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ORIGINAL ARTICLE

Inhibition of NF-κB-mediated transcription and induction of apoptosis by melampolides and repandolides

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Abstract
Purpose Nuclear factor-κB (NF-κB) plays a crucial role in the regulation of inflammatory processes, cell proliferation, and apoptosis. Blocking NF-κB signaling may represent a therapeutic strategy in cancer and inflammation therapy. The aim of this study was to investigate the effects of sesquiterpenes isolated from Asteraceae, namely melampolides (enhydrin, tetraludin A) and repandolides (repandins A, B, D and E) on the activation of NF-κB, cell growth of cancer cells, cell cycle progression and apoptosis. In addition, their effects on the activity of cyclooxygenase-2 (COX-2) enzyme were also evaluated.
Methods Cell-based reporter gene assay was conducted in SW1353 cells. COX-2 enzyme activity and cell growth inhibition was determined by enzyme immunoassay and MTT assay respectively. Cell cycle analysis was carried out by flow cytometry and apoptosis was observed by DAPI staining assay.
Results In SW1353 cells, transcription mediated by NF-κB was inhibited by enhydrin, tetraludin A and repandins A, B, D and E, while Sp-1 mediated transcription was not affected. COX-2 enzyme activity was inhibited by enhydrin, repandin A and E, but not by tetraludin A, repandin B and D. These compounds were effective in inhibiting the growth of a panel of human tumor cell lines in a concentration-dependent manner. Cell cycle analysis and DAPI staining indicated cell cycle arrest in G_2/M phase and induction of apoptosis.
Conclusions Enhydrin, tetraludin A and repandins A, B, D and E inhibited tumor cell growth and induced cell cycle arrest and apoptosis. These effects may be related to inhibition of NF-B activation.

Keywords NF-κB · Cyclooxygenase-2 · Sesquiterpene lactone · Melampolide · Repandolide · Apoptosis

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Introduction

Sesquiterpene lactones (SLs) are the active constituents of several medicinal plants used in traditional medicine for treatment of inflammation. A variety of different structural types of SLs are particularly common in the family Asteraceae (sunflower family). The SLs typically contain an α-methylene-γ-lactone moiety and many SLs also contain α, β-unsaturated esters and ketones as well as epoxide moieties [8]. These functional groups represent reactive receptor sites for biological nucleophiles, in particular, thiol and amino groups and, therefore, cause a wide range of biological activities, including mutagenic, genotoxic, anti-inflammatory, and anti-tumor activities [5, 20, 30].

The four species of the genus *Tetragonotheca* (Asteraceae) are a rich source for melampolide-type SLs and their biogenetic relatives, the repandolides [7].

The isolation and structure determination of repandolides (repandin A, B, D and E), and melampolides (tetraludin A and enhydrin) included in this study had been reported earlier from *Tetragonotheca repanda*, *Melampodium longipilum* and *Tetragonotheca ludoviciana* [10, 17, 23, 28]. Although the biological activities of repandins and tetraludin A have not been studied before, the structurally related melampolide enhydrin, has been reported for antidiabetic, antifungal and anti-inflammatory properties [12, 22, 25]. Other SLs (parthenolide and helenalin) have shown significant anticancer effects besides their anti-inflammatory action [5, 30]. Although various modes of action have been suggested for the biological activities of SLs, the mechanism of their anticancer activity is not sufficiently explained. Dirsch et al. [5] reported that sesquiterpene lactone helenalin triggers apoptosis in leukemia Jurkat T cells and the induction of apoptosis is dependent on caspase activation, but not dependent on the CD 95 system. Recently, inhibition of nuclear factor kappa B (NF-κB) activation has been reported to be involved in the anti-inflammatory action of parthenolide and helenalin [11, 15].

The nuclear factor kappa B (NF-κB) is an inducible, ubiquitous transcriptional regulator. It acts as a central mediator of the human immune response and regulates the expression of a variety of genes involved in immune and inflammatory response, including the COX-2 gene [1]. Cyclooxygenase (COX) is a key enzyme involved in the synthesis of prostaglandins, and two isoforms COX-1 and COX-2 have been identified. COX-1 is expressed constitutively in a number of cell types, and is responsible for various cytoprotective prostanoids in many organs, whereas COX-2 is usually absent under physiological conditions and is induced by stimuli like mitogens and cytokines [26]. However, it is constitutively expressed in a number of human cancer cell lines [6], and therefore, inhibition of COX-2 may also inhibit tumor cell growth. NF-κB has been implicated in the pathogenesis of a number of human cancers including head and neck cancers, pancreatic, colon cancer and breast cancers, T-cell leukemia and Hodgkin's lyphoma [19]. NF-κB also plays a critical role in the control of cellular growth and apoptosis [3]. Specific blockade of NF-κB signaling may cause cell growth inhibition and induce apoptosis and thus NF-κB seems to be an important target for cancer therapy. In this study we have utilized a cell based reporter gene assays to study the effects of enhydrin, tetraludin A and repandins A, B, D and E on NF-κB- and COX-2-promoter mediated transcription. Inhibition of COX-2 enzyme activity in macrophages as well as their growth inhibitory effects in a panel of human cancer cell lines was also studied. Furthermore, the cell cycle arrest and the mode of cell death were also evaluated (Fig. 1).

