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Involvement of extrinsic and intrinsic apoptotic pathways together with endoplasmic reticulum stress in cell death induced by naphthylchalcones in a leukemic cell line: Advantages of multi-target action

Evelyn Winter [a], Louise Domeneghini Chiaradia [b], Adny Henrique Silva [a], Ricardo José Nunes [b], Rosendo Augusto Yunes [b], Tânia Beatriz Creczynski-Pasa [a,*]

[a] Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina, 88040-900 Florianópolis, SC, Brazil
[b] Departamento de Química, Universidade Federal de Santa Catarina, 88040-900 Florianópolis, SC, Brazil

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ABSTRACT

Chalcones, naturally occurring open-chain flavonoids abundant in plants, have demonstrated anticancer activity in multiple tumor cells. In a previous work, the potential anticancer activity of three naphthylchalcones named R7, R13 and R15 was shown. In this study, the mechanism of actions of these chalcones was originally shown. The chalcones presented concentration and time-dependent cytotoxicity. To determine the type of cell death induced by chalcones, we assessed a series of assays including measurements of the caspase-8, -9 and -12 activities, expression of important apoptosis-related genes and proteins, changes in the cell calcium concentration and cytochrome c release. The activities of caspase-8, -9 and -12 increased after the treatment of L1210 cells with the three compounds. Chalcones R7 and R13 induced an increase of pro-apoptotic proteins Bax, Bid and Bak (only chalcone R13), as well as a decrease in anti-apoptotic Bcl-2 expression. These chalcones also induced an increase in Fas and a decrease in p21 and p53 expression. Chalcone R15 seems to act by a different mechanism to promote cell death, as it did not change the mitochondrion-related proteins, nor did it induce the cytochrome c release. All compounds induced an increase in cell calcium concentration and an increase in CHOP expression, which together with an increase in caspase-12 activity, suggest that chalcones could induce an endoplasmic reticulum (ER) stress. Taken together, these results suggest that chalcones induce apoptosis by different pathways, being an interesting strategy to suggest for cancer therapy.

1. Introduction

Cancer is a leading cause of death worldwide and the World Health Organization estimates that by 2030 there will be 13 million cancer related deaths. Acute lymphoblastic leukemia (ALL) is a malignant disorder that can originate from one single hematopoietic precursor committed to the B- or the T-cell lineage. Acquisition by the precursor of a series of genetic abnormalities in its normal maturation process can cause differentiation arrest and proliferation of the immature cell (Graux, 2011). ALL has a peak prevalence between the ages of two and five years, and is the most common form of childhood cancer (Faderl et al., 2003).

Childhood ALL can be successfully treated with multiple-agent chemotherapy, but many patients still develop serious acute and/or late complications due to the side effects of the drugs. Studies have shown that the risk of death from cardiac causes or other forms of cancer is higher in ALL survivors (Diller, 2011; Faderl et al., 2003; Mertens et al., 2008; Reulen et al., 2010). In addition, children with ALL are at risk of low bone mineral density as a result of the high doses of glucocorticoids and intrathecal methotrexate included in almost every ALL treatments (Kaste et al., 2006; Thomas et al., 2008).

Aberrant regulation of apoptosis mechanisms is an important pathological factor in a variety of major human diseases. Failure to appropriately engage this pathway is one of the hallmarks of cancer development, and many cancer cells exhibit significant resistance to apoptosis signaling (Sayers, 2011).

In mammalian cells, apoptosis occurs through two distinct molecular pathways, which are regulated by caspases. The intrinsic or mitochondrial pathway is activated by intracellular events, and depends on the release of pro-apoptotic and anti-apoptotic factors

* Corresponding author. Tel.: +55 48 3721 2212; fax: +55 48 3721 9542.
E-mail addresses: tania.pasa@ufsc.br, taniabcp@gmail.com (T.B. Creczynski-Pasa).

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from the mitochondria, such as the Bcl-2 family proteins, cytochrome c and APAF-1, among others. The extrinsic pathway is initiated by the binding of an extracellular death ligand to its cell-surface death receptor (Kiechle and Zhang, 2002; Riedl and Shi, 2004). The extrinsic pathway can crosstalk to the intrinsic pathway through the caspase-8-mediated cleavage of BID (a member of the Bcl2 family of proteins) (Billen et al., 2008).

The endoplasmic reticulum (ER) is an essential intracellular organelle, and in conjunction with Ca^{2+}, plays a vital role in the synthesis, folding and post-translational modification of proteins. If the ER becomes overwhelmed, either due to a problem with protein folding or by an overproduction of proteins, the cell triggers a specific ER stress response mostly resulting in apoptosis through caspase-12 activation (Berridge, 2002; Groenendyk and Michalak, 2005; Sitia and Braakman, 2003).

Some authors have concluded that because of the alterations' heterogeneity found in leukemia, new apoptosis-inductive agents are required, for better efficacy and side effects reduction (Debatin, 2004; Nicholson, 2000; Zhang et al., 2008).

Natural products are the source of most of the active compounds in medicines. More than 80% of drugs are obtained from natural sources or are based on natural compounds (Harvey, 2008). Chalcones are essential intermediate compounds in flavonoid biosynthesis in plants. Many studies have demonstrated anti-leukemic (Orlikova et al., 2011; Pedrini et al., 2010; Winter et al., 2010), anti-angiogenic (Kim et al., 2010), anti-infective (Nowakowska et al., 2008) and hypotensive (Ogawa et al., 2007) activities for chalcones, among other pharmacological effects.

In a previous work we demonstrated the general effects of naphthylchalcones R7, R13 and R15 (Fig. 1) on a lymphoblastic leukemia cell line, by monitoring the oxidative stress, and their influence on energetic metabolism (Winter et al., 2010). In this study it is being reported the molecular targets, using gene and protein expression, caspase activities, calcium concentration and cytochrome c release evaluation, Chalcones R7 and R13 triggered apoptosis by intrinsic and extrinsic pathways. Moreover, they induced endoplasmic reticulum stress triggered by changes in intracellular calcium concentration. The apoptosis induced by Chalcone R15 did not seem to be related to the mitochondrion, and was triggered by extrinsic pathway and ER stress.

