.0, 131.9, 129.8, 128.9, 128.7, 128.6, 128.3, 124.2, 111.6, 111.4, 102.2, 71.4. HRMS (ESI-FT-ICR) *m/z*: 409.0263 $[M-H]^-$; calcd. for $C_{22}H_{16}O_3{}^{81}Br$: 409.0262.

2.2. Cell culture and cytotoxicity assays

U-937, HL-60, K-562, NALM-6 and MOLT-3 cells were from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). U-937/Bcl-2 cells were kindly provided by Dr. Jacqueline Bréard (INSERM U749, Faculté de Pharmacie Paris-Sud, Châtenay-Malabry, France) and K-562/ADR, a cell line resistant to doxorubicin was provided by Dr. Lisa Oliver (INSERM, Nantes, France). The U-937 is a pro-monocytic, human myeloid leukaemia cell line which was isolated from a histiocytic lymphoma. HL-60 is an acute myeloid leukaemia cell line. K-562 is a chronic myeloid leukaemia cell line. NALM-6 is a human B cell precursor leukaemia. MOLT-3 is an acute lymphoblastic leukaemia cell line.

Cells were cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin, incubated at 37 °C in a humidified atmosphere containing 5% CO_2 as described [18]. The doubling times of the cell lines were 30 h in U-937, U-937/Bcl-2, K-562 and K-562/ADR, 24 h in HL-60 and 40 h in NALM-6 and MOLT-3. K-562/ADR was cultured in presence of 200 ng/ml doxorubicin. Human peripheral blood mononuclear cells (PBMC) were isolated from heparin-anticoagulated blood of healthy volunteers by centrifugation with Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). PBMC were also stimulated with phytohemagglutinine (2 µg/ml) for 48 h before the experimental treatment. The trypan blue exclusion method was used for counting the cells by a hematocytometer and the viability was always greater than 95% in all experiments. The

cytotoxicity of chalcone **3** (6´-benzyloxy-4-bromo-2´-hydroxychalcone, CHAL) was evaluated by colorimetric 3-(4,5-dimethyl-2-thiazolyl-)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay as previously described [19]. Chalcone **3** was dissolved in DMSO and kept under dark conditions at 25 ºC. Before each experiment the chalcone was dissolved in culture media at 37 ºC and the final concentration of DMSO did not exceed 0.3% (v/v).

2.3. DNA fragmentation

Cells were harvested by centrifugation, washed twice with PBS, resuspended in 30 µl of lysis buffer [50 mM Tris-HCl (pH 8.0) 10 mM EDTA and 0.5% SDS] and incubated sequentially with 1 µg/µl de RNase A for 1 h at 37 ºC and 1 µg/µl of proteinase K for 1 h at 50 ºC. Two µl of bromophenol blue (0.25%) was added and DNA was extracted with 100 µl of the mixture [phenol: chloroform: isoamyl alcohol (25: 24: 1)]. Samples were centrifuged at 12,000x *g* for 1 min at 25 ºC and the aqueous phases were extracted with 100 µl chloroform. Five µl of loading buffer [10 mM EDTA (pH 8.0), with 1% (w/v) of low melting point agarose, 0.25% bromophenol blue and 40% sucrose] was added and the mixture was incubated at 70 ºC during 10 min. The samples were separated in a 1.8% agarose gel by electrophoresis at 5 V/cm during 3-4 h, in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The gel was stained with ethidium bromide (1 µg/ml) during 20 min, exposed to UV illumination and the image captured by the DC290 Zoom Digital Camera (Kodak, Rochester, NY, USA).

2.4. Fluorescent microscopy analysis

Cells were washed with PBS and fixed in 3% paraformaldehyde for 10 min at room temperature. The paraformaldehyde was removed by centrifugation (12,000x *g*, 1 min, 25 ºC) and the samples were stained with 20 µg/ml of bisbenzimide trihydrochloride (Hoechst 33258) in PBS at 25 ºC during 15 min. An aliquot of 10 µl of the mixture was used to observe the stained nuclei with a fluorescent microscopy (Zeiss-Axiovert).

2.5. Quantification of hypodiploid cells and flow cytometry analysis of annexin V-FITC and propidium iodide-stained cells

Flow cytometric analysis of propidium iodide-stained cells was performed as previously described [20]. Briefly, cells (2.5 x 10^5) were centrifuged for 10 min at 500x *g,* washed with cold PBS, fixed with ice-cold 75% ethanol and stored at -20 ºC for 1 h. Samples were then centrifuged at 500x *g* for 10 min at 4 ºC, washed with PBS, resuspended in 200 µl of PBS containing 100 µg/ml RNase A and 50 µg/ml propidium iodide and incubated for 1 h in the dark. The DNA content was analyzed by flow cytometry with a BD FACSVerse™ cytometer (BD Biosciences, San Jose, CA, USA). Flow cytometric analysis of annexin V-FITC and propidium iodide-stained cells was performed as described [20].

2.6. Assay of caspase activity

Caspase activity was determined by measuring proteolytic cleavage of the chromogenic substrates Ac-DEVD-*p*NA (for caspase-3 like protease activity), Ac-IETD-*p*NA (for caspase-8 activity) and Ac-LEHD-*p*NA (for caspase-9 activity) as previously described [20].

2.7. Western blot analysis

Cells were harvested by centrifugation (500x *g*, 10 min, 4 ºC) and pellets were resuspended in lysis buffer [1% Triton X-100, 10 mM sodium fluoride, 2 mM EDTA, 20 mM Tris-HCl (pH 7.4), 2 mM tetrasodium pyrophosphate, 10% glycerol, 137 mM NaCl, 20 mM sodium β-glycerophosphate], with the protease inhibitors phenylmethylsulfonyl fluoride (PMSF, 1 mM), aprotinin, leupeptin, and pepstatin A (5 µg/ml each) and kept on ice during 15 min. Cells were sonicated on ice five times (5 s each, with intervals between each sonication of 5 s) with a Braun Labsonic 2000 microtip sonifier and centrifuged (11,000x *g*, 10 min, 4 ºC). Bradford´s method was used to determine protein concentration. The samples that were loaded in sodium dodecyl sulphate-polyacrylamide gel (from 7.5 to 15% depending on the molecular weight of interest) were prepared with the same amount of protein and boiled for 5 min. The proteins were transferred to a poly(vinylidene difluoride) membrane for 20 h at 20 V. The membrane was

blocked with 10% nonfat milk in Tris-buffered saline [50 mM Tris-HCl (pH 7.4), 150 mM NaCl] containing 0.1% Tween-20 (TBST) for 1 h, followed by incubation with specific antibodies against caspase-3, caspase-4, caspase-6, caspase-7, caspase-8, caspase-9, Bax, Bcl-2, β-actin and poly(ADP-ribose)polymerase overnight at 4 ºC. Membranes were washed three times with TBST and incubated for 1 h with the specific secondary antibody and the antigen-antibodies complexes were visualized by enhanced chemiluminescence using the manufacturer´s protocol.