Materials and methods

Chemicals and reagents

Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 (DMEM/F12), fetal bovine serum (FBS) and RPMI 1640 medium was purchased from JRH Bioscience (Lenex, KS, USA), Atlanta Biologicals and Gibco. The human recombinant TNF-α, phorbol myristate acetate (PMA), 4′-6-diamidino-2-phenylindol (DAPI), 3-[4, 4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), ribonuclease-type1A were from Sigma Chemical Co. Isoton II, carrier fluid used in flow cytometry, was from Becton Dickinson. PGE_2 EIA kit was from Cayman Chemical Co. Repandolides and melampolides included in this study were isolated from different plant species as described earlier [17, 18, 24].

Cell culture

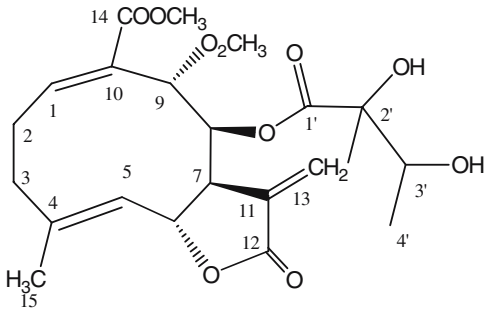
All cell lines used in this study were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The SW1353 human chondrosarcoma cells were cultured in 1:1 mixture of DMEM/F12. SK-MEL (human malignant, melanoma), MCF-7 and BT549 (human breast carcinoma), SK-OV3 (human ovary carcinoma) and KB (human epidermal carcinoma) cells were grown in DMEM, while RAW 264.7 (mouse macrophages) and HL60 (human leukemia) cells were grown in RPMI 1640 medium. The above media were supplemented with 10% FBS, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin. All cells were grown at 37°C in an atmosphere containing 5% CO_2 and 95% humidity.

Plasmids

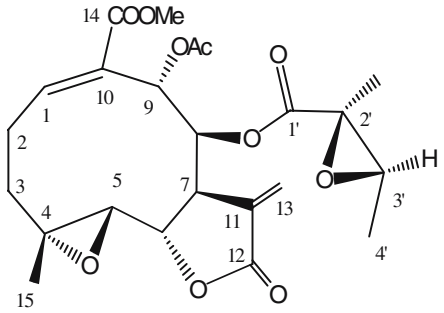
The COX-2 promoter construct was described previously [27]. The nuclear factor-κB reporter construct contained two copies of the element from the immunoglobulin K promoter (p BIIXLUC) and was a gift from Dr. Riccardo Dalla-Favera. The Sp-1 reporter plasmid (pGL3-promoter) was obtained from Promega.

Cell transfection and reporter gene assays

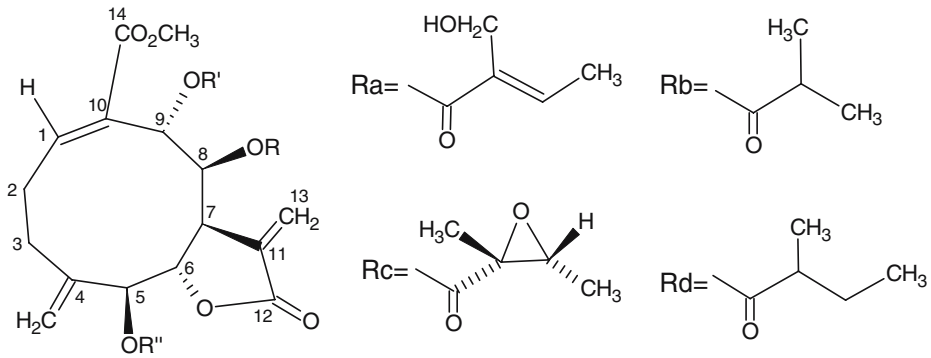
This assay was carried out as described previously in SW1353 cells [27]. Cells (1.2×10^7) were washed once



Tetraludin A



Enhydrin



Compound	R	R'	R''
Repandin A	Ra	Rc	H
Repandin B	Ra	Rd	H
Repandin D	Rb	Rd	H
Repandin E	Ra	Ac	H