2. Materials and methods

2.1. Chemicals

The cell culture medium was purchased from Cultilab (São Paulo, SP). Serum and antibiotics were purchased from GIBCO (Grand Island, NY). The ApopNexin™ FITC Apoptosis Detection Kit was purchased from Millipore (Billerica, MA); the DNase I, TRIZOL reagent and the primers were purchased from Invitrogen (Carlsbad, CA); the High-Capacity cDNA Reverse Transcription Kit and the Power SYBR-Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA); the fluorogenic substrate caspase-12 was purchased from Biovision (Milpitas, CA); the Fluo-3AM was purchased from Biovision (San Francisco, CA); the phycoerythrin-conjugated monoclonal antibodies were purchased from BD (Becton Dickinson, Franklin Lakes, NJ) and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Naphthylchalcones R7, R13 and R15 were synthesized as previously described (Winter et al., 2010).

2.2. Cell culture and treatments

Murine L1210 lymphoblastic leukemia cells, murine fibroblast (NIH/3T3) and monkey kidney cells (VERO) were obtained from the American Type Culture Cell (ATCC). L1210 cells were cultured in RPMI 1640 medium and NIH/3T3 and VERO cells were cultured in DMEM medium, both supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES. The cell culture was maintained at 37 °C in a 5% CO_2 humidified atmosphere and pH 7.4. Every two days, cells were passaged by removing 90% of the supernatant and replacing it with fresh medium. In all experiments, viable cells were checked at the beginning of the experiment by Trypan Blue exclusion. The concentration of chalcones used in all experiments were the CC_{50} of them after 24 h of incubation (concentration of the compounds, which results in 50% of cell viability). The CC_{50} values were determined by MTT method in a previous work (Winter et al., 2010). Sigmoidal dose–response curves were fitted to plot the percentage of viable cells versus log of compounds concentrations (0–100 μM) using GraphPad Prism 5. Chalcone R7 presented a CC_{50} value of 30 μM and chalcones R13 and R15 presented a CC_{50} value of 40 μM.

2.3. Calculation of selectivity index (SI)

The selectivity index (SI) corresponds to CC_{50} of chalcones on non-tumoral cell lines (VERO and NIH/3T3) divided by CC_{50} determined for cancer cells (L1210). A SI higher than one indicate that the compound is more selective for cancer cells. The CC_{50} of chalcones in all cell lines was calculated after 24 h of incubation according to the methodology described above.

2.4. Annexin V/PI staining for cell apoptosis

Cell apoptosis was assessed by measuring membrane redistribution of phosphatidylserine using an ApopNexin™ FITC Apoptosis Detection Kit (Millipore, Billerica, MA), according to the manufacturer's protocol. Briefly, cells (1×10^6/well) were plated and treated with the CC_{50} concentration for each compound, 30 μM of chalcone R7, 40 μM of chalcones R13 and R15 or solvent control (DMSO) for 12 h, washed twice with chilled phosphate-buffered saline (PBS), resuspended in the binding buffer, and stained with staining solution containing Annexin V-FITC and PI. After incubation in the dark for 15 min, cells were analyzed by flow cytometer FACSCanto (Becton Dickinson, Franklin Lakes, NJ) and the results were analyzed using WinMDI 2.9 software.

2.5. Determination of caspase activities

To determine the activity of caspases, 1×10^7 L1210 cells were incubated with the compounds for 4 h at 37 °C. Cells were then washed with PBS and lysed with lysis buffer containing 50 mM

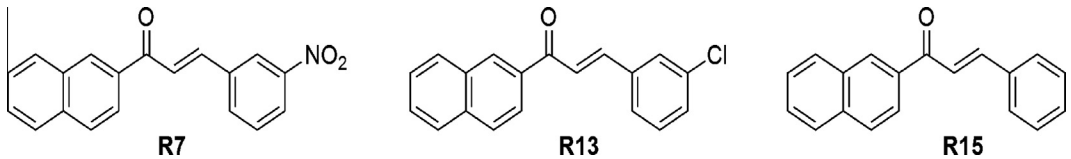

Fig. 1. Chemical structures of naphthylchalcones R7, R13 and R15.

HEPES pH 7.4, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 5 μg/ml aprotinin, 5 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and 5 mM dithiothreitol (DTT) at 4–8 °C for 10 min. An aliquot of the extract (about 100 μg of protein) was then added to a buffer containing 20 mM HEPES pH 7.4, 0.1% CHAPS, 2 mM EDTA, 5% sucrose and 5 mM DTT. The reaction medium was supplemented with 100 μM of Ac-LEHD-AFC, a fluorogenic substrate for caspase-9, or with 25 μM of Ac-IETD-AMC, a fluorogenic substrate for caspase-8, or with 25 μM of Ac-ATAD-AFC, a fluorogenic substrate for caspase-12. After incubation of the lysates with the respective substrates at 37 °C for 2 h, caspases activities were monitored using a spectrofluorimeter (Perkin Elmer LS55) by the production of fluorescent AMC or AFC. The fluorescence of blanks containing the cells without fluorescent substrates was subtracted from the sample values. Protein content was determined using a spectrofluorimeter at 280 nm for excitation and 340 nm for emission. The caspases activities were presented as a percentage taking into account the values of fluorescent units per μg of protein.

