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Induction of apoptosis and cell cycle arrest in L-1210 murine lymphoblastic leukaemia cells by (2*E*)-3-(2-naphthyl)-1-(3′-methoxy-4′-hydroxy-phenyl)-2-propen-1-one

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Abstract

Objectives New compounds with biological targets and less cytotoxicity to normal cells are necessary for cancer therapy. In this work ten synthetic chalcones derived from 2-naphtaldehyde were evaluated for their cytotoxic effect in murine acute lymphoblastic leukemia cells L-1210.

Methods A series of ten chalcones derived from 2-naphtaldehyde and corresponding acetophenones were prepared by aldolic condensation, using methanol as solvent under basic conditions, at room temperature for 24 h. The cell viability was determined by MTT colorimeter method. The cell cycle phase analysis was carried out by flow cytometry after propidium iodide staining. The apoptosis induction was assessed by exposure to phosphatidylserine (ANNEXIN V-FITC). Cytometric analysis was performed to evaluate the expression of p53, Bcl-2 and Bax protein. The caspase-3 expression was studied by immunoblotting analysis.

Key findings A preliminary screening of a series of ten chalcones derived from 2-naphtaldehyde showed that chalcone **8**, (2*E*)-3-(2-naphtyl)-1-(3′-methoxy-4′-hydroxy-phenyl)-2-propen-1-one, had the highest cytotoxic effect (IC50 of 54 μM), but not in normal human lymphocytes. To better understand the cytotoxic mechanism of chalcone **8**, its effect on cell cycle and apoptosis was assessed. Our results showed that chalcone **8** caused cell cycle arrest in the G2/M phase and a significant increase in the proportion of cells in the subG0/G1 phase. Our results also demonstrated that chalcone **8** promoted a modification in Bax : Bcl-2 ratio and increased p53 expression and caspase-3 activation.

Conclusions The studied chalcone **8** has cytotoxic effect against L-1210 lymphoblastic leukaemic cells, and this effect is associated with increase of p-53 and Bax expression.

Keywords apoptosis; chalcone; leukaemia

Introduction

Chalcones are precursors of flavonoids in higher plants and display a wide variety of pharmacological effects. Many studies have demonstrated that chalcones and chalcone derivatives present cytotoxic activity against cancer cells and may have potential applications in cancer treatment .[1–5] The suggested mechanism of this anti-tumour effect is that the chalcones interfere with the microtubule polymerization.[6]

Several reports have demonstrated that apoptotic processes play a key role in the control of haematopoietic tissue. Studies carried out over the past twenty years have dramatically improved our understanding of the role of apoptotic mechanisms in the control of differentiation, proliferation and survival of normal and leukaemic cells.[7]

Targeted therapies that are designed to induce apoptosis in leukaemic cells are currently the most promising anti-leukaemia strategies. These strategies are aimed at targeting and killing the leukaemia cells specifically with no, or limited, collateral damage to normal haematopoi-

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etic progenitor cells. The elucidation of the molecular apoptotic machinery and its defects in acute leukaemia forms the basis for developing new drugs able to trigger apoptosis of leukaemia cells.[8]

Scientific and technological advances in recent decades have made it possible to understand the pathways behind apoptosis in tumour cells. This knowledge permits the development of new drugs that are synthesized from plant-derived active principles and their semi-synthetic and synthetic analogues that are able to interfere with these pathways and potentially act as antineoplasic agents.[9]

In a previous study we showed that synthetic hydroxychalcones induce apoptosis in B16-F10 melanoma cells via ATP and GSH depletion.[10] Although some mechanisms have been suggested, the exact pathways involved in the cytotoxic effects of chalcones are not completely elucidated in the case of leukaemic cells.

In this study, we assessed the cytotoxic effect of ten synthetic chalcones in the murine acute lymphoblastic leukaemia cell line (L-1210). In addition, we have investigated the cytotoxic mechanism of chalcone **8**, (2*E*)-3-(2-naphthyl)-1-(3′-methoxy-4′-hydroxy-phenyl)-2-propen-1-one.

Materials and Methods

Synthesis

Reagents used were obtained commercially from Sigma-Aldrich and solvents from Vetec. A series of ten chalcones derived from 2-naphtaldehyde and corresponding acetophenones were prepared by aldolic condensation, using methanol as solvent under basic conditions, at room temperature for 24 h. The chalcone **8**, (2*E*)-3-(2-naphtyl)-1-(3′-methoxy-4′-hydroxy-phenyl)-2-propen-1-one, was synthesized by condensation of 2-naphtadehyde and 4-hydoxy-3-methoxy-acetophenone, under the same conditions. All structures, including chalcone 8, were identified by melting point, infrared spectroscopy (IR), ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) and elementary analysis as previously described by us.[10,11]

Cell culture and viability assay (MTT assay)

Murine L-1210 acute lymphoblastic leukaemia cells (ATCC, Manassas, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, São Paulo, Brazil) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES, pH 7.4 at 37°C in a 5% CO_2 humidified atmosphere in plastic culture flasks. Every two or three days, cells were passaged by removing 90% of supernatant and replacing it with fresh medium.