Fig. 1 Chemical structures of enhydrin, tetraludin A, and repandin A, B, D and E

in an antibiotic and FBS-free DMEM/F12, and then resuspended in 500 μL of antibiotic-free DMEM/F12 containing 2.5% FBS. A luciferase plasmid construct was added to the cell suspension at a concentration of 50 μg/mL and incubated for 5 min at room temperature. The cells were electroporated at 160 V and one 70-ms pulse using BTX disposable cuvettes model 640 (4-mm gap) in a BTX Electro Square Porator T 820 (BTX I, San Diego, CA, USA). Following electroporation, 1.25×10^5 cells per well were plated in 200 μL of DMEM/F12 containing 10% FBS and antibiotics. After 24 h, cells were exposed to different concentrations of test compounds for 30 min and then induced with PMA (70 ng/mL) or TNF-α (50 ng/mL) for 8 h. Cells were lyzed by adding 40 μL of a 1:1 mixture of lucLite reagent and PBS containing 1 mM calcium and magnesium (Packard Instrument Company, Meriden, CT, USA). Light output was detected in a TopCount microplate scintillation counter in a single-photon counting mode (Packard). Relative luciferase units (RLU) were 12 to 15-folds higher in PMA or TNF-α induced cells versus controls. IC_{50} were estimated from dose response curves. Statistical analysis was performed by unpaired Student *t* test using PA Stats.

Determination of COX-2 enzyme activity

Mouse macrophages (RAW 264.7) were plated in a 96-well plate (50,000 cells/well) and incubated at 37°C for 24 h. Cells were treated with lipopolysaccharide (LPS, 5 μg/mL) for 16 h to induce the production of COX-2. Induced cells were treated with test compounds for 2 h. Arachidonic acid (300 μM) was added and cells were further incubated with for 30 min. COX-2 activity was determined by measuring the level

of prostaglandin E_2 (PGE_2) produced in the medium using enzyme immunoassay.

Cell growth inhibitory assay

The growth inhibition of SK-MEL, MCF-7, BT549, SK-OV-3, and KB cells were carried out by MTT assay [16]. Briefly, cells in logarithmic growth were seeded at a density of 25,000 cells/well in a 96-well plate and grown for 24 h. They were then incubated with various drug concentrations for 48 h at 37°C. After the addition of 50 μL of MTT (1 mg/mL) per well, the plates were incubated at 37°C under 5% CO_2 for 4 h. The blue crystals formed were dissolved in 150 μL of DMSO and absorbance was measured at 540 nm using a Bio-Kinetics (EL 312 e) microplate reader (Bio-TEK, Instruments). HL 60 leukemia cells were seeded at 100,000 cells/well in 24-well plates and incubated for 24 h. Test compounds were added and cells were further incubated for 48 h at 37°C. Viable cells were counted by trypan blue exclusion method [4]. The cell growth inhibitory activity of compounds was expressed as IC_{50} defined as the concentration that caused inhibition of growth by 50% compared to control.

Cell cycle analysis by flow cytometry

Cells (1×10^5 mL^{-1}) were seeded in 25-cm^2 tissue culture treated flasks (7 mL/flask) and grown for 24 h prior to the addition of test compounds. After 6, 12, 24 and 48 h incubation with 3 μM of test compound or vehicle control at 37°C, approximately 2×10^5 cells were collected by centrifugation at 1,000 rpm for 5 min. Cell pellets were fixed in 1 mL ice-cold ethanol (70%) and stored at −20°C. Samples were washed twice with PBS before cell cycle assay. Routine DNA staining was performed by addition of 200 μL propidium iodide (50 μg/mL in PBS) and 50 μL RNase (final concentration 40 μg/mL) to each sample. Cellular DNA content was measured by a Facscan flow cytometer. Approximately 1×10^4cells were analyzed for each DNA content histogram. The samples were excited at 380–410 nm and the resulting fluorescence was measured at wavelengths >550 nm. Analysis of the percentage of cells in G1, S and G2+M phases of the cell cycle was made by the Cellquest computer program (Dickinson, CA, USA).

DAPI staining

DAPI staining assay was used to determine whether the mechanism of growth inhibition was related to induction of apoptosis. After treatment of cells with the test compound (3 μM) for 48 h, cells were collected by centrifuging at 2,000*g* for 15 min and washed once with ice-cold PBS buffer, and fixed in a solution of 3.7% formaldehyde for 10 min. Fixed cells were stained with 4 μg/ml DAPI for 15 min. The nuclear morphology of cells was observed under a fluorescence microscope.