2.6. RNA extration

For RNA isolation, 3×10^6 L1210 cells were incubated with the compounds for 4 h at 37 °C. Total RNA was prepared using Trizol reagent (Invitrogen). 0.5 ml of Trizol was added to each tissue sample and homogenized. Samples were incubated for 5 min at room temperature, and then an aliquot of chloroform was added. Samples were further incubated for 2 min at room temperature and centrifuged at 12,000×*g* for 15 min at 4 °C. The upper aqueous phase was transferred to a RNase-free tube and the RNA was precipitated by adding an aliquot of isopropanol. The samples were incubated at room temperature for 10 min and centrifuged at 12,000×*g* for 10 min at 4 °C. The resulting RNA pellet was washed in 75% ethanol and resuspended in RNase-free water.

2.7. Real-Time quantitative RT-PCR

The reaction was performed on an ABI Prism 7900 (Applied Biosystems). In summary, total RNA (2 μg) was used for cDNA synthesis, according to the manufacturer's instructions for the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, EUA), using random hexamers. This cDNA was subsequently used for quantitative PCR to quantify the gene expression levels. Power SYBR-Green PCR Master Mix (Applied Biosystems) was used to employ quantitative PCR. The sequences of specific primers used for each gene studied, including the endogenous control GAPDH, are shown in Table 1. Briefly, a reaction contained SYBR GreenPCR Master Mix, forward and reverse primers (final concentration 0.15 μM) and the template cDNA. The PCR was performed using ABI 7900 at 50 °C for 2 min, at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 57 °C for 1 min. The specificity of SYBR Green assay is dependent on the specificity of the primers used in each reaction. Nonspecific amplification and primer/dimer artifacts must be minimized in order to obtain accurate results. Thus, a dissociation curve reaction for each primer pair (95 °C for 15 s, 60 °C for 20 s and 95 °C for 15 s) is required, and has to be started immediately after the completion of the real-time PCR run. The gene-specific product was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and quantified using the comparative (ΔCt) Ct method as previously described (Livak and Schmittgen, 2001).

2.8. Measurement of proteins expression by flow cytometry

The expression levels of the proteins Bcl-2, p53 and Fas were analyzed by flow cytometry (Becton Dickinson FACSCantoII). In brief, cells were incubated with the chalcones for 4 and 12 h, washed with PBS and permeabilized in the BD Cytofix/Cytoperm™ Buffer (Becton Dickinson, Franklin Lakes, NJ) for 30 min at 4 °C. The cells were washed and resuspended in BD Perm/Wash™ buffer containing the phycoerythrin-conjugated monoclonal antibodies. Analysis of all the antibodies was carried out separately. After 30 min of incubation at 4 °C, the cells were washed and analyzed. Background fluorescence was measured by using an immunoglobulin isotype control antibody. For the Fas antibody, the first stage of permeabilization was not performed because this protein is located in the cytoplasmic membrane.

2.9. Measurement of intracellular calcium concentration

The changes in intracellular Ca^{2+} concentration were determined by the fluorescent dye, Fluo-3AM (Biovision, San Francisco, CA). Cells were incubated with chalcones or with A23187 (1 μM), a calcium ionophore used as positive control, at 37 °C for 4 h. In summary, the cell culture medium was replaced and the cells were incubated at 37 °C for 20 min with a fresh medium containing 3 μM Fluo-3 and 0.02% of Pluronic F127. Four times the volume of HBSS with 1% of fetal bovine serum was then added, and incubated for a further 40 min. Cells were then washed and analyzed using a spectrofluorimeter. Protein content was determined using a spectrofluorimeter at 280 nm for excitation and 340 nm for emission. Concentration of intracellular calcium was expressed as a percentage taking into account the values of fluorescent units per μg of protein.

2.10. Cell cycle analysis

L1210 cells (1×10^6) were treated with chalcones or Paclitaxel (1 μM) for 12 h. Cells were washed with cold PBS and resuspended in 70% ice ethanol to fix them. After 30 min, PBS with 2% bovine serum albumin was added. The cell pellets were collected by centrifugation, resuspended in hypotonic buffer (0.1% Triton X-100 in

Table 1
The Sequence of specific primers for each gene studied including the endogenous control (GAPDH[a]).

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)
p21	CTGTCTTGCACTCTGGTGTCT	GGCACTTCAGGGTTTTCTCT	149
p53	GTAAACGCTTCGAGATGTTCC	GACTGGCCCTTCTTGGTCT	123
Fas	TCTGCGATGAAGAGCATGGTT	GCAGCGAACACAGTGTTCACA	121
Bid	GAGATGGACCACAACATCCA	AGGCTGTCTTCACCTCATCAA	126
Bax	ACAGGGGCCTTTTTGCTAC	GAGACACTCGCTCAGCTTCTT	125
Bak	TACCTCCACCAGCAGGAAC	GACCCACCTGACCCAAGA	125
Bcl2	AGAGACTCACCAGGGTCTGC	GCACTACCTGCGTTCTCCTC	113
GAPDH	GTGTCCGTCGTGGATCTGAC	GGAGACAACCTGGTCCTCAG	132
CHOP	GGAAGTGCATCTTCATACACCACC	TGACTGGAATCTGGAGAGCGAGGGC	315

[a] GAPDH = Glyceraldehyde-3-phosphate dehydrogenase.

PBS and 100 μg/ml RNase A) with PI (20 mg/ml). Fluorescence emitted from the PI–DNA complex was quantified by flow cytometry (FACSCanto, Becton Dickinson), and the results were analyzed using WinMDI 2.9 software.

2.11. Determination of cytochrome c release

L1210 cells (5×10^6) were treated with chalcones for 4 h and then washed with PBS. Cells were harvested and permeabilized with 30 μmol/l digitonin for 30 min at 4 °C in a solution containing 10 mg/ml Tris–HCl (pH 7.0), 3 mg/ml ethylene glycol, tetra-acetic acid (EGTA) and 50 μg/ml sucrose. The cells were centrifuged at 10,000×*g* for 30 min. Cytochrome c release was evaluated by the method described by Appaix et al. (2000). The absorbance of the supernatants was recorded at 414 nm, using the medium as a blank.