2.8. Subcellular fractionation

Cells were harvested by centrifugation (500x *g*, 10 min, 4 ºC) and washed two times with cold PBS at 4 ºC. The pellets were resuspended in buffer [20 mM HEPES (pH 7.5), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol, and 5 µg/ml leupeptin, aprotinin, and pepstatin A] with 250 mM sucrose. The samples were incubated during 15 min on ice and lysed 10 times with a 22-gauge needle. The lysates were centrifuged (1,000x *g*, 5 min, 4 ºC). The supernatants were centrifuged at 105,000x *g* for 45 min at 4 ºC, and the resulting supernatant was used as the soluble cytosolic fraction and analysed by immunoblotting with specific antibodies against Smac/DIABLO and cytochrome *c*.

2.9. Analysis of mitochondrial membrane potential ($\Delta\Psi_m$) and intracellular reactive oxygen species (ROS) determination

The mitochondrial membrane potential was measured by flow cytometry using the fluorochrome 5,5´,6,6´-tetrachloro-1,1´,3,3´-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and intracellular ROS were detected by flow cytometry using the probe 2´,7´-dichlorodihydrofluorescein diacetate (H_2-DCF-DA). Flow cytometric analysis was carried out using a BD FACSVerse™ cytometer (BD Biosciences, San Jose, CA, USA). All of the methods have been described in detail elsewhere [21].

2.10. Statistical methods

Statistical differences between means were tested using (i) Student's *t*-test (two samples) or (ii) one-way analysis of variance (ANOVA) (3 or more samples) with Tukey's test used for *a posteriori* pairwise comparisons of means. A significance level of $P < 0.05$ was used.

3. Results

3.1. The synthetic chalcone, 6′-benzyloxy-4-bromo-2′-hydroxychalcone, inhibits the viability of human leukaemia cells

Preliminary studies on U-937 cells revealed that the 2′-hydroxychalcone derivative containing a bromine atom at position 4 and a benzyloxy at position 6′ (CHAL) displays a higher cytotoxic potency [IC_{50} (50% inhibition of cell growth) = 4.4 ± 1.1 μM] than the 2′-hydroxychalcone (IC_{50} = 42.0 ± 6.5 μM) and also than the analogue containing a bromine atom at position 4 (IC_{50} = 17.0 ± 2.0 μM). The 2′-hydroxychalcone containing a benzyloxy group at position 6′ displays a similar cytotoxic potency than CHAL (IC_{50} = 5.0 ± 2.6 μM). Since we were interested in the study of halogen chalcones, CHAL was selected to determine its cytotoxic properties in several human leukaemia cells. In addition, the precursor 4-bromobenzaldehyde for CHAL synthesis is more stable than benzaldehyde itself used in the synthesis of 6′-benzyloxy-2′-hydroxychalcone. Synthesis of CHAL was carried out using a standard strategy for the generation of chalcones and involved an aldol condensation reaction in basic media to generate the desired chalcone **3** (Figure 1A) [22,23].

Table 1. Effects of CHAL on cell viability of human leukaemia cell lines

IC_{50} (μM)						
U-937	U-937/Bcl-2	HL-60	K-562	K-562/ADR	NALM-6	MOLT-3
4.4 ± 1.1	5.0 ± 0.2	4.9 ± 0.5	7.2 ± 0.8	8.9 ± 1.8	4.3 ± 0.1	2.7 ± 0.3

Cells were cultured for 72 h and the IC_{50} values were calculated as described in the Experimental Section. The data shown represent the mean ± SE of 3-5 independent experiments with three determinations in each.

CHAL was found to have strong cytotoxic properties with IC_{50} values of approximately 5 μM against the seven human leukaemia cell lines tested (Table 1), including the human histiocytic lymphoma U-937, the human chronic myeloid leukaemia K-562, the human acute myeloid leukaemia HL-60, the human B cell precursor leukaemia NALM-6, the acute lymphoblastic

leukaemia MOLT-3 and cell lines over-expressing Bcl-2 (U-937/Bcl-2) and the glycoprotein P (K-562/ADR). For comparison, the standard antitumor agent etoposide was included as a positive control for U-937 (IC_{50} = 1.2 ± 0.3 μM), HL-60 (IC_{50} = 0.3 ± 0.1 μM) and MOLT-3 (IC_{50} = 0.2 ± 0.1 μM). Treatment with this chalcone resulted in a concentration-dependent inhibition of cell viability (Figure 1B) and induced significant morphological changes and an important reduction in the number of cells (Figure 1C). In addition, quiescent and proliferating peripheral blood mononuclear cells were more resistant than U-937 cells, even at 30 μM CHAL (Figure 1D). These results indicate that CHAL displays strong cytotoxic properties against leukaemia cells but has only weak cytotoxic effects against peripheral blood mononuclear cells (PBMCs) and also that the overexpression of the Bcl-2 protein and glycoprotein P did not confer resistance to CHAL-induced cytotoxicity.