Cell viability was assessed using MTT (3-(4,5-dimethiazol-zyl)-2-5-diphenyltetrazolium bromide; Sigma Chemical Co., St Louis, USA) assay.[12] Chalcones were added to murine L-1210 cells in different concentrations (10–100 μM) and incubated for 12, 24 or 48 h. For compounds soluble in DMSO, the same volume of the solvent was added to control wells. After incubation, MTT was added to each well followed by 3 h incubation. The cells were centrifuged and the supernatant was discarded. The formazan precipitated was dissolved with 100 μl of an isopropyl alcohol–HCl 0.04 N solution, and the absorbance was determined at 540 nm using a microplate reader. All assays were performed in triplicate.

Normal lymphocytes were collected from healthy donors and human peripheral blood mononuclear cells were isolated by Ficoll/Hypaque gradient. The normal lymphocytes were cultured under the same conditions described for leukaemia cell lines.

Cell cycle analysis

To assess cell cycle arrest, PI/RNase solution kit (Immunostep, Salamanca, Spain) was used. Cells (5×10^5 cells/well) were incubated with vehicle or chalcone **8** at 100 μM. After 24 h of incubation, cells were harvested and cell cycle analysis was assessed according to the kit protocol. Briefly, cells were harvested, washed with phosphate-buffered saline (PBS) and centrifuged for 5 min at 300 *g*. The supernatant was removed and the cells were fixed with 70% ethanol for 30 min at 4°C. Cells were washed once with PBS supplemented with 2% bovine albumin, centrifuged for 5 min at 300 *g* and the supernatant was removed. To the cell pellet was added 500 μl PI/RNase solution and the cells were incubated for 15 min at room temperature. After incubation the analysis was performed by flow cytometry (FACSCalibur, Becton Dickinson Immunocytometry Systems). The data were analysed by WinMID software.

Analysis of the apoptotic effects

For determination of apoptotic death an Annexin V-FITC Apoptosis Detection kit was used according to the manufacturer's instructions. Briefly, 5×10^5 cells/well were incubated with vehicle or chalcone **8** at 100 μM. After 6 h of incubation, cells were harvested, washed with PBS buffer, annexin buffer (1 : 10) and double-stained with Annexin V-FITC solution and PI/RNase solution. After incubation, 300 μl of annexin buffer was added and fluorescence was analysed by flow cytometry. Analysis was performed by flow cytometry (FACSCalibur, Becton Dickinson Immunocytometry Systems). The data were analysed by WinMID software.

Effect of chalcone on the expression of p53, Bcl-2 and Bax

For the analysis of Bcl-2, Bax and p53 proteins, 5×10^5 cells were incubated with vehicle or chalcone **8** at 100 μM for 24 h. At the end of the incubation, cells were washed with PBS and permeabialized with ethanol 70%. Subsequently, the cells were incubated in the dark at room temperature, with anti-p53-PE, anti-Bcl-2-FITC or anti-Bax-FITC, for the detection of p53, Bcl-2 and Bax, respectively. After incubation, the cells were washed with PBS/albumin 2% and re-suspended in PBS for cytometric analysis. Analysis was performed by flow cytometry (FACSCalibur, Becton Dickinson Immunocytometry Systems). The data were analysed by WinMID software.

Immunoblotting analysis of caspase-3

L-1210 cell lines were incubated in the presence of the chalcone **8** (100 μM) for 6, 9 and 12 h. Cells were harvested and lysed in the presence of protease and phosphatase inhibitors.

The protein content of cell extracts was determined by Bradford's method.[13] Cell lysates were denatured in Laemmli's sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) and heated in a boiling water bath for 5 min. Samples (50 μg total protein) were resolved by 12% SDS-PAGE[14] and proteins were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, San Francisco, USA). Membranes were blocked with Tween-TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween-20) containing 1% bovine serum albumin and probed with the specific primary antibody: rabbit anti-caspase-3 (1 : 1000; Cell Signaling Technology, Danvers, USA). After extensive washing in Tween-TBS, nitrocellulose sheets were incubated with HPN-conjugated anti-rabbit IgG antibody (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, USA) for 1 h. The blots were visualized by chemiluminescence using enhanced chemifluorescence (ECF) substrate (Amersham Pharmacia Biotech, Amersham, UK).