Results and discussion

The activation of NF-κB is involved in many cellular processes leading to inflammation and development of cancer [1, 2, 3, 19]. NF-κB is normally composed of a p50 and a p65 subunit and sequestered in the cytoplasm of cells where it is bound by a family of inhibitory proteins known as IκB [1]. These proteins (IκB-α, IκB-β and IκB-ε) contain multiple ankinrin repeats that are critical for their inhibitory function. The ability of the IκB to mask the nuclear localization signal of NF-κB prevents the nuclear translocation of these proteins. A variety of stimuli including TNF-α, interleukin-1 (IL-1), phorbol esters, LPS, viral infections, ultraviolet radiation and free radicals rapidly activate the IκB kinases, IKKα and IKKβ. The latter phosphorylate the inhibitory protein IκB to result in the ubiquitination and degradation of NF-κB. The active NF-κB is translocated from the cytoplasm to the nucleus where it binds to its target genes to initiate transcription. Using PMA and TNF-α as inducers, the effects of melampolides and repandolides on NF-κB-dependent gene transcription were investigated in SW1353 cells. Enhydrin, tetraludin A and repandin A, B, D, and E inhibited NF-κB-dependent transcription induced by PMA and TNF-α in a concentration-dependent fashion (Figs. 2a, 3a). The effects of melampolides and repandolides on transcription induced by the COX-2 promoter were also studied in SW1353 cells. Enhydrin, tetraludin A and repandin A, B, D, and E inhibited PMA and TNF-α-mediated transcription of COX-2 promoter activity in concentration-dependent fashion (Figs. 2b, 3b). The IC_{50} values are summarized in Table 1. PMA activation of both NF-κB and COX-2 promoter dependent transcription was more sensitive to the inhibitory effects of these compounds than activation by TNF-α ($P < 0.05$) except for tetraludin A. A luciferase construct with binding sites for Sp-1 was used as a control because this transcription factor is relatively unresponsive to inflammatory mediators. Hence, measurement of Sp-1-mediated luciferase expression is useful for detecting agents that nonspecifically inhibit luciferase expression because of cytotoxicity, inhibition of luciferase enzyme activity or light

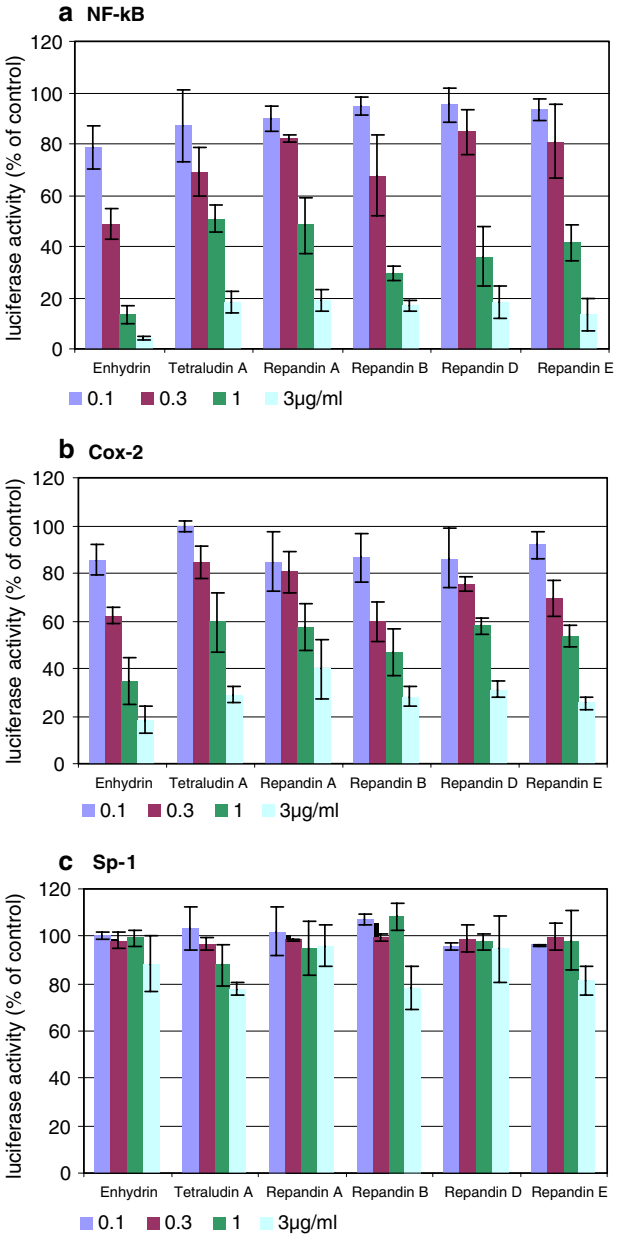


Fig. 2 Effect of melampolides and repandolides on PMA induced NF-κB (**a**), Cox-2 promoter (**b**) and Sp-1 (**c**) mediated transcription in SW1353 cells

