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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

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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

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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

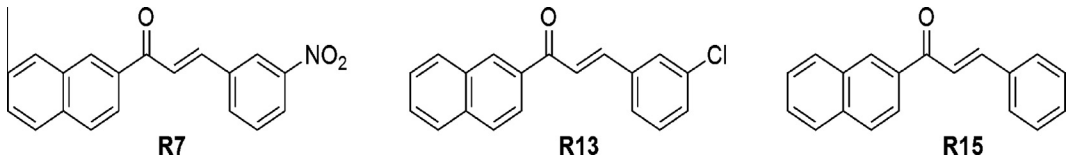

Fig. 1. Chemical structures of naphthylchalcones R7, R13 and R15.