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Cytotoxic Effects Induced by Combination of Heliantriol B2 and Dequalinium against Human Leukemic Cell Lines

M. Soledad Vela Gurovic,[1,2]* A. María Díaz Lanza,[3] María del Carmen Boyano Adánez,[1] M. Cristina Estañ Omaña,[1] Irene Gañán Gómez,[1] A. Paula Murray[2] and Pilar Sancho López[1]

[1]Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Campus Universitario, Universidad de Alcalá, 28871 Alcalá de Henares, Madrid, Spain
[2]INQUISUR-CONICET, Departamento de Química, Universidad Nacional del Sur, Av. Alem 1253 B8000CPB Bahía Blanca, Buenos Aires, Argentina
[3]Departamento de Farmacología, Facultad de Farmacia, Campus Universitario, Universidad de Alcalá, 28871 Alcalá de Henares, Madrid, Spain

Natural occurring compounds are considered an important source of antitumoral agents. In the present study, the cytotoxic potential of three pentacyclic triterpenes isolated from *Chuquiraga erinacea* (Asteraceae), against the human leukemic cell lines NB4 and K562 was assessed. Heliantriol B2 (HB2) showed the highest cytotoxic activity after 24 h treatment showing IC_{50} values of 1.98 ± 0.12 μM and 3.52 ± 0.14 μM for NB4 and K562 cells, respectively. This activity was higher than that of the reference compound dequalinium (DQA). Apoptosis and necrosis induced by HB2 in both NB4 and K562 cell lines were analysed by Annexin V/PI labeling. Mitochondrial alterations including reactive oxygen species (ROS) production and mitochondrial transmembrane potential ($\Delta\Psi m$) were also tested. The results demonstrated that HB2 induced cell death by apoptosis and necrosis and showed enhanced cytotoxic effects in combination with DQA. Besides, HB2 induced ROS overproduction in NB4 cells and a slight decrease of $\Delta\Psi m$. Consequently, our findings prompt further studies on the HB2 mechanism of action and its selectivity to tumor cells in order to assess the potential of HB2 as an agent for cancer treatment. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: heliantriol B2; NB4 cell line; K562 cell line; leukemia; dequalinium; apoptosis.

INTRODUCTION

Most of the antitumoral agents used in chemotherapy are aimed at inducing malignant cell death in order to eradicate the tumor, thus limiting its growth and spread. However, the lack of specificity for tumor cells exhibited by these agents causes undesirable side effects that have led to the investigation of new therapeutic strategies designed specifically to target malignant cells and trigger selective cell destruction.

Delocalized lipophilic cations (DLCs) accumulate selectively in cancer cells and into mitochondria in response to negative inside transmembrane potentials. Most DLCs are toxic to mitochondria at high concentrations. Their mechanism of action involves inhibition of NADH-ubiquinone reductase and F1-ATPase, and a consequent decrease in cellular ATP production, which finally leads to inhibition of mitochondrial energy metabolism (Modica-Napolitano and Aprille, 2001). Antitumoral properties of the DLC dequalinium (DQA) were first described by Weiss *et al.* (1987). Its selectivity towards carcinoma cells was studied by Modica-Napolitano *et al.* (2003). We have reported that DQA induces cell death by a mixture of apoptosis and necrosis in human leukemic cell lines such as NB4, derived from acute promyelocytic leukemia, and K562, derived from chronic myeloid leukemia in blastic crisis (Galeano *et al.*, 2005). DQA disturbs mitochondrial function inducing cytochrome c release to cytoplasm, superoxide anion ($O_2^{\bullet -}$) overproduction and ATP depletion (Sancho *et al.*, 2007). It has been demonstrated previously that the effectiveness of DLCs can be enhanced by combining drug therapies (Modica-Napolitano and Aprille, 2001).

Natural occurring compounds became an important source in the search for new anticancer agents (Li *et al.*, 2005). In this field, pentacyclic triterpenes have been investigated widely and shown to be active against human leukemia cells (Harikumar *et al.*, 2010) including the NB4 and K562 cell lines (Fernandes *et al.*, 2003; Xia *et al.*, 2005; Lee *et al.*, 2007; Liu and Jiang, 2007; Calviño *et al.*, 2010). Many pentacyclic triterpene alcohols isolated from plants belonging to the Asteraceae family possess cytotoxic activity against human cancer cell lines (Ukiya *et al.*, 2002; García and Delgado, 2006). *Chuquiraga erinacea* D. Don (Asteraceae), an endemic species from Argentina, contains several pentacyclic triterpenes which possess a large number of biological activities including antiinflammatory and chemoprotective (Vela Gurovic *et al.*, 2010). The present work first evaluated the cytotoxic potential of the pentacyclic triterpenes, calenduladiol, faradiol and heliantriol B2 (HB2), isolated from *C. erinacea* (chemical structure shown in Fig. 1), against the human leukemic cell lines

* Correspondence to: M. Soledad Vela Gurovic, INQUISUR-CONICET, Departamento de Química, Universidad Nacional del Sur, Av. Alem 1253 B8000CPB Bahía Blanca, Buenos Aires, Argentina.
E-mail: svela@uns.edu.ar

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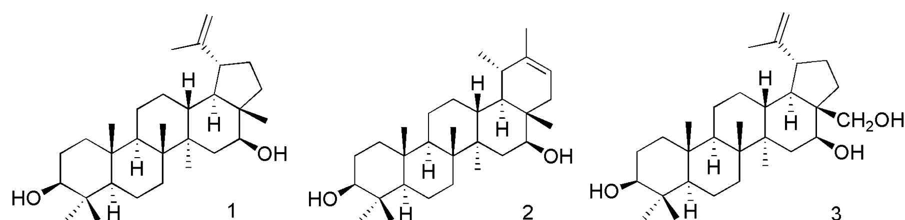

Figure 1. Structure of the pentacyclic triterpene alcohols calenduladiol (**1**), faradiol (**2**) and heliantriol B2 (**3**), isolated from *C. erinacea*.