Statistical analysis

Statistical significance was assessed by the Kruskal–Wallis test followed by post-hoc analysis; $P < 0.05$ was considered statistically significant. Cell cycle data were analysed by Mann–Whitney U-test ($P < 0.05$).

Results

Synthesis

Ten naphthylchalcones were prepared by aldolic condensation between the 2-naphthaldehyde and the corresponding acetophenones (Figure 1). All structures have been previously published by us.[10,11] The chalcone **8**, (2*E*)-3-(2-naphtyl)-1-(3′-methoxy-4′-hydroxy-phenyl)-2-propen-1-one, was obtained by condensation of 2-naphtadehyde and 4-hydoxy-3-methoxy-acetophenone, and purified with a yield of 39%.

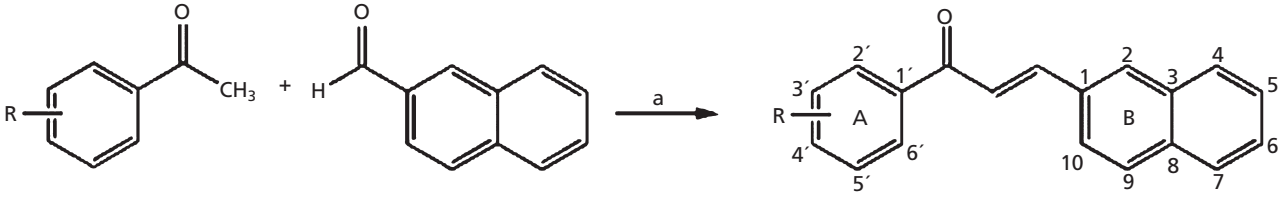
Cytotoxic effect

Initially, the cytotoxicity of different concentrations of naphthylchalcones toward murine L-1210 lymphoblastic leukaemia cells was determined through cell viability using the MTT assay.[12] After incubation of L-1210 cells with increasing concentrations (10–100 μM) of the chalcones for 24 h, only **1**, **8** and **10** reduced the percentage of viable cells in a concentration-dependent manner, when compared with the control (non-treated cells). A high level of cytotoxicity was observed at a concentration of 100 μM, and the percentage of viable cells was 62 ± 4.2%, 39% ± 2.0% and 54 ± 4.0%, respectively, for chalcones **1**, **8** and **10** (Figure 2). No expressive effect was observed for any of the other chalcones. Chalcone **8** was chosen for further study because it caused significant cell death at all concentrations tested (Figure 2c).

For treatment of L-1210 cells with 100 μM of compound **8** for 0, 12, 24 and 48 h, the greatest cell death was observed after 48 h (Figure 3), showing that the chalcone cytotoxic effect occurred in a time- and concentration-dependent manner with an IC50 of 54 μM. Interestingly, the same concentrations that were cytotoxic to leukaemia cells (L-1210) were ineffective against normal human lymphocytes (results not shown). Chalcones **1** and **10** showed IC50 values higher than 100 μM (results not shown).

Effect on cell cycle

The effect of chalcone **8** (100 μM) on the cell cycle distribution of L-1210 murine cells at 24 h was studied by DNA content analysis through flow cytometry (Figures 4a and 4b). The data listed in Figure 4c were obtained by separation of cell cycle phases by setting adjacent cursors without decon-



1 R: 2′-OH-4′,6′-diOCH_3
2 R: 4′--Br
3 R: H
4 R: 2′-OH
5 R: 4′-OCH_3
6 R: 4′-NO_2
7 R: 3′-NO_2
8 R: 3′-OCH_3-4′-OH
9 R: 3′,4′-diOCH_3
10 R: 2′,5′-diOCH_3

Figure 1 Synthesis of naphthylchalcone. Reagents and conditions: a, 50% KOH v/v and methanol, magnestic agitation at room temperature for 24 h.