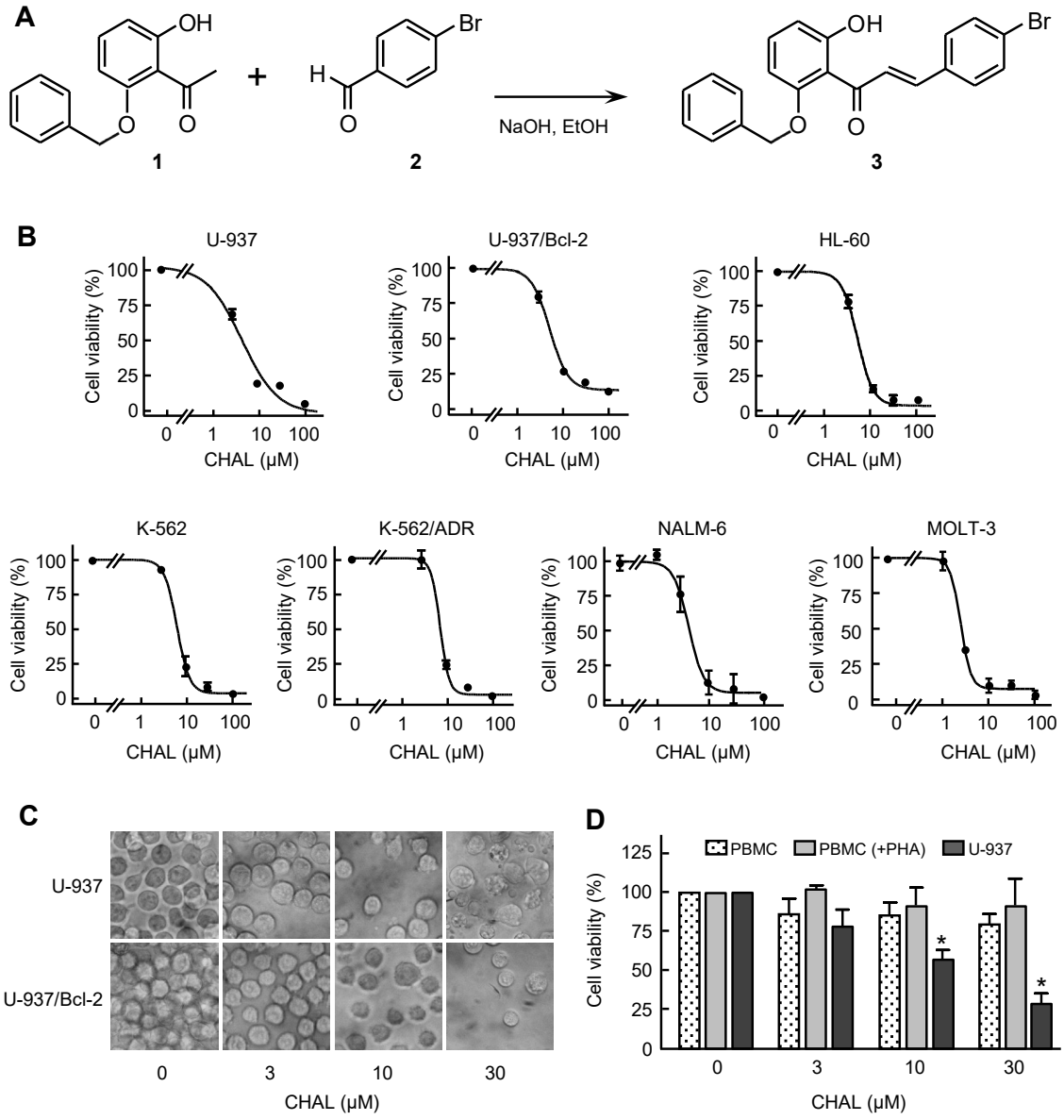

Figure 1. Synthesis and effects on cell viability of CHAL. (A) Synthesis and chemical structure of CHAL. (B) Dose-response study of CHAL on human leukaemia cells viability. Cells were cultured in the presence of increasing concentrations of CHAL for 72 h, and thereafter cell viability was determined by the MTT assay. (C) Photomicrographs of morphological changes visualized with an inverted phase contrast microscope after treatment with CHAL for 24 h. (D) Differential effects of CHAL on cell viability of normal peripheral blood mononuclear cells (PBMC) versus U-937 cells. Human leukaemia, and quiescent and phytohemagglutinine-activated PBMC [PBMC (+PHA)] cells from healthy human origin were cultured in the presence of the specified concentrations of CHAL for 24 h. Values represent means ± SE for

three independent experiments each performed in triplicate. *P<0.05, significantly different from the corresponding control.

3.2. CHAL induces apoptosis on human leukaemia cells

To elucidate the mechanism responsible for inhibition of cell viability, we investigated whether CHAL induces apoptosis. To this end, we analysed the nuclei of treated cells (10 μM CHAL for 24 h) using fluorescent microscopy after staining with the fluorochrome bisbenzimide trihydrochloride (Hoechst 33258) and observed condensation and fragmentation of chromatin in U-937 cells but not in U-937/Bcl-2 cells (Figure 2A). Agarose gel electrophoresis showed typical DNA fragmentation caused by internucleosomal hydrolysis of chromatin after treatment with 10 μM CHAL for 24 h (Figure 2B). Evaluation of the percentage of sub-G_1 (hypodiploid) cells by flow cytometry showed that the percentage of apoptotic cells increased approximately 25-fold in CHAL-treated U-937 compared with control cells after 24 h exposure at concentration as low as 10 μM. In contrast, 3 μM CHAL for 24 h was not sufficient to trigger apoptosis (Figure 2C). Interestingly, treatment with 10 μM CHAL for 24 h did not induce apoptosis in PBMC (results not shown). Time-course experiments revealed that apoptotic cells were already detected at 6 h of treatment, however, in U-937/Bcl-2 cells, the percentage of sub-G_1 cells did not increase significantly after 24 h of treatment with 10 μM CHAL (Figure 2D). CHAL (10 μM, for 24 h) also led to the exposure of phosphatidylserine on the outside of the plasma membrane as detected by annexin V-FITC staining in U-937 cells (Figure 2E). The results indicate that CHAL is a potent apoptotic inducer in human myeloid leukaemia cells and that the overexpression of Bcl-2 does not confer protection against apoptosis induction.