NB4 and K562. It also evaluated the cytotoxic effects induced after combination of DQA and HB2, the most active triterpene among those tested. In order to assess whether HB2 induces cell death by altering mitochondria, ROS production and the mitochondrial transmembrane potential were tested.

MATERIALS AND METHODS

Preparation of triterpenes and DQA stock solutions. Calenduladiol (lup-20(29)-en-3β,16β-diol), faradiol (taraxast-20-ene-3β,16β-diol) and heliantriol B2 (HB2) (lup-20(29)-ene-3β,16β,28-triol) were isolated from *Chuquiraga erinacea* D. Don subsp. *erinacea* as described previously (Vela Gurovic *et al.*, 2010). These triterpenes were dissolved in DMSO at a concentration of 25 mM for both calenduladiol and faradiol and 20 mM for HB2, filter sterilized and stored at –80°C until used. Dilutions before each experiment were made with cell culture medium. Final DMSO concentrations in the experiments did not exceed 1% throughout the study.

DQA was prepared as described previously (Galeano *et al.*, 2005). Briefly, a 10 mM dequalinium chloride (Sigma Chemical Co., St Louis, MO, MW 527.6) stock solution was prepared in methanol in a round bottom flask. The organic solvent was removed with a rotary evaporator. The DQA-film obtained was resuspended in 5 mM HEPES, pH 7.4, sonicated for 1 h, and centrifuged (1000 × **g**, 5 min) to remove metal particles from the probe as well as larger DQA aggregates. This procedure yielded an opaque solution of liposome-like DQA vesicles, which was then filtered using a 0.2 μM filter. The DQA concentration was determined by fluorimetry (Perkin-Elmer LS-50 B Spectrofluorimeter, excitation λ = 335 nm, emission λ = 360 nm). The DQA standard curve was found to be linear between 0 and 10 μM DQA (r^2 = 0.998).

Cell cultures. The human leukemic cell lines NB4 and K562 were grown in RPMI 1640 medium supplemented with 5% heat inactivated fetal calf serum, penicillin/streptomycin (1%) (Gibco-Life Technologies, Scotland, UK) and 80 μg/mL gentamycin (Schering-Plough, UK). The cells were seeded at a density of 2–3 × 10^5 cells/mL and maintained at 37°C in a humidified 5% CO_2 atmosphere.

Cytotoxicity assay. NB4 and K562 cell growth inhibition was measured using the MTT assay (Roche Mannheim, Germany) which detects mitochondrial dehydrogenase activity. Viable cells, with functional mitochondria, were able to reduce the tetrazolium ring to a blue formazan product, whereas dead cells remained uncolored. 1–2 × 10^4 cells were seeded into 96-well microculture plates in a final volume of 100 μL culture medium and exposed to triterpenes or DQA at seven concentrations at two-fold serial dilutions in cell culture medium, starting from a high of 100 μM, for either 24 or 48 h. IC_{50} is defined as the compound concentration that induced a 50% loss of metabolic activity. The IC_{50} values were determined using the software Prism 5 (GraphPad).

Necrotic cell death evaluation. Necrosis was determined by the loss of cell membrane integrity using propidium iodide (PI) free influx in non-permeabilized cells. For this study, 1 × 10^6 cells/mL were washed with PBS and incubated with 50 μg/mL of PI and the emitted fluorescence was analysed by flow cytometry in a FACScan (Becton Dickinson, San Jose, CA) with an FL-2 detector (620 nm band pass filter). Under these conditions, necrotic cells are brightly stained by PI and appear as a peak at very high fluorescence values. Apoptotic cells appear as a dimly fluorescent population. Since apoptosis ultimately leads to a loss of plasma membrane integrity, the necrosis determined here includes the late apoptosis.

Apoptotic cell death evaluation. The characteristic decrease in DNA content in the apoptotic process was analysed by flow cytometry of permeabilized PI-stained cells. Samples containing 1 × 10^6 cells/mL were washed with PBS and incubated with 0.5 mg/mL of RNase A for 30 min. The cells were then permeabilized with 0.1% Nonidet P-40 and incubated with 50 μg/mL of PI. Cell cycle analysis was carried out by flow cytometry (FL-2 detector in a linear mode) using the Cell Quest Pro software (Becton Dickinson, San Jose, CA). Permeabilization of cells causes the leakage of the cleaved low MW DNA fragments that are produced during apoptosis. As a consequence, apoptotic cells are identified as a hypodiploid peak, while healthy cells generate a typical cell cycle histogram. Non-apoptotic, primary necrotic cells are generally found among the healthy ones.