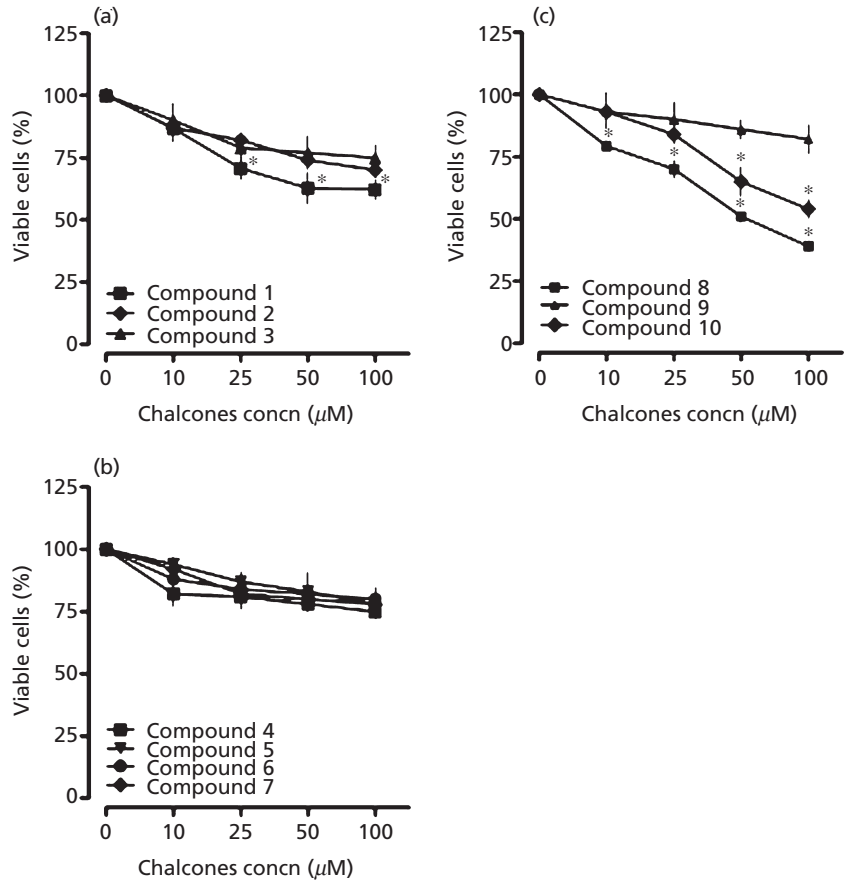


Figure 2 Toxicity of naphthylchalcones towards murine L01210 lymphoblastic leukaemia cells. The chalcones (100 μM) were incubated with the cells (1×10^5) for 24 h. The cell viability was monitored through the MTT assay. Optical density of control groups (c) was taken as 100% of cell viability. The cell viability was checked at the beginning of the experiment by Trypan Blue exclusion. The resultsare the mean ± SEM of at least three independent experiments. $^*P < 0.05$ compared with control group (Kruskal–Wallis followed by post-hoc analysis).

volution of overlapping G0/G1, S and G2/M phases. In comparison with the control group (without treatment), treated cells showed a significant increase in the proportion of cells in the subG0/G1 (45.59%) and G2/M (38.15%) phases, accompanied by a significant decrease in the G0/G1 (42.05%) and S (19.80%) phases (Figure 4c).

Apoptotic effect

To evaluate whether compound **8** induced cell death via an apoptosis or necrosis pathway, the cells were treated with the chalcone (100 μM) for 6 h and then marked with Annexin V-FITC and propidium iodide (PI) and analysed by flow cytometry. This double-staining method allowed live non-apoptotic cells (Annexin $V^{negative}/PI^{negative}$) to be distinguished from early apoptotic cells (Annexin $V^{positive}/PI^{negative}$) and late apoptotic cells (Annexin $V^{positive}/PI^{positive}$).[15,16] As illustrated in Figure 5, chalcone **8** induced cell death via an apoptosis pathway, bringing about a significant increase in the percentage of annexin $V^{positive}$ cells (23.5%) when compared with the control group (7.0%).

Effect on apoptotic signalling pathway

To investigate the mechanisms whereby compound **8** induces apoptosis, the levels of Bcl-2, Bax, p53 and activated caspase