2.12. Statistical analysis

The results were presented as means ± standard error of mean (SEM) of triplicates from at least three-independent experiments. Statistical significance was assessed by ANOVA followed by Dunnett's test, and a p value less than 0.05 was considered significant.

3. Results

3.1. Chalcones induced cytotoxicity and cell death by apoptosis

The potential cytotoxicity of naphthylchalcones R7, R13, and R15 (Fig. 1) in L1210 cells was shown in a previous study by our group (Winter et al., 2010). These chalcones induced concentration and time-dependent cytotoxicity with CC_{50} values after 24 h of incubation of 30 μM for R7 and 40 μM for R13 and R15. To characterize the cell death induced by the compounds, flow cytometry analysis with Annexin V/PI staining was employed. Following FACS, fluorescence of PI was plotted over Annexin V-FITC fluorescence (Fig. 2). As shown in Fig. 2a, healthy cells presented low FITC fluorescence and low PI fluorescence (Q1); early apoptotic cells presented high FITC fluorescence but low PI fluorescence (Q2); late apoptotic cells presented high FITC fluorescence and high PI fluorescence (Q3); necrotic cells presented low FITC fluorescence but high PI fluorescence (Q4). As shown in Fig. 2b, the percentage of apoptotic cells (Q2 + Q3) induced by the chalcones was significantly higher than the percentage of necrotic cells (Q4).

3.2. Chalcones selectivity

Chalcones presented a selectivity index (SI) higher than one indicating that chalcones present higher selectivity to leukemic cells when compared to non-tumoral cells (VERO and NIH/3T3). The only exception was chalcone R15 that presented the same toxicity in L1210 and VERO cells. For a better evaluation of chalcones toxicity, *in vivo* studies should be performed (see Table 2).

3.3. Chalcones induced apoptosis via an activated caspases-dependent pathway

Initiators caspases 8 and 9 are closely related to apoptotic signals. Once activated, these caspases cleave and activate downstream effector caspases (including 3, 6, and 7), which in turn cleave cytoskeletal and nuclear proteins and finally induce apoptosis (Riedl and Shi, 2004). Treatment of leukemic cells with chalcones for 4 h resulted in an increase in caspases 3, 8 and 9 activities (Fig. 3).

3.4. Chalcones induced gene expression alterations

The effect of the three chalcones on the expression of apoptosis-related genes such as the death receptor Fas, p21, p53, family-Bcl2 proteins like Bid, Bcl2, Bax and Bak and the transcription factor CHOP involved in endoplasmic reticulum stress were evaluated. Chalcones R7 and R13 induced an increase in Fas, Bid and Bax expression, did not alter the Bak and p53 expression, and decreased the p21 and Bcl2 expression. Chalcone R15 showed a different profile because it induced an increase in Fas, did not alter the Bax, Bak, Bid and Bcl2 expression, and decreased the p53 and p21 expression. All chalcones induced an increase in CHOP expression (Fig. 4).

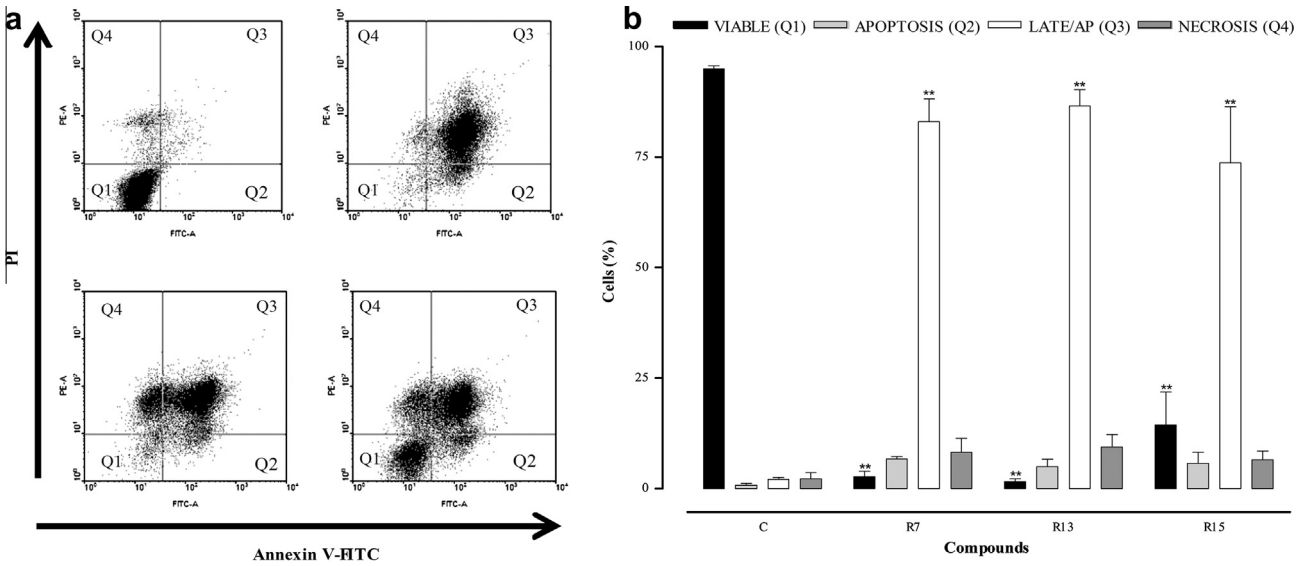

Fig. 2. Apoptosis induction in L1210 cells by chalcones. The apoptosis was measured by Annexin-V-FITC/PI) staining. L1210 cells were treated with chalcones for 12 h, stained with the dyes and analyzed by flow cytometry. The horizontal and vertical axes represent labeling with Annexin-V-FITC and PI, respectively. Q1 represents live cells, Q2 represents early apoptotic cells (positive for Annexin V only), Q3 represents late apoptotic cells (Annexin-V and PI positives) and Q4 represents necrotic cells (positive for PI only). The dot plots shown (a) are representative of one experiment, and the graph (b) shows the mean ± SEM of three independent experiments. ** $p < 0.01$ compared with the control.