Apoptosis was also assessed by the characteristic exposure of phosphatidyl serine (PS) on the cell surface. For this study 1 × 10^6 cells/mL were double-stained with annexin V-FITC and PI (AnnexinV-FITC Apoptosis Detection Kit, Calbiochem, US) and analysed by flow cytometry (FACScan, Becton Dickinson, San José, CA).

Measurement of superoxide anion. The intracellular accumulation of superoxide anion ($O_2^{\bullet-}$) was determined using the fluorescent probe dihydroethidium (DHE, Molecular Probes, Eugene, OR). The cells were

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Table 1. IC_{50} values of three natural occurring pentacyclic triterpenes, calenduladiol, faradiol and heliantriol B2, in the human leukemic NB4 and K562 cell lines treated for 24 or 48 h with increasing (0.8–100 μM) concentrations of the different compounds. For comparative reasons, IC_{50} values for the antitumoral dequalinium are also shown. Data are presented as the mean (μM) ± SEM from at least three separate experiments.

	NB4 cells		K562 cells	
	24 h	48 h	24 h	48 h
Calenduladiol	63.59 ± 7.60	22.49 ± 2.23	130.30 ± 4.69	42.07 ± 9.26
Faradiol	32.47 ± 1.08	30.60 ± 2.61	132.80 ± 7.50	60.15 ± 14.02
Heliantriol B2	1.98 ± 0.12	1.33 ± 0.19	3.52 ± 0.14	2.12 ± 0.04
Dequalinium	7.73 ± 0.54	nd	14.15 ± 1.87	nd

nd, not determined.

incubated with 2 μM DHE during the last 15 min of treatment. The fluorescence intensity was measured by flow cytometry (FL-2, 620 nm band pass filter).

Measurement of mitochondrial transmembrane potential (ΔΨm). Cells (0.5×10^6) were washed with PBS and incubated for 15 min at 37°C with 1 μg/mL of rhodamine 123 (Sigma Chemical Co., St Louis, MO). After washing, the cells were resuspended in 0.5 mL of PBS and the fluorescence was measured by flow cytometry (FL-1 detector).

Statistics. Statistical analyses were performed using Student's *t*-test. Differences were considered significant if $p < 0.05$.

RESULTS

Effect of heliantriol B2 on cell metabolic activity

In order to assess the cytotoxic activity of the triterpenes calenduladiol, faradiol and heliantriol B2 (HB2) on the NB4 and K562 human leukemic cell lines, a concentration- and time-response study was initially carried out. Mitochondrial dehydrogenase activity in metabolically active cells was determined by the MTT test. The cells were treated with increasing concentrations (from 0.5 to 100 μM) of test compounds for either 24 or 48 h. Experiments using DQA as a reference antitumoral drug were also carried out. The IC_{50} values obtained are shown in Table 1. The three compounds assayed show a loss of metabolic activity that was concentration- and time-dependent in both cell lines, proving the NB4 cells to be more sensitive than the K562 cells. In addition, the results indicate that HB2 is the most effective of the three compounds assayed, with IC_{50} values even lower than those obtained for DQA. Concerning these results, further studies on NB4 and K562 cell death induced by the triterpene triol HB2 were performed.

Considering that the effectiveness of DLCs can be enhanced in combination with other anticancer agents (Modica-Napolitano and Aprille, 2001), apoptosis and necrosis induced after NB4 and K562 cell treatment with HB2 alone or in co-treatment with DQA were assessed. Experimental conditions selected were 24 h of incubation at 2 μM HB2 for NB4 cells, 3 μM HB2 for K562 cells and 10 μM DQA for both NB4 and K562 cells. Concentrations were chosen by considering the IC_{50} values. In this manner, studies were assessed at a concentration sufficient to observe cytotoxic effects, but not too high to avoid difficulties in interpretation of results due to excessive cell death.

HB2-induced apoptosis and necrosis

The ability of HB2 to induce NB4 or K562 cell death by either apoptosis or necrosis was analysed subsequently by flow cytometry. Apoptosis was evaluated by cell cycle analysis obtained after PI staining of previously permeabilized cells. The characteristic loss of cell membrane integrity in necrotic processes was analysed by PI staining of cells. Figure 2A shows representative flow cytometry histograms of NB4 and K562 cells obtained from the controls and cells incubated with HB2 and DQA alone and in combination. The typical cell cycle histograms with two-well defined peaks corresponding to the G_0/G_1 and G_2/M phases were clearly observed in control cells. Necrotic cells were detected as a peak at very high fluorescence intensity in the flow cytometry histograms. The percentage of apoptosis, obtained from cells in the sub-G_0/G_1 region of histograms, as well as the percentage of necrosis, obtained from necrotic region of histograms, at the different experimental conditions, is shown in Fig. 2B.

A clear increase of NB4 cells in the apoptotic and necrotic regions was seen after each treatment (Fig. 2A). The percentage of apoptosis in NB4 cells treated with 2 μM HB2 was 10.6%, which increased up to 28.3% after combination with 10 μM DQA (Fig. 2B). Taking into account that the percentage of apoptotic cells induced by DQA was about 15.4%, the apoptosis detected by the combination of HB2 and DQA could be considered as the sum of individual effects. All treatments induced necrosis in NB4 cells in comparison with the controls, although the variation of the results did not allow the observation of substantial differences between them (Fig. 2B).