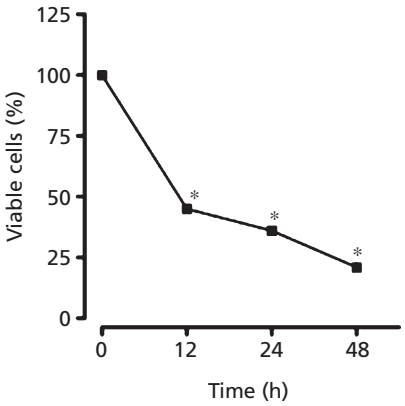
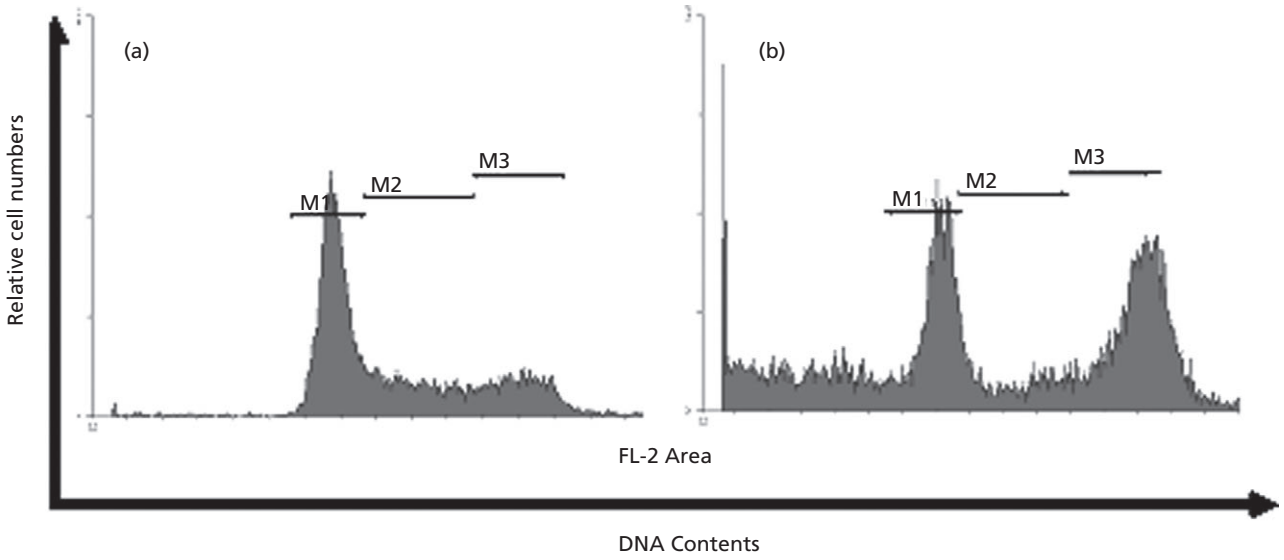


Figure 3 Cytotoxic effect of chalcone **8** on murine acute lymphoid leukaemia cells (L-1210). The cytotoxic effect of chalcone (100 μM) on L-1210 cells was determined. The cells were incubated for 0, 12, 24 and 48 h. The cell viability was assessed by the MTT colorimetric method. The optical density of the control group corresponds to 100% of cell viability. The results are the mean ± SEM of at least three independent experiments. $^*P < 0.05$ compared with control (Kruskal–Wallis ($P = 0.0156$) followed by post-hoc analysis).



(c)

	G0/G1	S	G2/M	Apoptosis (Sub G0/G1)
Control (% ± SEM)	54.23	24.85	20.91	8.21
	±0,15	±0,59	±0,40	±0,94
Chalcone 8 (100μM) (% ± SEM)	42.05*	19.80*	38.15*	45.59*
	±1,40	±0,20	±0,85	±2,00

Figure 4 Effect of chalcone **8** on the cell cycle of murine acute lymphoid leukaemia cells (L-1210). L-1210 cells were incubated in the presence or absence of 100 μM chalcone and the percentage of cells in each phase of the cell cycle was examined through DNA content. (a, b) The phases of the cell cycle are well represented: cells in apoptosis (Sub-G0/G1), cells in the G0/G1 phase (M1), cells in the S phase (M2) and cells at G2/M (M3). (a) Cells treated for 24 h in the absence of chalcone. (b) Cells treated for 24 h in the presence of chalcone. This figure is representative of three independent experiments. (c) Mean (% ± SEM) in each phase of the cell cycle. *$P < 0.05$ compared with control (Mann–Whitney *U*-test).

3 were assessed. For this study, the cells were pretreated with chalcone (100 μM) and analyzed after 24 h for Bcl-2, Bax and p53 and after 6, 9 and 12 h for caspase 3. Incubation with the compound significantly increased the expression of Bax, p53 and cleaved caspase 3 (Figure 6b and 6c, Figure 7).

Discussion

In the last few decades many pharmacological properties have been attributed to chalcones and their derivatives, due to their antioxidant,[17] anti-angiogenic,[18] anti-metastatic,[19] antiproliferative and cytotoxic activity.[27] Although many studies have demonstrated an important in-vitro cytotoxic effect of chalcones on tumour cells,[1–5] the exact mechanisms by which these compounds exert their cytotoxicity remain unclear.

In this study, the cytotoxic activity of 10 naphthylchalcones toward murine L-1210 lymphoblastic leukaemia cells was evaluated. Compounds **1**, **8** and **10** significantly reduced the viable cells at a concentration of 100 μM (Figure 2).