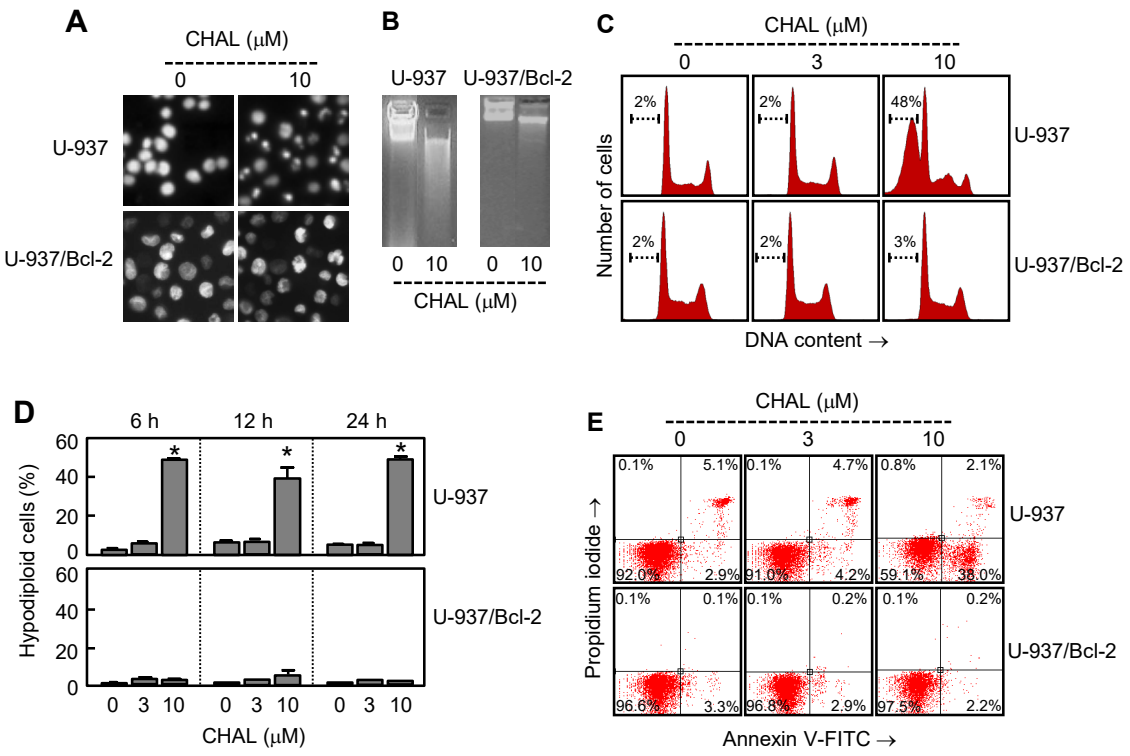

Figure 2. Effects of CHAL on apoptosis induction in human myeloid leukaemia cells. (A) Photomicrographs of representative fields of cells stained with bisbenzimide trihydrochloride to evaluate nuclear chromatin condensation after treatment with 10 μM CHAL for 24 h. (B) Cells were incubated with 10 μM CHAL for 24 h and genomic DNA was extracted, separated on an agarose gel and visualized under UV light after ethidium bromide staining. (C) Cells were incubated in the presence of the indicated concentrations of CHAL and subjected to DNA flow cytometry using propidium iodide labelling. Representative histograms and the percentage of hypodiploid cells (apoptotic cells) are shown. (D) Cells were incubated in the presence of the specified concentrations of CHAL for different periods of time and the percentage of hypodiploid cells was determined by flow cytometry using the propidium iodide labelling method. Values represent means±SE from three different experiments performed in triplicate. *indicates $P<0.05$ for comparison with untreated control. (E) Flow cytometry analysis of annexin V-FITC and propidium iodide (PI)-stained U-937 and U-937/Bcl-2 cells after treatment with the indicated concentrations of CHAL for 24 h. Cells appearing in the lower right quadrant show positive annexin V-FITC staining, which indicates phosphatidylserine translocation to the cell surface, and negative PI staining, which demonstrates intact cell

membranes, both features of early apoptosis. Cells in the top right quadrant are double positive for annexin V-FITC and PI and are undergoing necrosis. Representative data from three separate experiments are shown.

To assess whether cell growth inhibition is also mediated by alterations in the cell cycle, cells were incubated with CHAL for different time periods (6-24 h), stained with propidium iodide and analyzed by flow cytometry. There were no obvious changes in the different phases of the cell cycle after treatment with different concentrations of CHAL (3-10 μM) across the different time periods (results not shown).

3.3. CHAL-induced apoptosis is mediated by a caspase-dependent pathway

U-937 and U-937/Bcl-2 cells were cultured for 24 h with 10 μM CHAL in order to investigate the influence of caspases in their responses to this chalcone. Lysates were analyzed by Western blot using specific antibodies (Figure 3A). The results showed that CHAL induced the cleavage of the initiator caspases-8 and -9, in U-937 but not in U-937/Bcl-2 cells. The chalcone also induced hydrolysis of pro-caspase-6 and -7, as well as the proteolytic processing of pro-caspase-3 only in U-937 cells. Pro-caspase-4 which has been involved in the endoplasmic reticulum stress was also processed. Poly(ADP-ribose) polymerase was effectively hydrolysed to the 85 kDa fragment after CHAL treatment, in accordance with the pro-caspase-3 activation. These results indicate that CHAL induces the processing of multiple caspases in U-937 but not in U-937/Bcl-2 cells.

The enzymatic activities of caspases-3/7, -8 and -9 were also evaluated since they are not always associated with pro-caspase processing. Cells were treated with CHAL (10 μM, 24 h) and lysates were assayed for the cleavage of the tetrapeptide substrates Ac-DEVD-*p*NA, Ac-IETD-*p*NA and Ac-LEHD-*p*NA as specific substrates of caspase-3/7, caspase-8 and caspase-9, respectively. The results showed a significant activation of these caspases after 24 h of treatment in U-937 but not in U-937/Bcl-2 cells (Figure 3B), in accordance with the

immunoblot experiments. These results indicate that CHAL is able to induce activation of caspases in leukaemia cells.