In K562 cells, the treatment with HB2 induced a slight apoptosis and necrosis at 3 μM, either in the absence or presence of DQA (Fig. 2A). Co-treatment with DQA did not significantly increase the percentage of apoptosis but did increase necrosis induced by HB2 alone ($^{+}p < 0.05$). An HB2-induced cell cycle arrest could be suggested from the cell cycle profiles (Fig. 2A). DQA induced neither necrosis nor apoptosis in K562 cells, which agrees with our previous studies in relation to the resistance of K562 cells to

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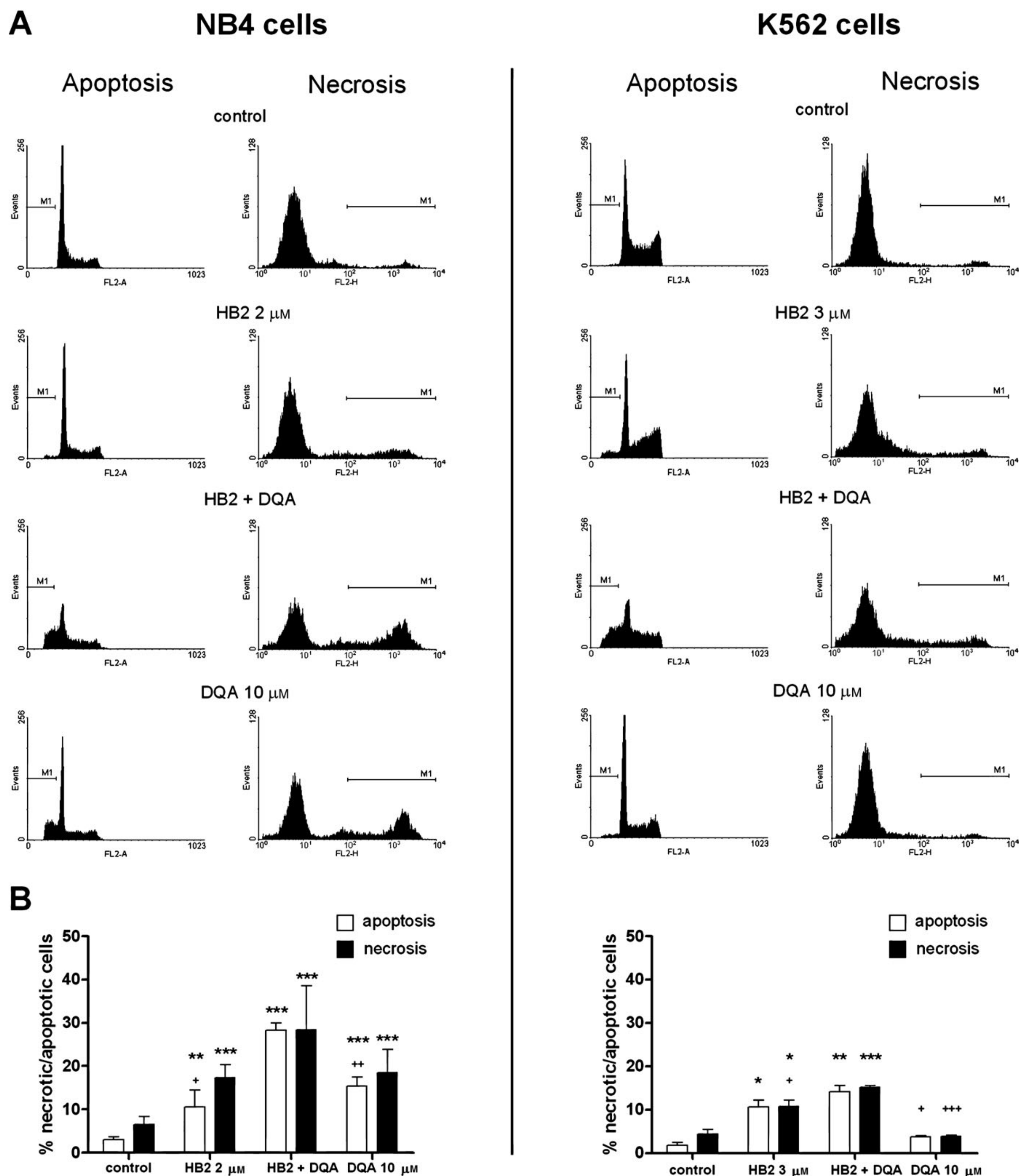

Figure 2. Effect of HB2 and DQA on NB4 and K562 cell death after 24 h treatment at the concentrations indicated. (A) Representative cell cycle distribution profiles of untreated (control) and treated cells as measured by flow cytometry after cell permeabilization and PI staining (apoptosis) or PI accumulation in non-permeabilized cells (necrosis). (B) Frequency of apoptosis and necrosis obtained from the corresponding sub-G_0/G_1 and necrotic regions of the flow cytometry histograms. Data are presented as the mean ± SEM of three to nine separate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the control group. + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$, compared with the HB2 + DQA group.

DQA-induced apoptosis (Galeano *et al.*, 2005). These results suggest that apoptosis and necrosis observed after combination of HB2 and DQA are due mainly to the presence of HB2.

All these results show that in both NB4 and K562 cell lines HB2 induces cell death at a lower concentration than DQA, and that co-treatment enhances the apoptosis and necrosis.