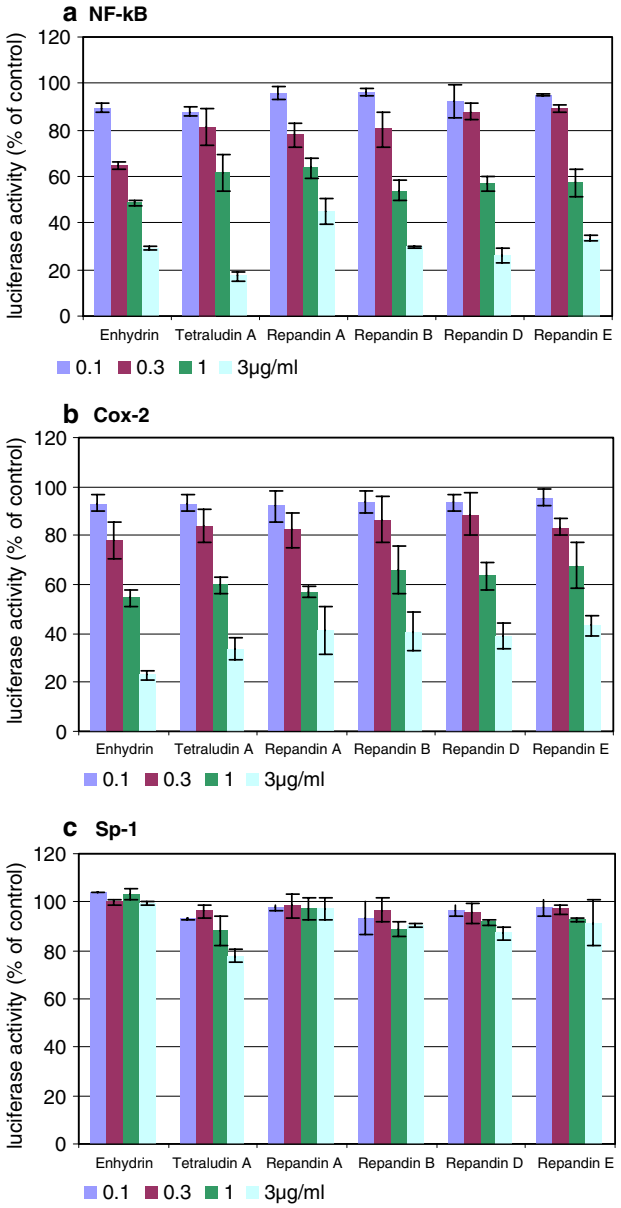


Fig. 3 Effect of melampolides and repandolides on TNF-α induced NF-κB (**a**), Cox-2 (**b**) promoter and Sp-1 (**c**) mediated transcription in SW1353 cells

output. None of these compounds inhibited Sp-1 dependent luciferase expression (Figs. 2c, 3c).

To determine whether melampolides and repandolides inhibited COX-2 enzyme activity, COX-2 mediated production of PGE_2 from exogenous arachidonic acid was measured in macrophages. Enhydrin and repandin A, and repandin E inhibited COX-2 mediated production of PGE_2 in LPS induced macrophages in a concentration-dependent manner with IC_{50} values of 23.0, 11.0 and 3.8 µM (Table 2). Tetraludin A, repandin B and repandin D did not affect COX-2 activity up to a concentration of 50 µM. This suggested that enhydrin, repandins A and E exert pharmacological effects by inhibiting both the activation of NF-κB and COX-2 enzyme activity, whereas tetraludin A, repandins B and D exert pharmacological activity by mainly inhibiting activation of NF-κB as also shown previously for some sesquiterpene lactones (diversifolin and derivatives) that inhibited NF-κB, but did not influence COX-2 enzyme activity [21]. Repandin A and E inhibited NF-κB and COX-2 activity, suggesting their mechanisms are different from other sesquiterpene lactones.