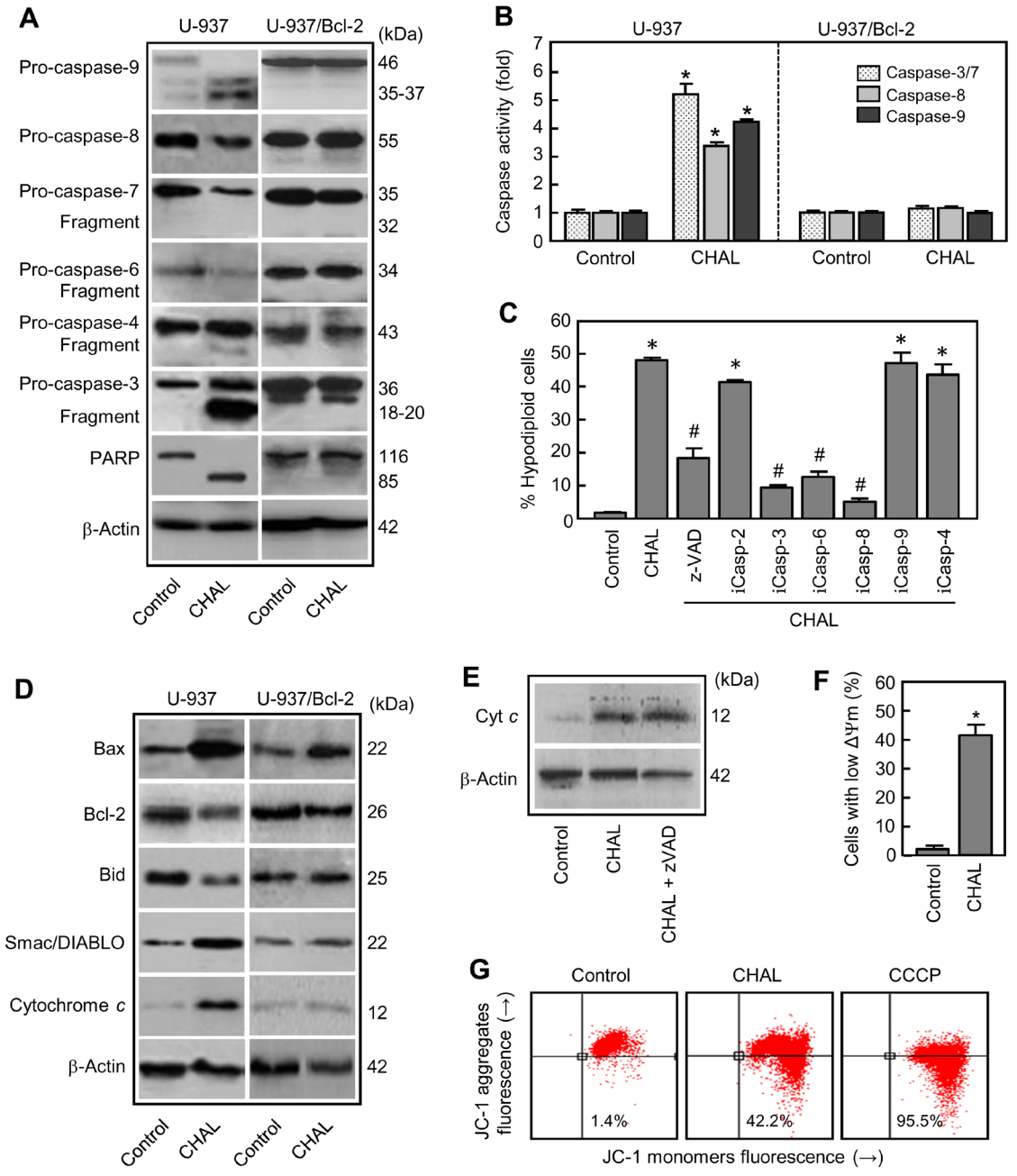

Figure 3. Processing, activation and role of caspases and mitochondrial proteins in CHAL-induced apoptosis on leukaemia cells. (A) Cells were incubated with 10 µM CHAL for 24 h and the cleavage of caspases and PARP was analyzed by immunoblotting. The protein β-actin was used as a loading control. (B) Caspase activation induced by CHAL on leukaemia cells. Cells

were treated as above and cell lysates were assayed for caspase-3/7, -8 and -9 activities. Results are expressed as n–fold increases in caspase activity compared with control. Values represent the means ± SE of three independent experiments each performed in triplicate. *indicates $P<0.05$ for comparison with untreated control. (C) Cells were pretreated with z-VAD-fmk (100 μM) or the selective caspase inhibitors z-VDVAD-fmk (iCasp-2, 50 μM), z-DEVD-fmk (iCasp-3, 50 μM), VEID-CHO (iCasp-6, 25 μM), z-IETD-fmk (iCasp-8, 50 μM), z-LEHD-fmk (iCasp-9, 50 μM) or LEVD-CHO (iCasp-4, 50 μM) for 1 h before the addition of CHAL (10 μM) during 24 h, and apoptotic cells were analyzed by flow cytometry. Bars represent the mean ± SE of three independent experiments each performed in triplicate. *indicates $P<0.05$ for comparison with untreated control. $^{\#}$ indicates $P<0.05$ for comparison with CHAL treatment alone. (D) Effect of CHAL on mitochondrial cytochrome *c* and Smac/DIABLO release and on Bcl-2 family members. Cells were treated with 10 μM CHAL and cytosolic fractions (in the case of cytochrome *c* and Smac/DIABLO) or whole cell lysates (for Bax, Bcl-2 and Bid) were analyzed by Western blot. β-Actin was used as a loading control. (E) Cytochrome *c* release is not inhibited by z-VAD-fmk. U-937 cells were pretreated with the caspase inhibitor z-VAD-fmk (100 μM, 1 h) and then with CHAL (10 μM, 24 h) and cytosolic fractions were subjected to immunoblotting. (F) CHAL reduces the mitochondrial membrane potential ($\Delta\Psi_m$). U-937 cells were treated with 10 μM CHAL, harvested at 6 h and $\Delta\Psi_m$ analyzed by flow cytometry after staining with the JC-1 probe. *indicates $P<0.05$ for comparison with untreated control. (G) Representative dot plots after staining with the JC-1 probe; as a positive control, cells were stained in the presence of 50 μM of CCCP (carbonyl cyanide *m*-chlorophenylhydrazone).

To determine whether caspases were involved in the apoptosis, U-937 cells were pretreated with the general caspase inhibitor z-VAD-fmk (100 μM) and cultured in the presence of CHAL for 24 h. As shown in Figure 3C, apoptosis was significantly reduced, suggesting a caspase-dependent pathway. To identify which caspases were important in CHAL-induced apoptosis,

the effect of specific cell-permeable caspase inhibitors was examined. The caspase-3/7 inhibitor (z-DEVD-fmk, 50 μM), the caspase-6 inhibitor (Ac-VEID-CHO, 25 μM) and the caspase-8 inhibitor (z-IETD-fmk, 50 μM) blocked the apoptosis induced by CHAL (10 μM) in U-937 cells. In contrast, neither the caspase-4 inhibitor (Ac-LEVD-CHO), nor caspase-9 inhibitor (z-LEHD-fmk, 50 μM) nor the caspase-2 inhibitor (z-VDVAD-fmk, 50 μM) blocked the apoptosis in U-937 cells (Figure 3C). These experiments demonstrate that the extrinsic apoptotic pathway played the main role in U-937 cells death induced by CHAL.

To determine the mitochondrial proteins release, cytosolic preparations were analyzed by Western blot after 24 h treatment with 10 μM CHAL. As shown (Figure 3D) cytochrome *c* levels increased in the cytosolic fraction in accordance with the processing and activation of pro-caspase-9 and also the substantial release of the proapoptotic mitochondrial protein Smac/DIABLO. Moreover, Bcl-2 levels clearly decreased after treatment with CHAL, while there was an increase in the proapoptotic factor Bax. CHAL also induced a decrease in Bid levels suggesting the processing of this protein due to caspase-8 activation (Figure 3D).