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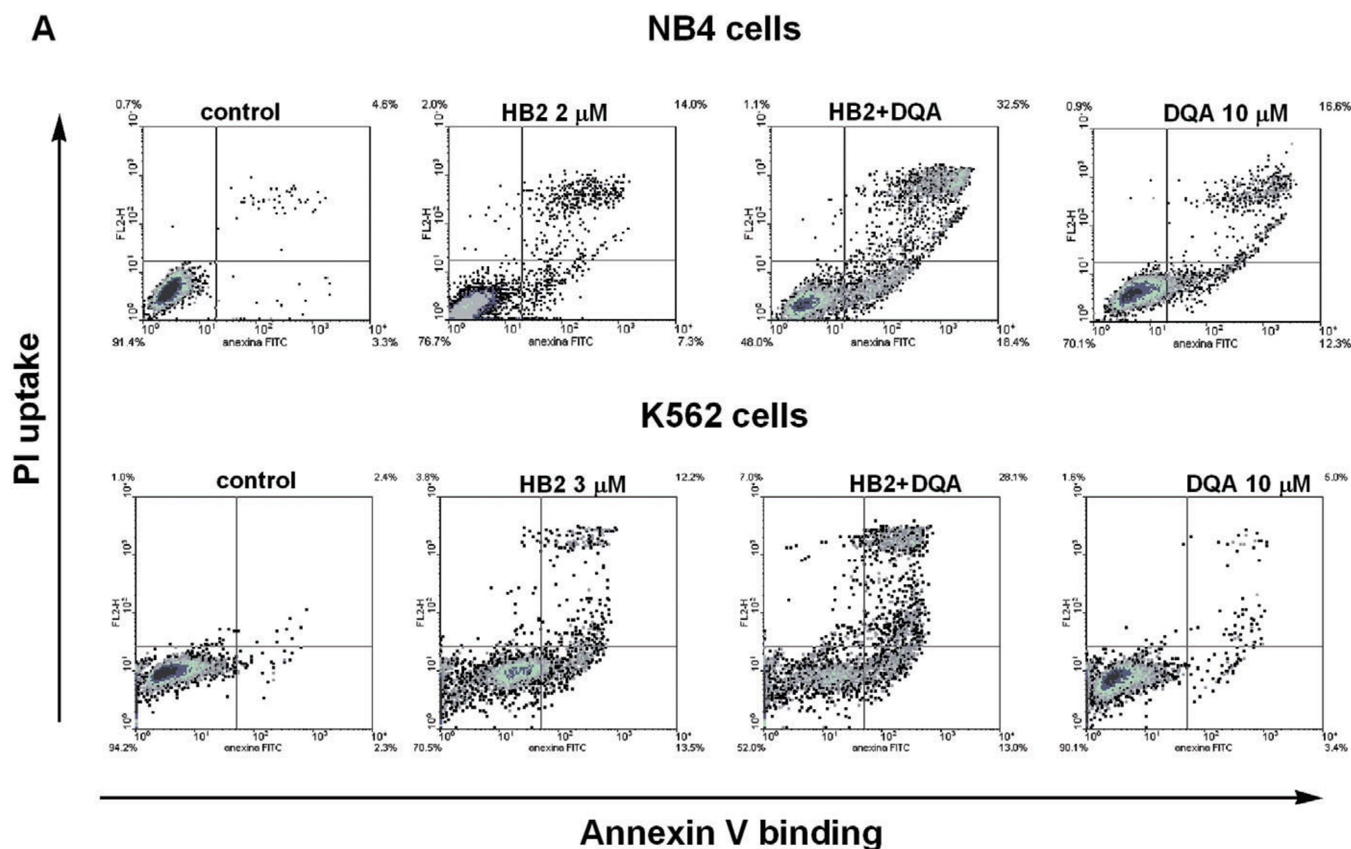