Table 1 Inhibition of NF-κB and COX-2 promoter mediated transcription by enhydrin, tetraludin A and repandins A, B, D and E in human chondrosarcoma (SW1353) cells induced with PMA or TNF-α

Test compounds	NF-κB		COX-2		Sp-1	
	PMA	TNF-α	PMA	TNF-α	PMA	TNF-α
Enhydrin	0.6 ± 0.0	1.9 ± 0.3*	2.6 ± 0.1**	4.3 ± 0.5*	NA	NA
Tetraludin A	2.4 ± 0.3	3.0 ± 0.4	5.8 ± 1.3**	5.5 ± 0.4**	NA	NA
Repandin A	2.6 ± 0.3	6.6 ± 1.1*	3.2 ± 0.3	7.0 ± 1.1*	NA	NA
Repandin B	1.1 ± 0.5	2.4 ± 0.6*	2.6 ± 0.3**	4.3 ± 0.1*, **	NA	NA
Repandin D	1.1 ± 0.2	2.7 ± 0.1*	2.5 ± 0.4**	4.0 ± 0.5*, **	NA	NA
Repandin E	1.3 ± 0.1	2.1 ± 0.1*	2.6 ± 0.2**	4.2 ± 0.4*, **	NA	NA

Values are IC50s in μM ± SD

NA no activity

*Significantly different from PMA induced cells ($p < 0.05$). **Significantly different from inhibition of NK-κB mediated transcription ($p < 0.05$)

Table 2 Effect of enhydrin, tetraludin A, repandin A, B, D and E on COX-2 enzyme activity in mouse macrophages (RAW 264.7)

Test compound	IC_{50} (μM)
Enhydrin	23.1 ± 2.3
Tetraludin A	NA
Repandin A	11.3 ± 2.3
Repandin B	NA
Repandin D	NA
Repandin E	3.8 ± 0.5

NA no activity

Furthermore, the growth inhibitory activities of melampolides and repandolides against a panel of human leukemia and solid tumor cell lines were evaluated. Enhydrin and repandin A, B, D and E inhibited the growth of HL-60, SK-MEL, KB, MCF-7, BT 549 and SK-OV3 cells in a concentration-dependent manner. The IC_{50} values are given in Table 3. In general, enhydrin, and repandin B, D and E showed stronger activity than tetraludin A and repandin A.

To study the effects of melampolides and rependolides on cell cycle arrest and apoptosis, enhydrin and repandin D were selected to further study their effect on the cell cycle. Treatment of HL 60 cells with repandin D (3 μM) for 12 h resulted in the accumulation of cells in G_2/M phase, with a corresponding decrease in the number of cells in G_1 (Fig. 4). At 48 h of drug treatment, most of the cells were dead, characterized by a dramatic increase in the hypodiploid rate (a peak before G_1). Similar effects were observed with enhydrin (data not shown). DAPI staining assay showed that cells treated with enhydrin or repandin D exhibited condensed and fragmented nuclei which are indicative of apoptosis. Representative figure is shown for repandin D (Fig. 5).

Based on the above, we conclude that enhydrin, tetraludin A, repandin A, B, D and E exhibit cell growth inhibition and induction of cell cycle arrest and apoptosis. These effects may be related to inhibition of NF-κB activation, which is consistent with previously reported anti-inflammatory activities of other sesquiterpene lactones provided by other investigators [13, 15, 21, 25]. However, repandin A and E also showed the inhibition of COX-2 enzyme activity, which is different from the other sesquiterpane lactones. In addition, although repandin A and E are structurally similar to repandin B and D, their activity on COX-2 is different. The relationship between the structure and activity of these repandins will be studied in detail.

With regard to the mechanism of action of melampolides and repandolides as regulators of NF-κB mediated transcription it was interesting to note that enhydrin,

Table 3 Inhibitory effects of enhydrin, tetraludin A, repandin A, B, D and E on the growth of a panel of human tumor cell lines in vitro (48 h drug exposure)

Cell line	IC_{50} (μM)					
	Enhydrin	Tetraludin A	Repandin A	Repandin B	Repandin D	Repandin E
HL-60	0.7 ± 0.1	1.3 ± 0.3	1.5 ± 0.1	0.3 ± 0.4	0.4 ± 0.0	0.4 ± 0.1
SK-MEL	13.2 ± 4.2	>22	>19	>19	10.6 ± 1.3	5.7 ± 0.8
KB	3.7 ± 0.6	5.1 ± 0.7	12.1 ± 0.3	4.5 ± 0.4	4.0 ± 0.3	5.0 ± 0.9
MCF-7	3.2 ± 0.4	7.0 ± 0.4	6.2 ± 0.1	3.6 ± 0.3	2.1 ± 0.0	3.4 ± 0.3
BT 549	1.3 ± 0.2	5.3 ± 0.3	6.6 ± 1.2	3.8 ± 0.4	2.7 ± 0.9	3.4 ± 0.7
SK-OV3	3.2 ± 0.7	4.2 ± 0.5	7.0 ± 0.5	3.0 ± 0.9	2.3 ± 0.5	3.2 ± 0.3