Table 2
Values of selectivity index (SI) of chalcones.

	SI (VERO/L1210)	SI (NIH3T3/L1210)
R7	1.7	1.4
R13	1.9	2.2
R15	1.0	1.3

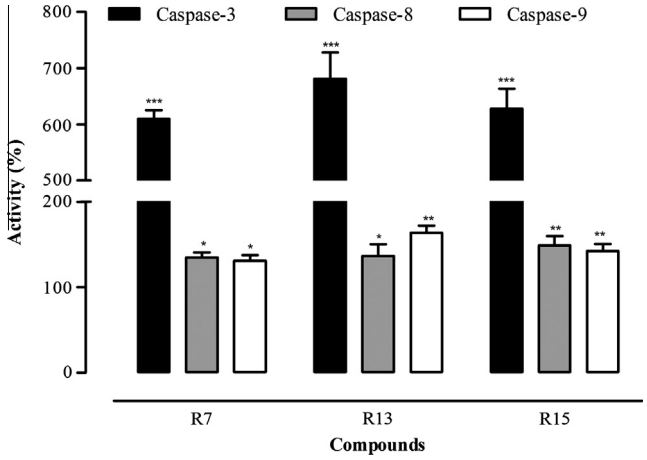

Fig. 3. Activation of caspase-3,-8 and -9 in L1210 cells by chalcones. L1210 cells were incubated with the compounds for 4 h. Caspases activities were measured by monitoring the cleavage of Ac-DEVD-AMC, a fluorogenic substrate for caspase-3, Ac-IETD-AMC, a fluorogenic substrate for caspase-8 and of Ac-LEHD-AFC a fluorogenic substrate for caspase-9. The activity is given as a percentage of the control considered 100%. * $p < 0.05$ and ** $p < 0.01$ compared with the control.

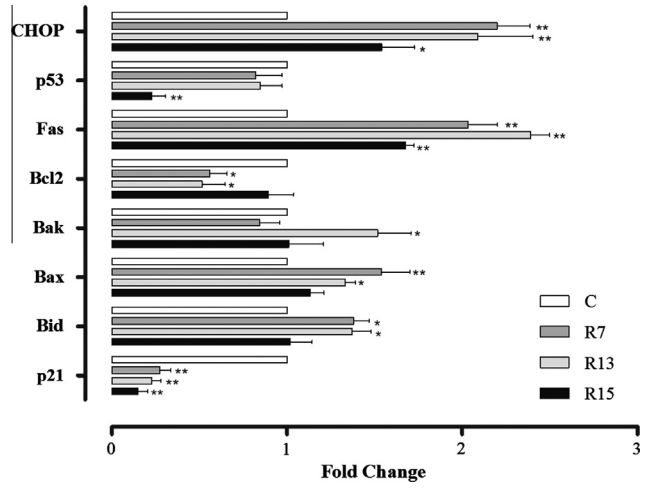

Fig. 4. Changes in expression of genes apoptosis-related induced by chalcones. Real time RT-PCR analyses of CHOP, p53, Fas, Bcl-2, Bak, Bax, Bid and p21 were performed using 2 μg total RNAs derived from cells treated with the chalcones for 4 h. GAPDH was utilized as endogenous control and the expression level of genes in untreated cells was designated as 1.0. * $p < 0.05$ and ** $p < 0.01$ compared with the control.

3.5. Chalcones induced protein expression alterations

To confirm the results obtained with the real time PCR, the proteins Bcl-2, Fas and p53 were analyzed by flow cytometry after incubation with the compounds for 4 h, 12 h and 24 h (only for Fas detection). The chalcones R7 and R13 induced a decrease and the chalcone R15 did not alter the Bcl-2 level, in agreement with the results obtained from gene expression assay (Fig. 5a). All

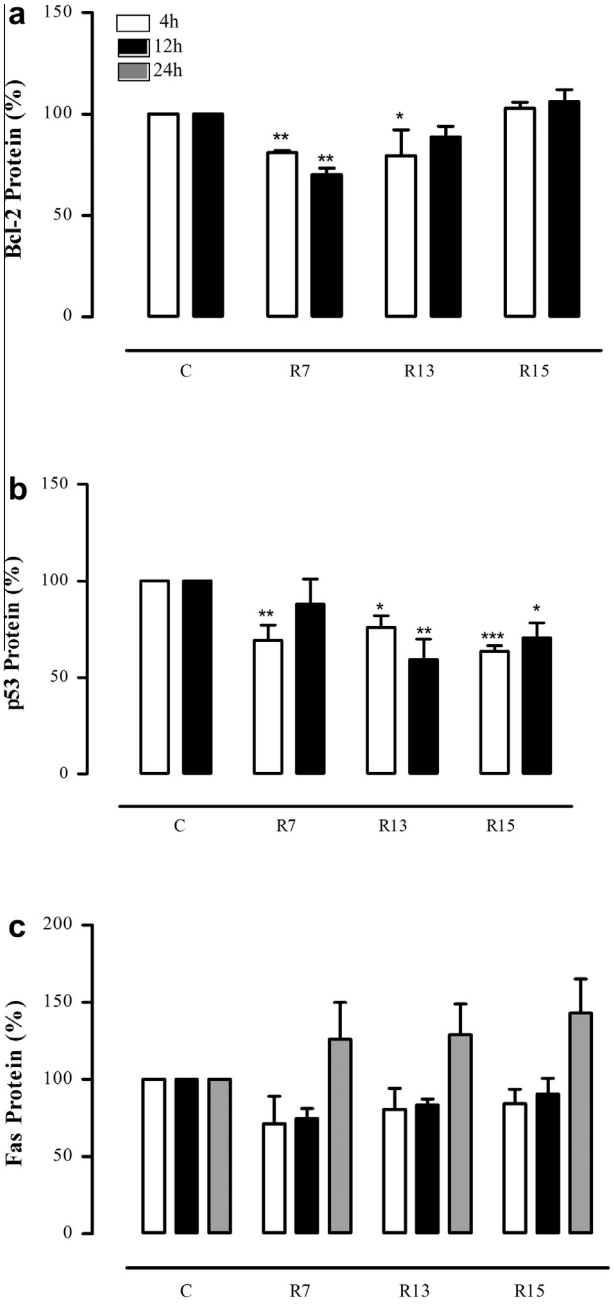

Fig. 5. Changes in expression of proteins apoptosis-related after incubation with chalcones. Cells were incubated with chalcones for 4 and 12 h. Analyses of Bcl-2 (a), p53 (b) and Fas (c) (4, 12 and 24h) performed by flow cytometry using specific antibodies. * $p < 0.05$ and ** $p < 0.01$ compared with the control.