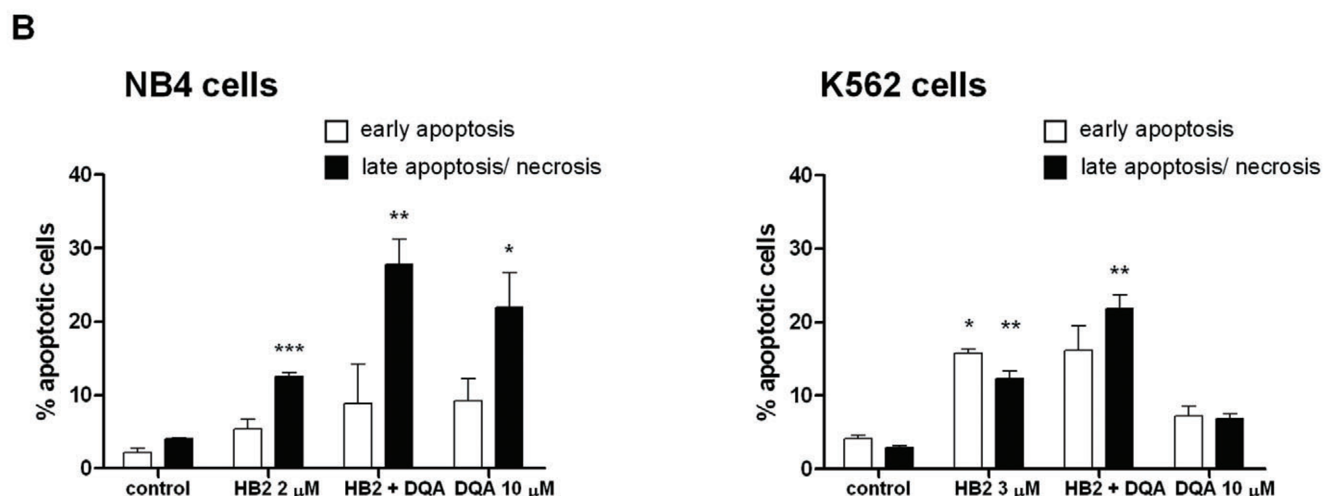

Figure 3. Effect of HB2 and DQA on apoptosis in NB4 and K562 cells after 24 h treatment at the concentrations indicated. (A) Representative dot plot diagrams obtained by flow cytometry of Annexin V-FITC/PI double-stained cells. Lower left (LL) $FITC^-/PI^-$ are live cells, lower right (LR) $FITC^+/PI^-$ are early apoptotic cells, upper right (UR) $FITC^+/PI^+$ are late apoptotic or necrotic cells and upper left (UL) $FITC^-/PI^+$ are necrotic cells. (B) Frequency of cells in early apoptosis and late apoptosis and necrosis obtained from the corresponding dot plot diagrams. Data are presented as the mean ± SEM of three to nine separate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the control group. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

Annexin V-FITC analyses

Apoptosis was also studied by determining the cell surface exposure of PS. Since the plasma membrane excludes viability dyes such as PI, the cells stained with Annexin V but not with PI are considered to be in early apoptosis (Annexin V^+, PI^-). In the absence of phagocytosis, the final stages of apoptosis involve necrotic-like disintegration of the total cell (Gorczyca, 1999). In late apoptosis, the cell membrane loses integrity allowing cell staining with both Annexin V and PI. Thus necrotic or apoptotic cells in terminal stages will be both Annexin V-FITC and propidium iodide positive and will be reported in the upper right-hand quadrant (Annexin V^+, PI^+). Figure 3A shows representative dot plot diagrams obtained for NB4 and K562 cells subjected to different treatments. The percentage of cells in early apoptosis (lower right quadrants) and late apoptosis or necrosis (upper right quadrants) obtained from the corresponding dot plot diagrams are shown in Fig. 3B.

The results show that the percentage of NB4 cells in early apoptosis after treatment with 2 μM HB2 was

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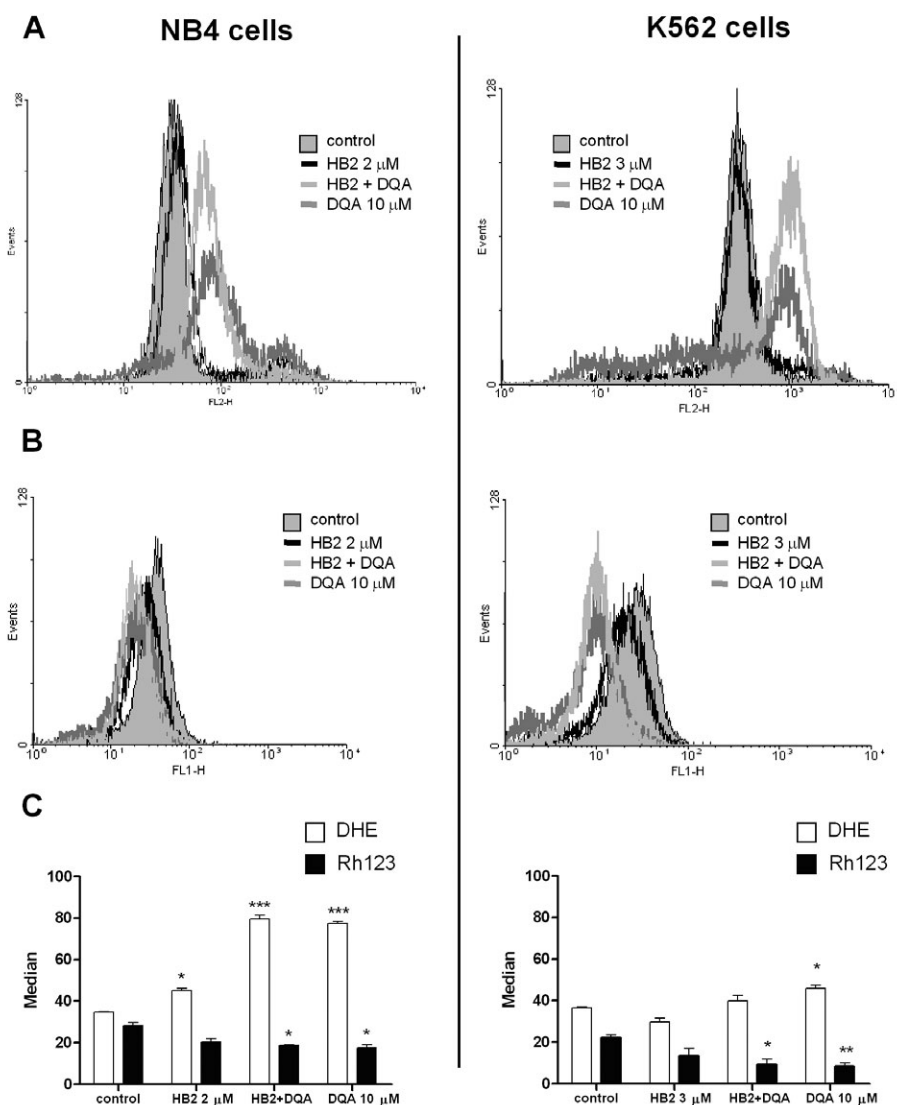