In general, chalcones that have hydroxyl substituents are more potent antioxidants and have a stronger cytotoxic effect on tumour cells.[17] Studies have shown that phloretin, a tetrahydroxychalcone, induced cell death through apoptosis in HL-60 human leukaemic cells[20] and isoliquiritigenin induced apoptosis and blocked the cell cycle progression in human hepatoma cells (Hep G2).[21] In our study, the chalcones that had a hydroxyl group at the A-ring demonstrated a cytotoxic effect on L-1210 cells (except compound **4**).

Chalcones with a 2-naphthyl group as the B-ring have also been identified as potent anti-angiogenic and anti-tumour compounds in an endothelial SRV cell line.[22] Our results for the 2-naphthaldehyde derivative, chalcone **8** ((2*E*)-3-(2-naphthyl)-1-(3′-methoxy-4′-hydroxy-phenyl)-2-propen-1-one), are in agreement with this since it showed a strong cytotoxic effect in a concentration- and time-dependent manner on leukaemic L-1210 cells (IC50 54 μM), as shown in Figure 3a and 3b. Besides, chalcone **8** did not have a cytotoxic effect in normal human lymphocytes (results not shown).

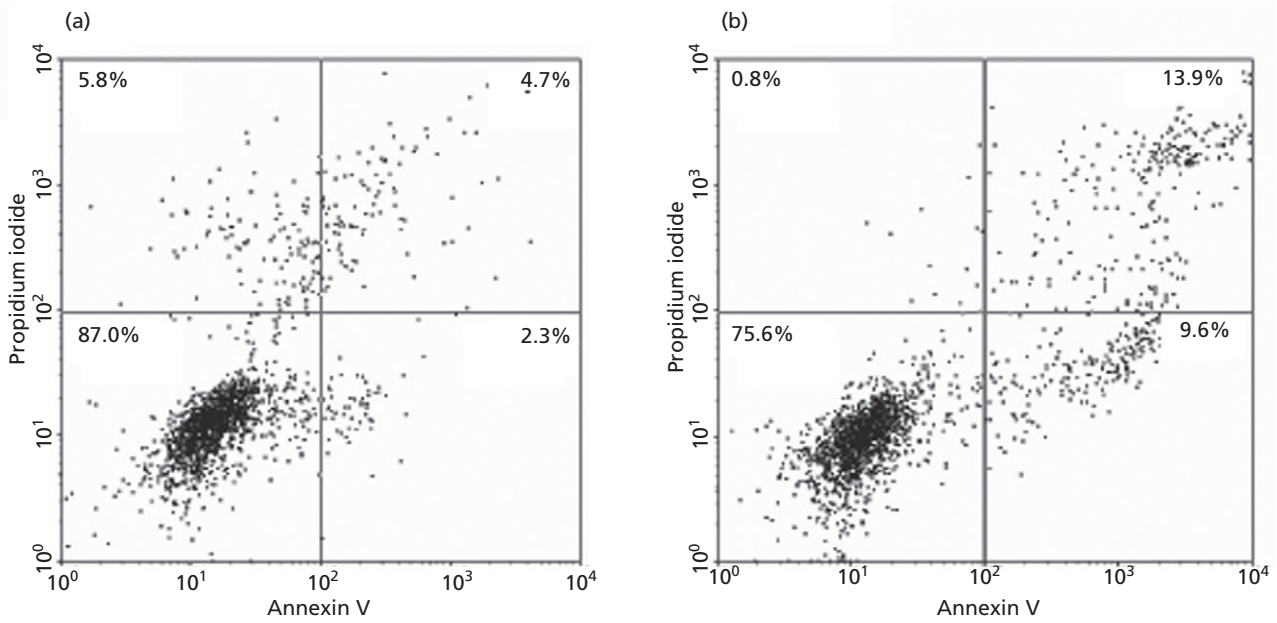


Figure 5 Induction of apoptosis by chalcone **8** on murine acute lymphoid leukaemia cells (L-1210). (a) L-1210 cells in DMEM medium without treatment. (b) L-1210 cells were incubated with chalcone (100 μM) for 6 h. Apoptosis was dermined by analysis of tagging cells with Annexin V-FITC/PI by flow cytometry. The results are the means ± SEM of at least three independent experiments. This figure is representative of three independent experiments. Lower left quadrant: Annexin $V^{negative}/PI^{negative}$. Lower right quadrant: Annexin $V^{positive}/PI^{negative}$. Upper left quadrant: Annexin $V^{negative}/PI^{positive}$. Upper right quadrant: Annexin $V^{positive}/PI^{positive}$.

To gain a better insight into the mechanism of cytotoxicity induced by compound **8**, its effects on cell proliferation and apoptosis were evaluated.