chalcones induced a decrease in p53 protein level after 4 h of incubation, but only the R15 had the same effect after 12 h of incubation in cell culture (Fig. 5b). A decrease in p53 gene expression was also observed in the results obtained by PCR (Fig. 4). In addition, the chalcones did not induce statistically significant alterations in Fas protein expression after 4 or 12 h of incubation, however a tendency for the expression of this protein to increase was observed after 24 h of incubation (Fig. 5c).

3.6. Chalcones induced cytochrome c release from mitochondria

Mitochondrial cytochrome c, which functions as an electron carrier in the respiratory chain, translocates to the cytosol in cells undergoing apoptosis, and together with APAF-1 mediates

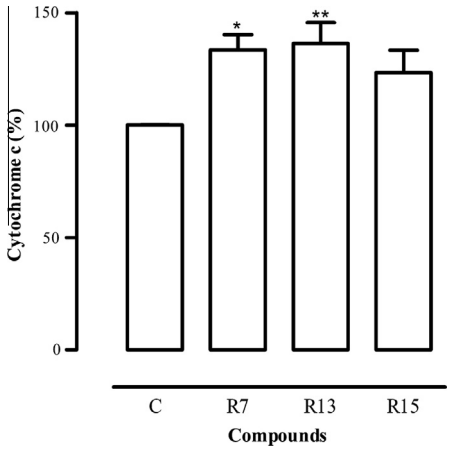

Fig. 6. Increase in cytochrome c release induced by R7 and R13. Cells were incubated with the chalcones for 4 h. Cytochrome c was measured spectrophotometrically (414 nm). The results are given as a percentage of the control. * $p < 0.05$ and ** $p < 0.01$ compared with the control.

caspase-9 activation. The apoptosis inhibitors from Bcl-2 family prevent the efflux of cytochrome c from mitochondria. A reduction in mitochondrial transmembrane potential ($\Delta\Psi m$) has been reported to accompany cytochrome c release (Coultas and Strasser, 2003). Chalcones R7 and R13 induced a statistically significant increase in cytochrome c release from mitochondria to cytoplasm (Fig. 6), which is probably related to changes in mitochondrial membrane potential ($\Delta\Psi m$) (Winter et al., 2010).

3.7. Chalcones induced calcium concentration and caspase-12 activity changes

ER stress can be induced by altering calcium concentration with or without the participation of the caspase-12, for this reason the effect of the three compounds on caspase-12 activity and on calcium intracellular changes was evaluated. The ionophore A23187 was used as a control for calcium release. All chalcones induced a large increase in intracellular calcium concentration compared to the control (Fig. 7a), and also increased the caspase-12 activity (Fig. 7b), indicating that the action of these compounds is related to ER stress.

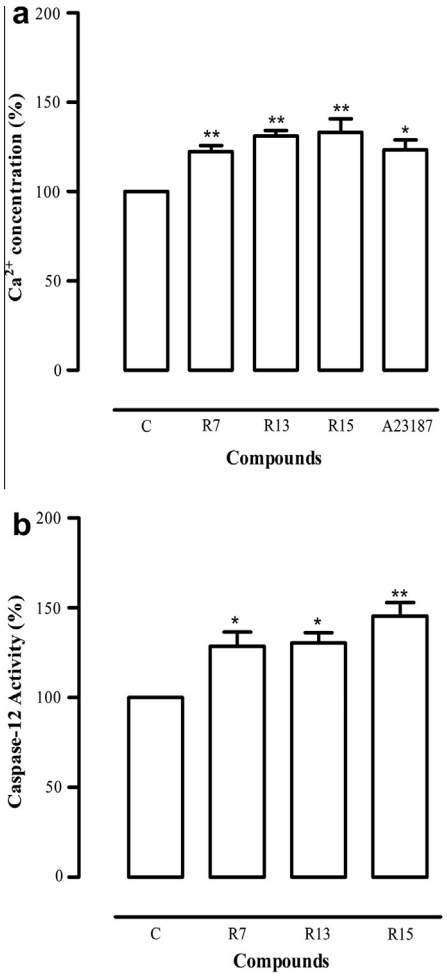

Fig. 7. Increase in caspase-12 activity and in intracellular calcium concentrations induced by chalcones. Cells were incubated with the chalcones or with A23187 (as a positive control for calcium analysis) for 4 h. Caspase-12 activity was measured by monitoring the cleavage of Ac-ATAD-AFC – a fluorogenic substrate for caspase-12. Calcium concentration was evaluated by fluorescent dye Fluo-3AM. The results are given as a percentage of the control. * $p < 0.05$ and ** $p < 0.01$ compared with the control.

3.8. Chalcones did not modify the cell cycle

To gain an insight into the mechanism of action of these naphthylchalcones, we evaluated their effect on cell cycle distribution. As can be seen in Fig. 8, exposure of L1210 cells to chalcones for 12 and 24 h did not result in the arrest of G0/G1, S or G2/M phases of the cell cycle in contrast to Paclitaxel – a mitotic inhibitor used in cancer chemotherapy, which was used as a positive control in this study. Paclitaxel induced a statistically significant enrichment of G2-M fraction after 12 and 24 h of incubation.