Figure 4. Effect of HB2 and DQA on mitochondrial function in NB4 and K562 cells treated for 24 h at the concentrations indicated. (A) Median of DHE-derived fluorescence, as indicative of radical superoxide production ($O_2^{\bullet-}$). (B) Median of mitochondrial transmembrane potential ($\Delta\Psi_m$) as determined by changes in fluorescence upon rhodamine 123 loading. (C) Average medians from fluorescence histograms of DHE and Rhodamine 123. Data are presented as the mean ± SEM from fluorescence histograms from at least three separate experiments.

lower than that obtained with DQA, the percentage being similar to that obtained with HB2 plus DQA. The percentage of cells in late apoptosis or necrosis was also lower after HB2 treatment than after DQA treatment but it was increased by combination of both compounds. It can then be suggested that the observed effect in NB4 cells after co-treatment is due mainly to DQA, and that HB2 increased the DQA-induced apoptosis.

The treatment of K562 cells with HB2 alone or in combination with DQA induced a similar percentage of early apoptosis (around 16%), while DQA produced neither apoptosis nor necrosis. However, the percentage of cells in late apoptosis and necrosis after the combination of HB2 and DQA was higher than that obtained after single treatments.

Effect of heliantriol B2 on superoxide anion and mitochondrial transmembrane potential

As has been described, DQA-induced cell death is mediated by mitochondrial alterations, including $O_2^{\bullet-}$ accumulation and loss of $\Delta\Psi_m$ (Galeano *et al.*, 2005). To determine if HB2 exerts its cytotoxic mechanism of action through the mitochondria, as does DQA, both the ROS accumulation and mitochondrial function were evaluated in NB4 and K562 cells after exposure to HB2 alone or in combination with DQA. ROS generation was studied by measuring the DHE-derived fluorescence by flow cytometry, as indicative of the $O_2^{\bullet-}$ levels. Representative flow cytometry histograms are shown in Fig. 4A. The $\Delta\Psi_m$ was evaluated by the fluorescent probe rhodamine 123 (Rh123). Representative flow cytometry histograms are shown in Fig. 4B. The average medians obtained from corresponding $O_2^{\bullet-}$ and $\Delta\Psi_m$ fluorescence histograms appear in Fig. 4C.

As can be observed in Fig. 4C, NB4 cell treatment with 2 μM HB2 induced an $O_2^{\bullet-}$ overproduction significantly different from that of the control cells. These $O_2^{\bullet-}$ levels were lower than those reached in co-treatment or with DQA alone, which were similar between them. The $\Delta\Psi_m$ of NB4 cells decreased after each treatment with respect to the control cells, the differences being signifi-

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cant either after the combination of HB2 with DQA or after DQA treatment (Fig. 4C). This behavior can be also observed in Figs 4A and 4B, which show how the treatment with HB2 produced fluorescent profiles near to the control cells for either DHE and Rh123 signals, while DQA or HB2 + DQA treatments produce a displacement of the signal to the right side for DHE (Fig. 4A) and to the left side for Rh123 (Fig. 4B).

K562 cells treated either with HB2 or with HB2 in combination with DQA increased the $O_2^{\bullet-}$ production in a not significant manner while DQA alone induced a significant $O_2^{\bullet-}$ overproduction. This suggests that HB2 could be exerting an antioxidant function in this cell line by decreasing the DQA-induced ROS production. Figure 4C also shows that $\Delta\Psi_m$ did not significantly decrease after 3 μM HB2 treatment but in combination with DQA decreased in a significant manner, due mainly to the presence of DQA. The effects on $O_2^{\bullet-}$ production and $\Delta\Psi_m$ were also observed by the shifts of the DHE and Rh123 signals with respect to the control signal (Figs 4A and 4B, respectively). The results suggest that HB2 did not significantly decrease the $\Delta\Psi_m$ in K562 cells at the concentration assayed, and that the observed effects after combination with DQA on $O_2^{\bullet-}$ overproduction and $\Delta\Psi_m$ decrease were due mainly to DQA.

DISCUSSION

The major aim of this work was to investigate the cytotoxicity of three natural pentacyclic triterpenes calenduladiol, faradiol and heliantriol B2 (HB2), isolated from *C. erinacea*, in human leukemic NB4 and K562 cell lines. It was demonstrated that HB2 induced a concentration- and time-dependent cytotoxic activity in both cell lines. These effects were more pronounced in NB4 than in K562 cells. The results show that triterpenediols calenduladiol and faradiol presented a very low cytotoxicity in either NB4 or K562 cells at 24 h treatment. This is consistent with data reported previously for calenduladiol in K562 cells (García and Delgado, 2006) but differs from the results reported by Ukiya *et al.* (2002) for faradiol. However, the trihydroxylated compound HB2 showed a high cytotoxicity against both cell lines at either 24 or 48 h treatment, with IC_{50} values between 1 and 3.5 μM (equivalent to 0.4 and 1.6 μg/mL).