Disorders in the cell cycle and regulatory proteins are frequently related to the development of neoplasias. Thus, knowledge of how chalcones affect these mechanisms is important for the development of new antineoplasic drugs. Nakatani *et al*. have demonstrated that C-benzylated dihydrochalcones cause cell cycle arrest in the G1 phase, the activation of caspase 3 and DNA fragmentation in HL-60 promyelocytic leukaemia cells.[23] Other studies have shown that chalcone derivatives induce cell cycle blockage in the G2/M phase in different tumour cell types.[24–26] In the same way, the compound **8** tested here caused cell cycle blockage in the G2/M phase.

Cell cycle arrest activates many DNA repair mechanisms[27,28] and if the DNA repair is impossible, the pro-apoptotic protein p53 induces the cell to apoptosis.[29–32] The cells are normally able to repair genomic damage although this is dependent on the extent of such damage. Sometimes, the DNA damage causes ATP depletion or circumvents the activation of the caspase cascade, impairing the cell death by apoptosis and, consequently, the cell progresses to necrosis.[33] The process of necrosis is related to the cytoplasmic content, extravasation and local inflammatory reaction. For this reason, there is great interest in new drugs that can induce tumour cells to apoptosis. As can be observed in Figures 4 and 5, chalcone **8** induces apoptosis of L-1210 cells, which can be confirmed by the sub G0/G1 phase (45.59%) of the cell cycle. The interruption of the cell cycle and the apoptosis caused by chalcones and their derivatives have been attributed to different mechanisms, such as tubulin polymerization inhibition,[26] p21 and p27 induction,[25,26] inhibition of cyclin B_1, cyclin A, Cdc_1[26] or cyclin D_1, cyclin E and cdks 2, 4 and 6 expression.[24]

The current understanding of the apoptosis signal transduction of chalcones and their derivatives is limited and perhaps each compound acts in a different way. While some authors have demonstrated that these compounds induce an increase in pro-apoptotic proteins, such as Bax and Bak, and a decrease in anti-apoptotic proteins, such as Bcl-2 and Bcl-xL,[24,25] others have concluded that only the Bcl-2 protein expression is reduced.[34] These findings suggest that the cytotoxic effect of chalcones is related to their structure and the cell type studied. For a better comprehension of which mechanism induces apoptosis, some molecular events involved in the apoptosis pathways were investigated. Since the p53 protein is mainly responsible for the first cell cycle checkpoint in the G1/S phase, the study began with this protein. When compared with the control group, chalcone **8** clearly caused an increase in p53 expression (Figure 6).

The decrease in the expression of pro-apoptotic members of Bcl-2 family, or overexpression of anti-apoptotic members of Bcl-2 family, is associated with enhanced oncogeneic potential and poor response rates to chemotherapy.[35–37] The members of the Bcl-2 family regulate the initiation of the mitochondrial apoptotic pathway. These proteins regulate cytochrome C release to the cytoplasm, which causes activation of caspase 9 and, consequently, the activation of downstream effector caspases.[38–41] In our study, Bcl-2 expression did not alter significantly, but an increase in Bax protein expression was observed in the