To determine whether cytochrome *c* release precedes caspase activation, U-937 cells were preincubated with the pancaspase inhibitor z-VAD-fmk (1 h, 100 μM) and then followed with 10 μM CHAL for 24 h. The broad-spectrum caspase inhibitor was unable to prevent cytochrome *c* release, which suggests that cytochrome *c* release is independent of caspase activation (Figure 3E).

To examine whether a disruption of the mitochondrial membrane potential ($\Delta\Psi_m$) is associated with the release of mitochondrial proteins, cells were stained with the fluorescent probe JC-1 and analysed by flow cytometry. It was found that the $\Delta\Psi_m$ dropped at 6 h of treatment, indicating that the dissipation of $\Delta\Psi_m$ is an early event in CHAL-induced apoptosis (Figure 3F). Representative dot plots of these experiments are shown, including the experiment that used protonophore CCCP as a positive control (Figure 3G).

3.4. Effect of cathepsins and mitogen-activated protein kinases (MAPKs) inhibitors and dependence on Reactive Oxygen Species in CHAL-induced cell death

Since lysosomal cathepsins have been associated with caspase–dependent and –independent apoptotic cell death and with the activation of the intrinsic apoptotic pathway, their involvement was investigated using the cathepsin inhibitors Mu-Phe-HPh-fmk (M4070, cathepsins B/L inhibitor) and pepstatin A (cathepsin D inhibitor). As shown in Figure 4A, cathepsins B/L inhibition significantly decreased apoptosis stimulated by CHAL suggesting that these types of proteases might be involved in cell death.

The mitogen-activated protein kinases (MAPKs) cascades play important roles in the regulation of apoptosis and growth of malignant hematopoietic cells. To determine whether activation of MAPKs plays a key role in CHAL-induced apoptosis, the effect of pharmacological inhibitors of MAPKs was investigated. Treatment of cells with inhibitors of mitogen-activated extracellular kinases 1/2 (MEK1/2), namely PD98059 or U0126 and the specific JNK/SAPK (c-Jun *N*-terminal kinases /stress activated protein kinase) inhibitor SP600125, had no influence on the percentage of hypodiploid cells induced by CHAL. In contrast, the $p38^{MAPK}$ (p38 mitogen-activated protein kinase) inhibitor SB203580 partially decreased the percentage of hypodiploid cells (Figure 4B).

Increased reactive oxygen species (ROS) production may lead to cell death in leukaemia cells [24]. To determine whether CHAL induces ROS, U-937 cells were treated with the chalcone, stained with the fluorescent dye 2′, 7′-dichlorodihydrofluorescein diacetate (H_2-DCF-DA) and analysed by flow cytometry. As shown in Figure 4C, in U-937 cells but not in PBMC, treatment with 10 μM CHAL induced a 2.5-fold increase in the H_2-DCF-DA-derived fluorescence as indicated by a rightward shift in fluorescence (Figure 4D). These results suggest that CHAL induces the generation of H_2O_2 and other peroxides. To investigate whether ROS is involved in the apoptosis induced by CHAL, U-937 cells were preincubated with the antioxidants *N*-acetyl-L-cysteine (10 mM), α-tocopherol (20 μM), trolox (2 mM), the NADPH oxidase inhibitor diphenyleneiodonium chloride (1 μM), superoxide dismutase (400 units/ml) and catalase (500

units/ml). None of these antioxidants, except catalase, blocked ROS generation (Figure 4E) or cell death (Figure 4F) as assessed by flow cytometry indicating that CHAL-induced apoptosis is dependent on ROS production.