To date, several natural occurring pentacyclic triterpene acids such as oleanoic, betulinic, pomolic and ursolic, are known to inhibit the growth of and induce apoptosis in human leukemic cell lines (Fernandes *et al.*, 2003; Liu and Jiang, 2007; Vasconcelos *et al.*, 2007). HB2 inhibited cell growth at lower concentrations than the triterpene acids mentioned, which showed cytotoxic activity for leukemic cells at concentrations ranging from 10 μg/mL to 30 μg/mL after 24 h treatment. Therefore, the aim was to elucidate the molecular events implied in the HB2-induced cytotoxicity in NB4 and K562 cells in order to evaluate its potential as an antitumoral agent.

First investigated was the implication of apoptotic and necrotic processes in cell death. The results indicate that the HB2-induced cell death was cell type- and concentration-dependent. After 24 h, low HB2 concentrations induced some apoptosis and necrosis in NB4 and K562 cells. In this and previous studies (Galeano *et al.*, 2005) an absence of apoptosis was shown in K562 cells treated with DQA, which is consistent with other studies reporting a resistance of these cells to apoptosis (McGahon *et al.*, 1994; Ceballos *et al.*, 2000). K562 cells are deficient in the pro-apoptotic protein p53 and express the fusion protein Bcr-Abl kinase that provides continuous cell survival signaling. The apoptotic resistance of K562 cells has been shown to be overcome by treatment with different drugs (Fang *et al.*, 2000). In our hands, the apoptosis resistance of K562 cells was slightly overcome by treatment with HB2. Therefore, we suggest that Bcr-Abl kinase activity could be implicated in the HB2 mechanism of action in K562 cells. Further experiments will be necessary to study this hypothesis. Since in many cases the activity of some chemotherapeutic compounds is increased after combination with other agents, experiments to study the effects induced by HB2 in combination with the antitumoral DQA were carried out. In both NB4 and K562 cell lines it was found that the combination of HB2 with DQA enhanced apoptosis induced by each agent, suggesting an additive effect. Annexin V-PI labeling assays confirmed that apoptosis induced by the combination of HB2 and DQA played a relevant role in cell death. Apoptosis was due mainly to DQA in NB4 cells and to HB2 in K562 cells. The additive effect exerted on apoptosis was clearly observed on necrosis in K562 but not in NB4 cells, probably due to the variation of the results obtained. In general, the HB2-induced cell death by apoptosis or necrosis was more pronounced in NB4 than in K562 cells showing it to be cell type-dependent.

It is well known that mitochondria play a critical role in initiating apoptotic cell death (Körper *et al.*, 2004). Therefore, we searched for HB2-induced mitochondrial alterations related to $\Delta\Psi_m$ and $O_2^{\bullet-}$ accumulation either in the absence or presence of DQA. It was shown previously that DQA induces mitochondrial alterations including loss of $\Delta\Psi_m$ and $O_2^{\bullet-}$ overproduction in both NB4 and K562 cell lines (Galeano *et al.*, 2005; Sancho *et al.*, 2007). In this work, it was shown that HB2 induces a slight $O_2^{\bullet-}$ overproduction in NB4 cells, suggesting a different mechanism of action in both cell lines, ROS being implicated only in the NB4 cell line. The results suggest that HB2 could be protecting K562 cells against ROS generation. In both cell lines the $\Delta\Psi_m$ decrease induced by HB2 was not statistically significant, but it was either after combination with DQA or after DQA alone. In this way, the effects observed after cotreatment could be due mainly to DQA.

Wang and Fang (2009) studied the properties of pentacyclic triterpenes as apoptotic agents. Several studies on the mechanism by which this kind of compounds exert their activity have been reported. Among them, alpha-hederin induces apoptosis by a mechanism that involves disruption of mitochondrial membrane potential and an increase of ROS (Swamy and Huat, 2003), the lupane-type betulinic acid triggers the mitochondrial pathway of apoptosis (Fulda, 2008). Other pentacyclic triterpene belonging to lupane-type induced apoptosis on human leukemia cells by mediating the cleavage of PARP and upregulation of Bax proteins (Chen *et al.*, 2008). Boswellic acid acetate induced apoptosis in myeloid human leukemia cells by increasing the levels of death receptors and indirectly activating caspase-8 (Xia *et al.*, 2005). The results support that

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HB2, a lupane-type triterpenoid with a chemical structure very similar to that of betulin and betulinic acid, could trigger apoptosis in NB4 and K562 cells by mechanisms other than those directly associated with mitochondrial function.

CONCLUSIONS

In summary, it was demonstrated that heliantriol B2 (HB2), a natural occurring tri-hydroxylated pentacyclic lupane-type triterpene, induced NB4 and K562 cell death by a mixture of apoptosis and necrosis at a lower concentration than the antitumoral agent DQA and other pentacyclic triterpene acids. HB2-induced apoptosis and necrosis were increased in combination with DQA. The mechanisms underlying HB2-induced effects seem to involve oxidative stress in NB4 cells exerting an antioxidant function in K562 cells. Although the $\Delta\Psi_m$ decrease was not statistically significant, its implication in HB2-induced cell death on both NB4 and K562 cell lines at the concentrations tested should not be discarded.

Considering the high potential demonstrated by HB2 as an antitumoral agent in human leukemia cell lines, further studies on the combination with agents other than DQA, the potential of HB2 against other cancer cell lines and the mechanism of action implied should be performed in order to explore the antitumor potential of this natural compound.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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