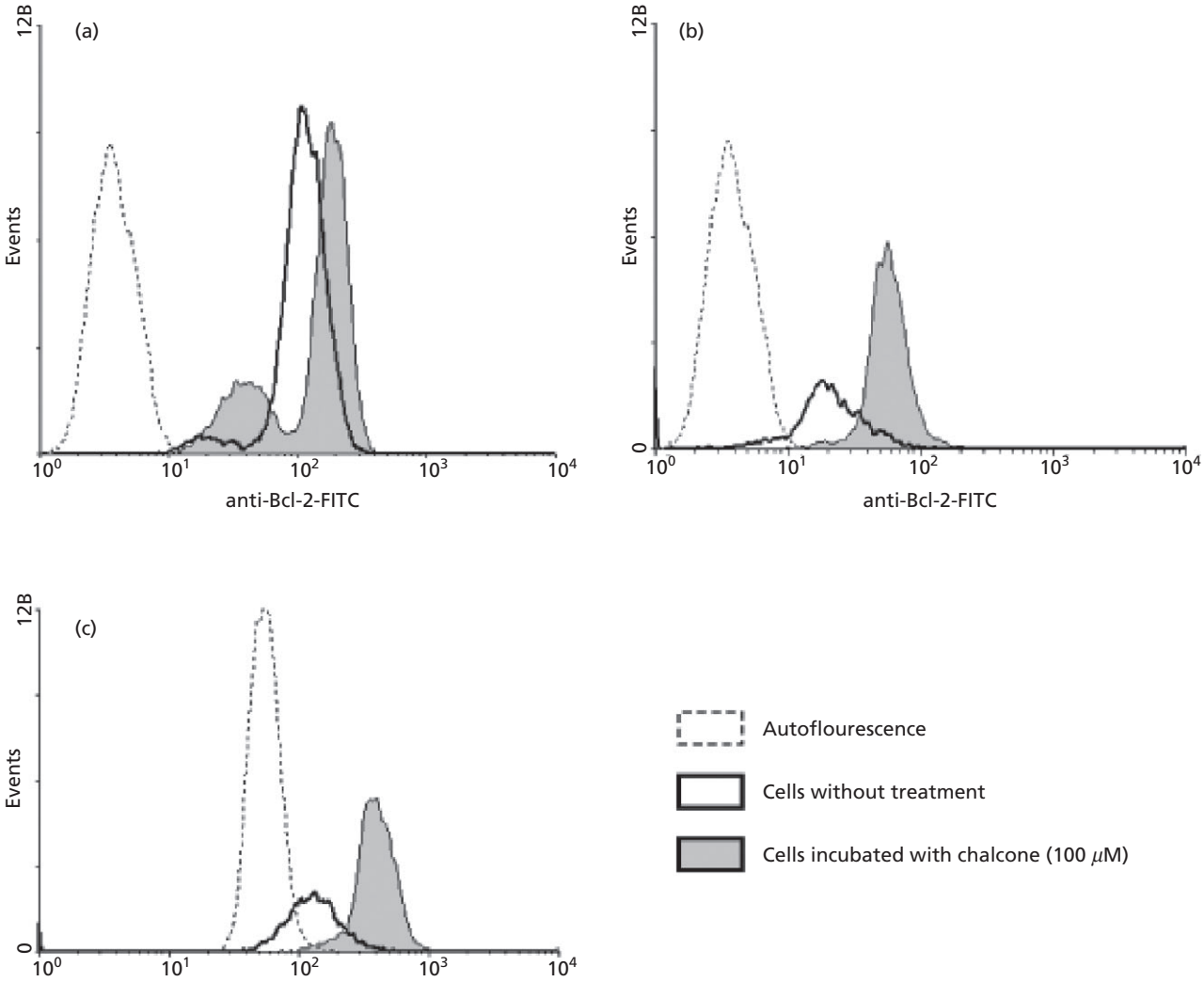


Figure 6 Effect of chalcone **8** on apoptotic signalling pathyway in murine acute lymphoid leukaemia cells (L-1210). L-1210 cells were incubated in the presence or absence of chalcone 100 μM and marked with anti-Bcl-2-FITC, anti-Bax-FITC or anti-p53-PE for the detection of Bcl-2, Bax and p53, respectively. (a) Expression of Bcl-2 anti-apoptotic protein. (b) Expression of Bax pro-apoptotic protein. (c) Expression of p53. This figure is representative of three independent experiments.

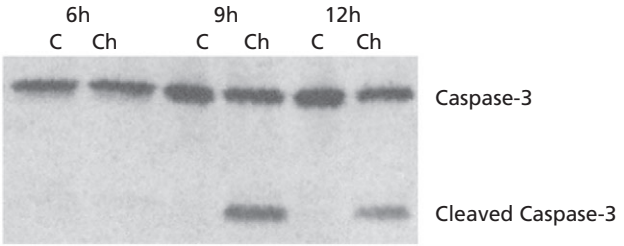


Figure 7 Effect of chalcone **8** on caspase-3 protein expression and its activity in murine acute lymphoid leukaemia cells (L-1210). L-1210 cells were incubated in the presence of absence of chalcone (100 μM) for 6, 9 and 12 h. Cells were immunolabelled with monoclonal rabbit anti-caspase-3, anti-rabbit IgG antibody, HPN-conjugated, and visualised by chemiluminescence using an enhanced chemifluorescence (ECF) substrate. This figure is representative of three independent experiments. C, control; Ch, chalcone.

L-1210 cells treated with compound **8** (Figure 6c), suggesting that it may have been mediated by p53. This change in the Bax : Bcl-2 ratio facilitates cell death by apoptosis.[42] Our findings corroborate this statement, since the tested chalcone **8** caused an increase in p53 and Bax expression (Figure 6b and 6c). Furthermore, it is known that the disequilibrium between pro- and anti-apoptotic proteins leads to cytochrome C release and, consequently, caspase 3 activation (Figure 7).

Conclusion

Together, these results show that chalcone 8, (2*E*)-3-(2-naphthyl)-1-(3′-methoxy-4′-hydroxy-phenyl)-2-propen-1-one, induces apoptosis in L-1210 cell line by cell cycle arrest in G2/M phase. In addition, it also increases Bax and p53 expression and caspase 3 activation.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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