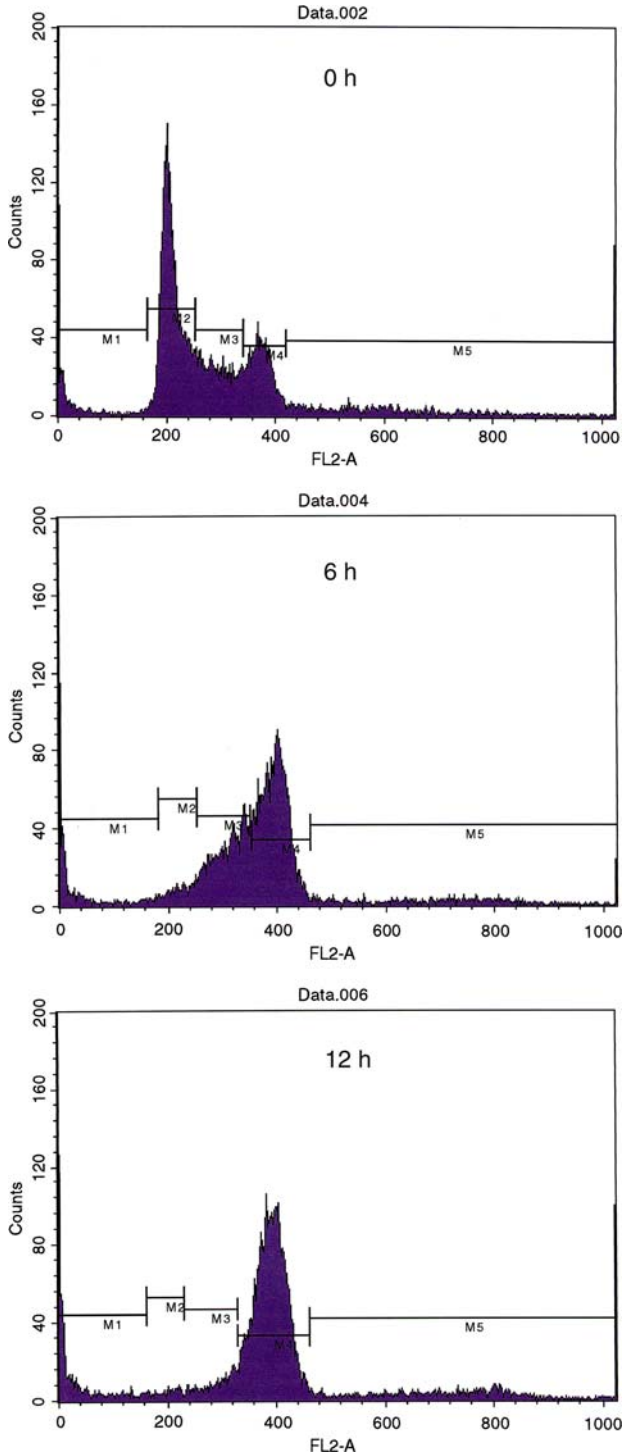


Fig. 4 Flow cytometry histograms of cell cycle distribution of HL 60 cells after treatment with repandin D (3 μM) in comparison to vehicle control (0.5% DMSO)