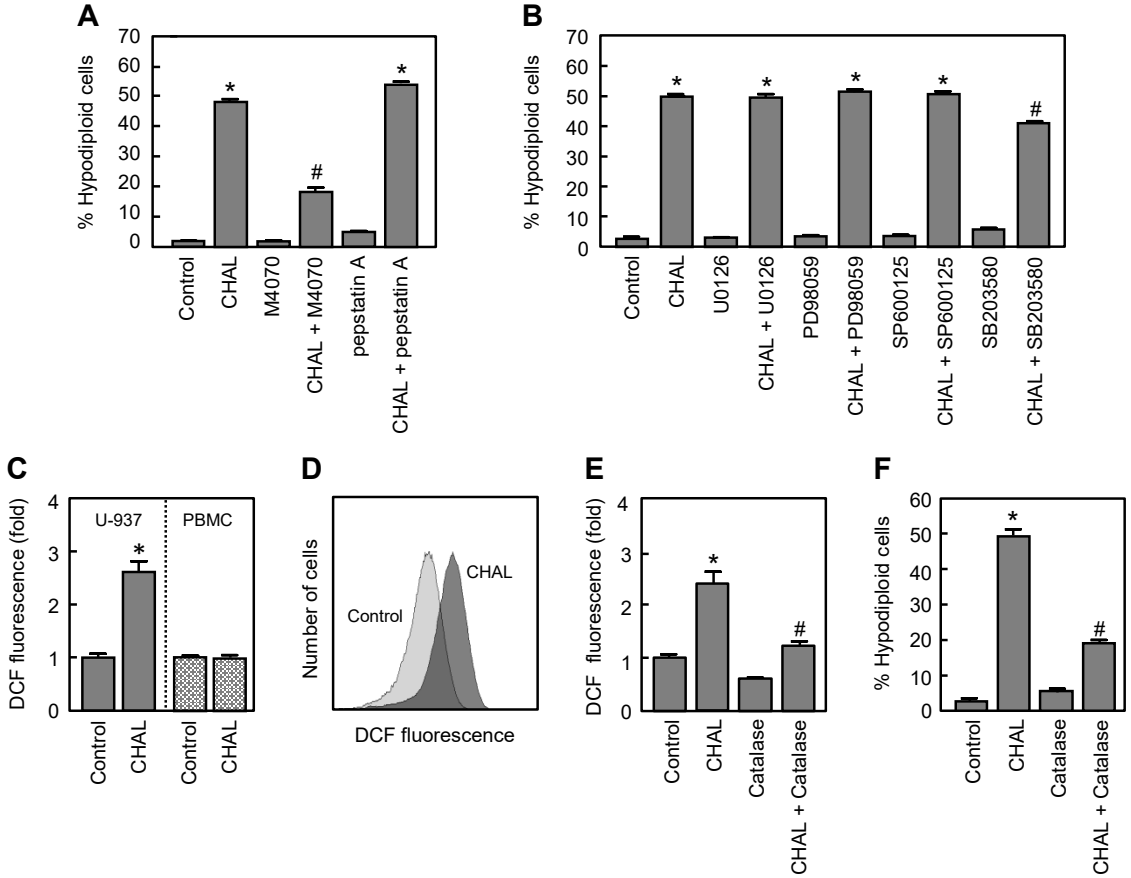

Figure 4. Effects of cathepsins and mitogen-activated protein kinase inhibitors and reactive oxygen species generation on CHAL-induced apoptosis. (A) Cells were pretreated with the cathepsin inhibitors Mu-Phe-HPh-fmk (M4070, 20 μM, 3 h) and pepstatin A (100 μM, 12 h) and then incubated with 10 μM CHAL for 24 h and apoptosis was quantified by flow cytometry. Bars represent the mean ± SE of three independent experiments each performed in triplicate. *indicates $P<0.05$ for comparison with untreated control. # indicates $P<0.05$ for comparison with CHAL treatment alone. (B) Cells were preincubated with U0126 (10 μM), PD98059 (10 μM), SP600125 (10 μM), and SB203580 (2 μM) for 1 h and then treated with CHAL (10 μM) for 24 h. Apoptosis was quantified by flow cytometry, and bars represent the means ± SE of three independent experiments each performed in triplicate. *indicates $P<0.05$ for comparison

with untreated control. # indicates $P<0.05$ for comparison with CHAL treatment alone. (C) U-937 and PBMC were incubated with CHAL (10 μM) for 2 h, and the fluorescence of oxidized H_2DCF was determined by flow cytometry. (D) A representative histogram is shown. Similar results were obtained from three independent experiments. (E, F) Cells were preincubated with catalase (500 units/ml) for 1 h and then treated for 24 h with 10 μM CHAL and the fluorescence of oxidized H_2DCF (E) and the percentage of hypodiploid cells (F) were determined by flow cytometry. Bars represent the means ± SE of three independent experiments each performed in triplicate. *indicates $P<0.05$ for comparison with untreated control. # indicates $P<0.05$ for comparison with CHAL treatment alone.

4. Discussion

Chemotherapy drugs used in the treatment of cancer are expensive, toxic and show limited efficacy in treating these diseases. Naturally occurring compounds or compounds inspired in natural products have attracted the attention as potential anticancer agents. Chalcones are compounds that can be easily obtained by synthetic methods and, in general, are less toxic than current chemotherapy. These compounds display a vast array of pharmacological and biological activities, including antitumor properties. Chalcones have been described as Notch signaling inhibitors. This signal transduction pathway is considered a rational target in potential anticancer therapy because Notch inhibition triggers antiproliferative and pro-apoptotic effects in several human T-cell acute lymphoblastic leukaemia cells [25]. Recently, anthraquinone-chalcone hybrids have been reported as strong cytotoxic and apoptotic inducers in human leukaemia cell lines [26]. The naturally occurring chalcone broussochalcone A has been described as a human cytochrome P450 2J2 inhibitor that is cytotoxic against human hepatoma HepG2 cells [27].

In this study, we designed a specific chalcone, 6′-benzyloxy-4-bromo-2′-hydroxychalcone, to examine the hypothesis that the introduction of one benzyloxy group at position 6′ in the A ring might enhance cytotoxicity against cancer cell lines (due to an increase in lipid solubility that would facilitate cell penetration). Neither the effects on cell viability nor the mechanism

involved in cell death induction of this specific chalcone have been investigated to date. Synthetic chalcone was screened for cytotoxicity against human tumour cells and found to be a potent cytotoxic compound against seven human leukaemia cell lines, including cells that overexpress the anti-apoptotic protein Bcl-2 and the glycoprotein P. Since P-glycoprotein-mediated multidrug resistance is a major limitation of the clinical efficacy of microtubule-targeting agents, it is necessary to find new compounds that may overcome this resistance. Experiments using P-glycoprotein-overexpressing K-562/ADR cells indicate that CHAL was also cytotoxic in these cells, showing similar IC_{50} values in both the multidrug resistant K-562/ADR (IC_{50} = 7.2 ± 0.8 μM) and the corresponding parent K-562 cells (IC_{50} = 8.9 ± 1.8 μM). Interestingly, dose-response studies revealed that quiescent PBMC and proliferating PBMC were resistant toward CHAL. One possible explanation why normal cells are resistant to CHAL could be that this chalcone targets the transcription factor nuclear factor κB (NF-κB) which is the major molecular target affected by some chalcones [2,28]. For example, the naturally occurring butein (2′,3,4,4′-tetrahydroxychalcone) blocks NF-κB through Iκ-B kinase inhibition, avoiding its nuclear translocation and therefore deregulating its downstream biomolecules. This inactivation of NF-κB is involved in apoptosis induction and the inhibition of proliferation, cell cycle progression, angiogenesis, invasion, metastasis and chemoresistance of various cancers [28]. NF-κB signaling is elevated in leukemic stem cells but not in the normal hematopoietic stem cells. The ability to target the NF-κB pathway could be the reason why CHAL selectively kills cancer and not normal cells. Normal cells might not be sensitive to CHAL because their basal NF-κB activity is often low or sometimes required for cell differentiation rather than oncogenesis [29,30]. In addition, the modulation of ROS production could explain the preferential toxicity towards cancer cells, since CHAL was unable to induce ROS generation in PBMC. NF-κB is involved in the processes of oxidative stress response and the increase in intracellular ROS in U-937 cells, as described here, could activate this transcription factor. Further studies are needed to determine whether NF-κB signaling is a target of CHAL.

A recent report describes how a novel 3′,5′-diprenylated chalcone inhibits the growth of the human leukaemia cells HEL and K-562 with IC_{50} values that were comparable with the chalcone described in this paper. However, this chalcone was also cytotoxic against human normal hepatocyte (LO2) cells. The authors concluded that 3′,5′diprenylated chalcone may inhibit the growth of leukaemia cells by inducing apoptosis and autophagy [31].