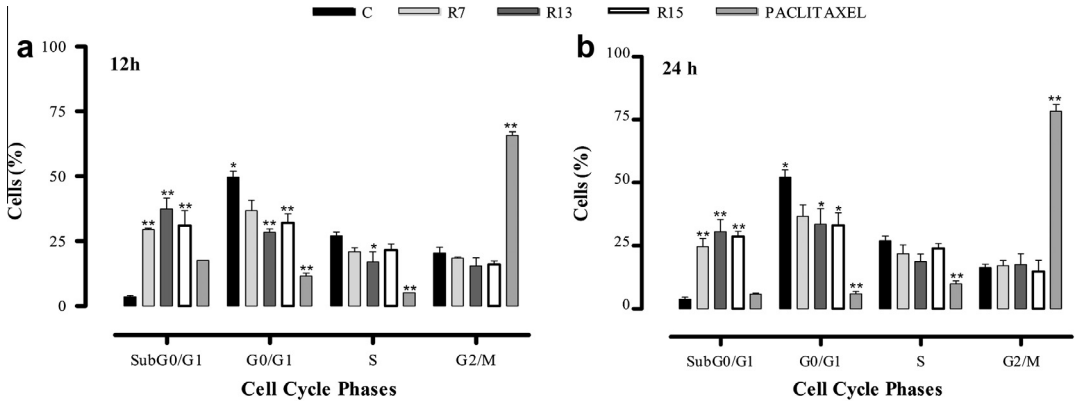

Fig. 8. Effect of chalcones on cell cycle distribution. L1210 cells were treated with chalcones and Paclitaxel for 12 (a) and 24 h (b) and stained with PI. Following flow cytometry, cellular DNA profile was further analyzed using the software WinMDI 2.9. Data represent the percentage of cell counts in each cell cycle phase. * $p < 0.05$ and ** $p < 0.01$ compared with the control.

The percentage of cells located on SubG0/G1 phase corresponds to the apoptotic cells. Chalcones R7, R13 and R17 induced an increase of cellular content in this phase, confirming the apoptosis process evaluated by other methods; Annexin V/PI staining (Fig. 2) and by caspases activities activation (Fig. 3).

4. Discussion

The development of new drugs for the treatment of cancer is based on the potential of compounds to block cell proliferation and/or induce apoptosis (Herr and Debatin, 2001). The anti-CD20 antibody Rituximab and the Bcr-Abl-selective kinase inhibitor Gleevec, respectively, are two examples of the success of targeted therapy. Targeting the apoptotic pathways of malignant cells has become an attractive concept. Apoptosis can be triggered by death ligands that bind to their death receptors (such as Fas or TRAIL) located in the cytoplasmic membrane, leading to the formation of a minimally homotrimeric ligand–receptor complex. Subsequently, this complex recruits further cytosolic factors, such as caspase-8, forming an oligomeric death-inducing signaling complex (DISC), which then cleaves and activates the effector caspase-3 (Kiechle and Zhang, 2002; Nakata et al., 2004). The death receptors, mainly TRAIL, have been shown to induce apoptosis selectively in many tumor cell lines, such as blood cancers, without affecting normal cells and tissues. A large number of human tumor cells have shown TRAIL resistance, and the TRAIL sensitization can induce the apoptosis of cancer cells (Ashkenazi and Herbst, 2008; Nakata et al., 2004). All chalcones tested induced an increase in caspase-8 activity (Fig. 3) and Fas expression (Fig. 4), and may therefore represent an important strategy for the treatment of TRAIL resistant cancer cells.

Apoptosis can be triggered in response to a wide range of death stimuli that are generated from the inside of cells, such as oncogene activation and DNA damage. The intrinsic pathway is mediated by mitochondria, and in response to apoptotic stimuli, several proteins are released from the intermembrane space of mitochondria into the cytoplasm. Some of the well-characterized proteins include cytochrome c, SMAC/DIABLO, AIF, EndoG and Bcl2-family proteins. Cytochrome c binds to the protein APAF1 and induces a conformational change that allows APAF1 to bind to ATP/dATP and to form the apoptosome, which mediates the activation of caspase-9, thereby triggering a cascade of caspase activation (Riedl and Shi, 2004; Sayers, 2011).

The majority of chemotherapies induce the intrinsic apoptosis, however the crosstalk between the extrinsic and intrinsic apoptosis pathway can result in a synergic and efficient induction of cell death. This synergy between the death receptor and the mitochondrial pathway has been observed in several cancer cells, including blood cancers (Nakata et al., 2004; Ray and Almasan, 2003).

The three chalcones evaluated induced an increase in caspase-9 activity, suggesting an engagement of the intrinsic pathway (Fig. 3). However, only chalcones R7 and R13 altered the Bcl2-family proteins expression (Figs. 4 and 5) and an increase in cytochrome c release (Fig. 6). Chalcone R15 did not change the Bcl2-family proteins expression (Figs. 4 and 5). For this reason, we suggest that chalcones R7 and R13 act via different cell death mechanisms compared to R15. In a previous work already mentioned, we also showed that the Chalcones R7 and R13 decreased the mitochondrial membrane potential and ATP concentration in L1210 cells (Winter et al., 2010). These previous results are in agreement with the results shown here, because R7 and R13 decreased the anti-apoptotic Bcl-2, increased the pro-apoptotic Bax and Bak expression (Fig. 4), and increased cytochrome c release, which are related to the mitochondrial damage and to the intrinsic pathway. These two chalcones also induced an increase in caspase-8 activity (Fig. 3) and Fas expression (Fig. 4), indicating the engagement of the extrinsic pathway beyond the intrinsic pathway. The link between the two pathways is made by Bid, a protein that is activated after caspase-8-mediated cleavage (Billen et al., 2008; Li and Yuan, 1998). The chalcones R7 and R13 induced an increase in Bid expression (Fig. 4).