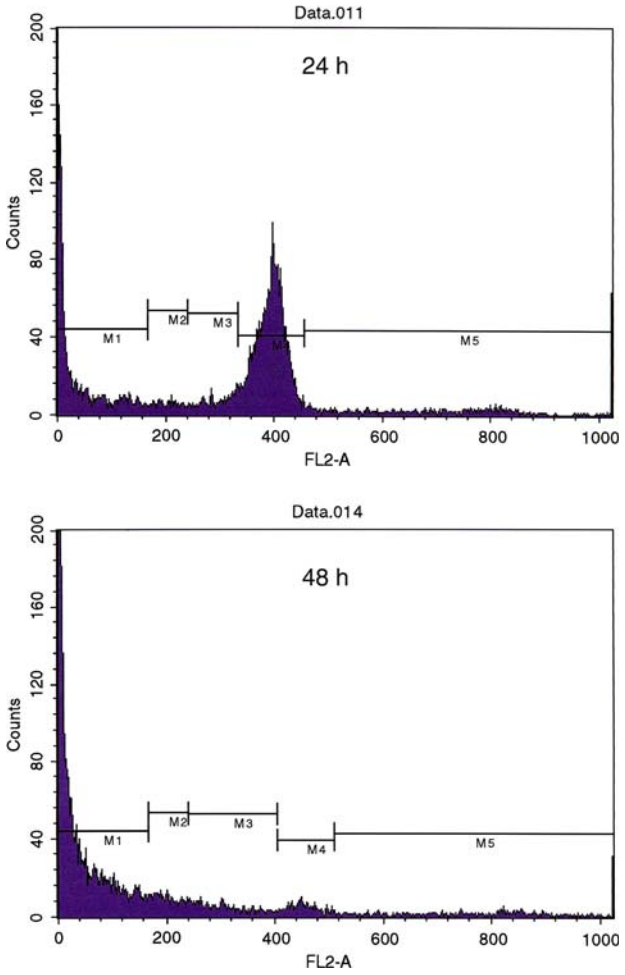


Fig. 4 continued

tetraludin A and repandins A, B, D and E inhibited the NF-κB-mediated transcription induced by either PMA or TNF-α, indicating that these lactones target a common step in these signal transduction pathways. Hehner et al. [10] reported that the SL parthenolide inhibited the activation of NF-κB by preventing the degradation of IκB-α and IκB-β. Koo et al. [13] also reported that constunolide and parthenolide suppressed NF-κB by targeting IκB phophorylation. However, Lyss et al. [15] provided a contradictory explanation that the sesquiterpene lactone helenalin neither inhibits IκB degradation nor prevents NF-κB nuclear translocation, but selectively alkylates the p65 subunit of NF-κB. Recent study further confirmed that parthenolide and other SLs act directly on NF-κB by alkylating cystein residue (Cys 38) in its p65 subunit. Although a slight inhibition of IκB degradation was detected for all SLs, the amount of remaining IκB was too low to explain the observed NF-κB inhibition [9, 21]. Many earlier studies showed that SLs containing α, β-unsaturated carbonyls such as α-methylene-γ-lactones or α, β-unsubstituted cyclopentenones are important. These functional groups are known to react with nucleophiles, especially with cystein sulfhydryl groups, in a Micheal-type addition [14, 20]. Enhydrin, Tetraludin A and repandin A, B, D and E contain α-methylene-γ lactone moiety, therefore,

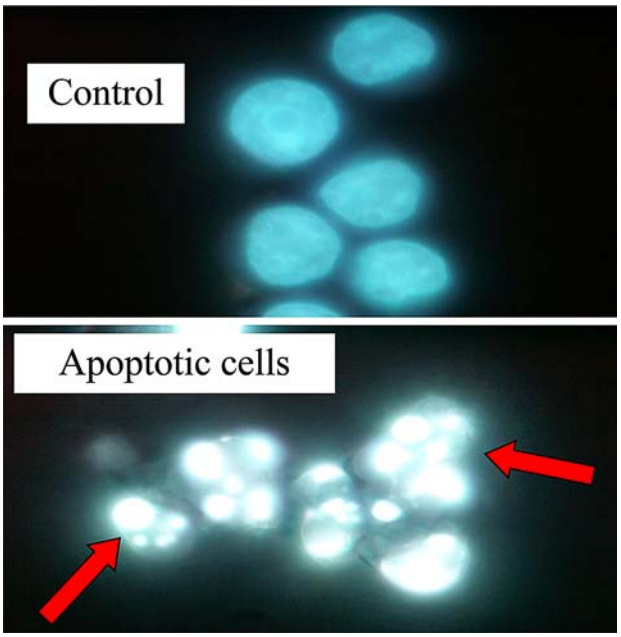


Fig. 5 Induction of apoptosis by repandin D in HL 60 cells. Cells were treated with vehicle (0.5% DMSO) or repandin D (3 μM) for 48 h, harvested and washed with ice-cold PBS, followed by fixation in 3.7% formaldehyde and methanol. Fixed cells were incubated with 1 μg/mL of DAPI, and nuclear morphology was examined by using a fluorescent microscope. *Arrow* indicates apoptotic cells with condensed and fragmented nuclei

it is likely that these lactones could alkylate directly cystein sulfhydryl groups in the p65 subunit of NF-κB, preventing DNA binding of active NF-κB. Further investigations are warranted for identification of alkylation of cystein sulfhydryl group by enhydrin, tetraludin A and repandins.

Since apoptosis is required for proper tissue homeostasis, defects in apoptosis signaling pathway contribute to carcinogenesis and chemoresistance. Thus apoptosis inducing activity seems to play significant role in the anticancer effects of these compounds similar to the action of parthenolide and helanalin described earlier [7, 29].

In conclusion, enhydrin, tetraludin A, and repandin A, B, D, and E suppressed the growth of tumor cells, induced a cell cycle arrest in G_2/M phase and apoptosis along with the inhibition of NF-κB and COX-2 promoter mediated transcription and inhibition of COX-2 activity. These results are in agreement with previously reported anti-inflammatory activities of other SLs. The precise mechanism will be further studied, and the relationship between the structure and activity of these SLs also needs to be studied in more detail.

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