The experiments described here support apoptosis as the main cytotoxicity pathway induced by CHAL in U-937 cells and that overexpression of the anti-apoptotic protein Bcl-2 does not confer resistance to apoptosis. The effects of CHAL in U-937 cells include condensation and fragmentation of chromatin, an increase in hypodiploid DNA content, caspase activation and a significant reduction in apoptosis by the pan-caspase inhibitor z-VAD-fmk, supporting a caspase dependent cell death mechanism. An effective blockage of CHAL-induced apoptosis by z-IETD-fmk was observed supporting a mechanism mediated by the initiator caspase-8. In contrast, the selective inhibitor against caspase-9 (z-LEHD-fmk) was unable to significantly block cell death suggesting a minor role of this initiator caspase in CHAL-induced cell death. Future experiments will be necessary to confirm the role of caspase-9 in the mechanism of cell death. Recently, we have described an effective block of cell death induced by the synthetic flavonoid 3',4'-dibenzyloxyflavonol by the selective inhibitor of caspase-9 but not by the selective inhibitor of caspase-8 [14]. Although CHAL is able to induce caspase-4 cleavage, the endoplasmic reticulum stress signalling pathway does not appear to be involved since apoptosis was not blocked by a caspase-4 inhibitor. However it is important to note that the experiments of caspase activation reveal inductions of both initiator caspases, caspase-8 and -9, activities in U-937 cells but there was not a significant activation of caspases in U-937/Bcl-2. Moreover, the results demonstrate i) that caspase-3/7 is activated in response to CHAL and ii) the inhibitors against caspase-3/7 (z-DEVD-fmk) and caspase-6 (VEID-CHO) were able to block cell death. Overexpression of Bcl-2 protein protects the cells, because it prevents the release of the pro-apoptotic protein cytochrome *c* from the mitochondria to the cytosol, apoptosome formation and the consequent activation of caspase-9 [32]. Our experiments demonstrate that CHAL is cytotoxic against human leukaemia U-937 cells that overexpress Bcl-2, as determined by the

MTT assay. We emphasize that the cytotoxicity experiments were performed with long incubation times (72 h), while those that evaluated apoptosis were carried out for much shorter times. It is possible that CHAL might affect the cell viability in U-937/Bcl-2 by a different mechanism to apoptosis. Although we did not observe any change in the percentages of cells in each phase of the cell cycle up to 24 h in both cell lines, U-937 and U-937/Bcl-2, one explanation might be the increase in the duration of each phase of the cell cycle, as described for the electron transport chain complex II inhibitor 2-thenoyltrifluoroacetone [33]. Further studies will be necessary to determine how inactivation or avoidance of Bcl-2 protein protection is achieved to yield similar IC_{50} values in both cell lines (U-937 and U-937/Bcl-2). In U-937 cells, this chalcone induces the release of the proapoptotic mitochondrial proteins cytochrome *c* and Smac/DIABLO, a decrease in Bcl-2 levels and up-regulation of Bax. A decrease in the ratio Bcl-2/Bax has been also observed in K-562 cells treated with the 3´-geranyl-mono-substituted chalcone xanthoangelol, however the concentrations of this chalcone (up to 30 μM) and exposure durations (up to 48 h) were higher than those used here [34].

Several death stimuli can induce the lysosomal pathway which is characterized by a partial rupture of lysosome membrane and subsequent release of cathepsins into the cytosol [35]. Experiments with cathepsins inhibitors revealed that these cysteine proteases are also involved in the mechanism of cell death. The effective blockage of apoptosis induction by an inhibitor of cathepsins B/L supports a model in which CHAL also induces cell death by a cathepsins B/L mediated mechanism. Futures studies will be necessary to determine whether additional pathways of cell death are involved in U-937 cells.

The mitogen activated protein kinase signalling pathway is involved in the control of cell proliferation, differentiation and apoptosis. The ERK1/2 (extracellular signal-regulated kinase) pathway is involved in cell proliferation and differentiation, whereas JNK/SAPK and $p38^{MAPK}$ pathways are activated in response to stress and growth factors and mediate signals that regulate apoptosis [36]. Neither the specific MEK 1/2 inhibitors nor the selective JNK/SAPK inhibitor SP600125 influenced or attenuated cell death, suggesting that activation of these protein kinases is not required for apoptosis triggered by CHAL. However, inhibition of $p38^{MAPK}$ was found to

decrease partially cell death suggesting that activation of this protein kinase is involved in part in CHAL induced-apoptosis. The activation of p38 mitogen-activated protein kinase signalling plays a key role not only in inhibiting but in promoting proliferation and in increasing resistance to chemotherapeutic agents [37]. It has been shown that inhibition of $p38^{MAPK}$ using the pharmacologic inhibitor SB203580 enhances all-*trans*-retinoic acid–dependent growth inhibition of acute promyelocytic leukaemia [38] and also reduces the apoptosis induced by the combination of arsenic trioxide and the isoflavone genistein in U-937 cells [39]. A similar attenuation of apoptosis by SB203580 has been observed in U-937 cells treated with cadmium, suggesting that $p38^{MAPK}$ activation is required, at least in part, for cadmium-induced apoptosis [40].

Reactive oxygen species (ROS) display a variety of biological effects including, among others, enhanced cell proliferation and cell death. Many antitumor compounds exhibit cytotoxic properties via ROS-dependent activation of apoptotic cell death in cancer cells including leukemic cells [41]. We demonstrate that the synthetic chalcone induced a fast ROS generation in U-937 cells. This elevated ROS production may affect the activity of a variety of apoptotic effectors such as the Bcl-2 family of proteins and the mitochondrial cytochrome *c* [42]. In addition, the increased levels of ROS might trigger the activation of death receptors [43]. The results shown here demonstrate that ROS play a key role in the mechanism of cell death triggered by CHAL, since the enzymatic antioxidant catalase was able to block ROS generation and cell death. Further experiments will be needed to determine whether CHAL is able to induce death receptors, including Fas, TNF receptor 1, TNF-related apoptosis inducing ligand receptor 1 and TNF-related apoptosis inducing ligand receptor 2.

5. Conclusions

CHAL displays potent cytotoxic properties against human leukaemia cells (U-937, HL-60, K-562, NALM-6 and MOLT-3) including Bcl-2-overexpressing U-937/Bcl-2 and P-glycoprotein-overexpressing K-562/ADR, but has only weak cytotoxic effects on peripheral blood mononuclear cells (PBMCs). Growth inhibition of U-937 cells was caused by induction of

apoptotic cell death and the overexpression of Bcl-2 conferred resistance to apoptosis. Cell death was associated with the activation of multiple caspases and was dependent on the generation of reactive oxygen species. It was prevented by the selective caspase-3/7, -6 and -8 inhibitors, and by a cathepsins B/L inhibitor. CHAL is a potent apoptotic inducer in human leukaemia cells that might be useful in the development of new strategies for targeted cancer therapies.

Appendix A. Supplementary data

Spectral data for 6′-benzyloxy-4-bromo-2′-hydroxychalcone (CHAL).

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Conflict of Interest

We declare no conflict of interest.

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