Although the synergistic effect of apoptosis by combined treatment of extrinsic and intrinsic apoptosis is well established, their underlying mechanisms are not completely understood (Schneider-Jakob et al., 2010).

Many mutations related to the source of cancer, such as loss of proapoptotic factors like p53 and Bax or the overexpression of anti-apoptotic factors like Bcl-2 and Mcl-1, reduce intrinsic apoptosis signaling and thus prevent the efficient elimination of transformed cells. Furthermore, standard chemotherapy and radiotherapy for cancer predominantly initiate apoptosis via the intrinsic pathway and thus might positively select cancer cells, which can evade intrinsic apoptosis signaling (Sayers, 2011). As the Chalcones R7 and R13 studied here induced the extrinsic and the intrinsic pathways concomitantly, decreasing anti-apoptotic like Bcl-2 and increasing pro-apoptotic factor Bax and cytochrome c, they can be considered important as a strategy for cancer treatment. These compounds could achieve the intrinsic pathway-resistant cancer cells and those cells that present mutation in apoptosis-related factors. Conversely, Dou et al. showed that the apoptosis induced in a leukemic cell line (HL60) was also via both intrinsic and extrinsic pathways, however they did not evaluate the possible changes in Bid (Dou et al., 2011), while Kim et al. demonstrated that the effect of the flavonoid genistein on cervical cancer cells is through intrinsic and extrinsic apoptosis pathways by an increase in Bax, a decrease in Bcl-2 and the activation of Bid (Kim et al., 2009).

In addition, it was also shown in our previous study that Chalcone R15 induced a strong ROS generation and did not change the mitochondrial membrane potential in L1210 cells (Winter et al., 2010). In this work, it was observed that R15 did not change the Bcl2-family proteins and Bid expression (Fig. 4). The cell death triggered by R15 also was attended by caspases 8 and 9 (Fig. 3), but it does not seem to be related to mitochondrial damage. The increase of caspase-9 can possibly be related to the ER stress induced by R15, because this compound did not induce the cytochrome c release.

The transcription factor p53 suppresses tumorigenesis and is directly mutated in over 50% of tumors. In the other 50% of cases, the gene of p53 itself is not mutated, however the gene p53 pathway is often partially inactivated (Cheok et al., 2011). In addition, the mutation of the p53 gene is related to a poor clinical outcome in childhood ALL (Kawamura et al., 1999). Usually, the studies aim to increase the p53 expression and to induce the apoptosis of the cancer cells (Liu et al., 2008; Martinez-Rivera and Siddik, 2012). In this study we found that the chalcones, mainly the R15, decreased the p53 protein expression. Although seemingly controversial at first sight, this effect deserves further studies, as it could be an important strategy for the treatment of patients who exhibit a p53-mutated tumor.

All three chalcones induced an increase in calcium concentration (Fig. 7a), caspase-12 activity (Fig. 7b) and CHOP expression (Fig. 4), indicating that these compounds lead to ER stress. The ER plays a key role in maintaining intracellular Ca^{2+} homeostasis, which directly influences protein folding and subsequently ER stress and apoptosis. The ER stress occurs when there is over production and accumulation of misfolded proteins, or in a situation of altered calcium homeostasis (Berridge, 2002). The result of prolonged ER stress can be the apoptosis activation through different mechanisms. One of the components of the ER stress is C/EBP homologous protein (CHOP) also known as GADD153. Elevated levels of CHOP leads to alterations in gene transcription involved with apoptosis as inhibition of anti-apoptotic Bcl-2, stimulation of death receptor 5 (DR5), increase of pro-apoptotic protein Bim, caspases

activation and mitochondrial events (Oyadomari and Mori, 2004; Schonthal, 2013). The caspase-12 is the main caspase involved in ER stress and after activation can activate the caspase-9, and consequently the caspase-3 (Groenendyk and Michalak, 2005; Nakagawa et al., 2000). Studies have been shown compounds with antitumoral activity inducing ER stress by CHOP similarly to chalcones studied in this work (Choi et al., 2013; Gills et al., 2007; Lovat et al., 2003; Sanchez-Lopez et al., 2013).

Most anticancer agents exhibit their inhibitory effects on tumor cell growth by inducing cell cycle arrest and apoptosis. Recent reports have demonstrated that chalcones induce cell cycle arrest in different cancer cell lines (Hsu et al., 2006; Mielcke et al., 2012; Rao et al., 2010). None of the compounds induced cell cycle arrest in this work (Fig. 8). This lack of effect on the cell cycle may be related to the strong decrease in p21 expression. The p21 protein is regulated by the p53 gene, and is best known for its ability to regulate the cell cycle (Sherr, 1996; Vermeulen et al., 2003). However, p21 has been classified as an anti-apoptotic protein that can inhibit the caspase-3 activation (Dotto, 2000; Suzuki et al., 2000). High expression of p21 can lead cells to display apoptosis resistance (Asada et al., 1999). The decrease of p21 expression caused by Chalcones R7, R13 and R15 probably promoted apoptosis, but not via the cell cycle arrest.

In conclusion, our study suggests that the Chalcones R7, R13 and R15 induced apoptosis by different mechanisms in a leukemic cell line and presented higher selectivity for leukemic cells than for non-tumoral cells. The mechanism of action of Chalcones R7 and R13 is related to mitochondrial signaling, to trigger the extrinsic and the intrinsic apoptosis pathways, which can be an advantage for the treatment of intrinsic pathway-resistant tumors, and also to ER stress. On the other hand, Chalcone R15 triggered the apoptosis by extrinsic pathway and ER stress. These results indicate that the compounds studied are promising in the research of new drugs for cancer treatment because they induce a minimum of two apoptosis pathways what could help to circumvent possible cancer cell strategies of resistance.

Conflict of Interest

The authors declare that there is no conflict of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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