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Influence of helenanolide-type sesquiterpene lactones on gene transcription profiles in Jurkat T cells and human peripheral blood cells: anti-inflammatory and cytotoxic effects

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Abstract

Sesquiterpene lactones (SLs) are known to have potent anti-inflammatory and cytotoxic properties. So far, the anti-inflammatory effects have mainly been attributed to their inhibition of DNA-binding of the transcription factor NF-κB (p65), which is a pivotal regulator of the cellular immune response. Since NF-κB is involved in the transcriptional control of several pro-inflammatory and regulatory genes, we investigated the effects of one bifunctional NF-κB (p65) inhibiting and two monofunctional NF-κB (p65) inactive helenanolide-type SLs on PMA and LPS-induced mRNA expression in $CD4^+$ Jurkat T and human peripheral blood mononuclear cells (PBMCs) with reverse transcription real-time PCR (RT-rt-PCR). The monofunctional SLs 11α,13-dihydrohelenalin acetate (DHAc) and chamissonolide significantly reduced mitogen-induced cytokine and iNOS mRNA levels in PBMCs and Jurkat T cells at low micromolar concentrations. DHAc also showed significant effects on the gene expression of the house-keeping genes GAP-DH and β-actin, as well as on NF-ATc, p65, I-κBα, bcl-2, and cyclin D1. The bifunctional NF-κB inhibitor helenalin not only effectively inhibited pro-inflammatory gene expression, but also strongly down-regulated all investigated mRNA levels in a time-dependent manner. Flow cytometry and caspase-8 and -3 assays revealed that helenalin strongly and DHAc moderately induced apoptosis in Jurkat T cells, whereas chamissonolide caused cytoprotective effects. In PBMCs, DHAc and chamissonolide did not inhibit NF-κB (p65) DNA-binding at concentrations effective on the transcriptome. Thus, it can be concluded that the biological effects of SLs are not only due to NF-κB inhibition, but must be coupled to other mechanisms.

Keywords: Sesquiterpene lactones; *Arnica*; Gene transcription; Anti-inflammatory effects; Apoptosis; Chamissonolide

1. Introduction

SLs, most widely distributed within the Asteraceae, form one of the largest groups of bioactive compounds of plant origin. It is generally accepted that most of their biological effects, e.g. cytotoxicity and anti-inflammatory activity, are mediated by alkylation of biological nucleophiles. The α,β-unsaturated carbonyl structures of SLs, such as α-methylene-γ-lactone and α,β-en-one groups, react with the sulfhydryl moieties of enzymes and functional proteins via a Michael type addition and thus interfere with the function of these macromolecules [1–3] (Fig. 1).

In particular, the potent cytotoxic and anti-inflammatory actions of SLs have received considerable attention for decades, and several studies have revealed the potent anti-inflammatory potential of SLs *in vitro* and *in vivo*. In the last years it has been shown that different SLs potently inhibited DNA-binding of the transcription factor NF-κB (p65) [4–9]. Because NF-κB is able to promote the expression of many pro-inflammatory genes the current opinion states that the inhibition of the activation of NF-κB is one of the main anti-inflammatory mechanism of action of SLs.

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Abbreviations: SLs, sesquiterpene lactones; PBMCs, peripheral blood mononuclear cells; RT-rt-PCR, reverse transcription real-time polymerase chain reaction; TNF-α, tumour necrosis factor; iNOS, inducible nitric oxide synthase; DHAc, 11α,13-dihydrohelenalin acetate; DEPC, diethyl pyrocarbonate; FAM, 6-carboxy-fluorescein; TAMRA, 6-carboxy-tetramethyl-rhodamine; C_T, threshold cycle number; PBS, phosphate-buffered saline; VCAM-1, vascular cell adhesion molecule-1; GM-CSF, granulocyte macrophage colony-stimulating factor; PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharides; FACS, fluorescence-activated cell sorter; ELISA, enzyme-linked immunosorbant assay.

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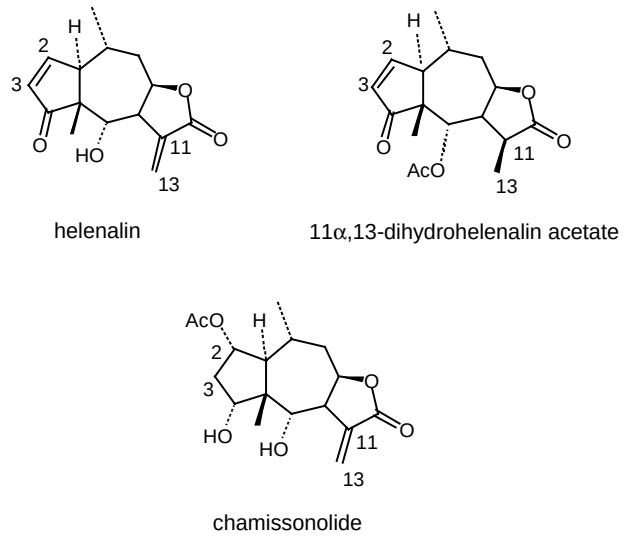

Fig. 1. Structures of the investigated sesquiterpene lactones.

A strong line of evidence suggests that certain SLs alkylate NF-κB p65 on the cysteine residue and so prevent NF-κB translocation to the nucleus [6,7]. It was further postulated that SLs inhibit NF-κB by preventing the degradation of I-κBα and I-κBβ [8]. Other studies have shown that SLs directly bind and inhibit I-κB kinase (IKKβ) and thereby disturb signalling that normally leads to the ubiquitination and subsequent proteasome-mediated degradation of I-κB [9].

To make a further contribution to clarify the molecular mechanism by which especially monofunctional SLs may exert their anti-inflammatory activity we investigated the effects of the bifunctional SL helenalin and the monofunctional 11α,13-dihydrohelenalin acetate and chamissonolide on NF-κB p65 binding, PMA- and LPS-induced pro-inflammatory mRNA levels, as well as their effects on house-keeping and regulatory mRNAs in human PMBCs and Jurkat T cells. All three compounds have previously been shown to cause anti-inflammatory effects *in vitro* and helenalin and DHAc also *in vivo*, helenalin being the most active [10,11]. Together with many other pseudoguaianolide-type SLs, they constitute the active principle of the alcoholic extracts from *Arnica montana* and *A. chamissonis* ssp. *foliosa* [12]. The mRNA levels of the cytokines IL-2, IL-6, GM-CSF, IFN-γ, the inducible nitric oxide synthase (iNOS), the transcription factors (or mRNAs related to transcription factors) NF-ATc, I-κBα, p65, and the cell homeostasis factors cyclin D1, bcl-2, β-actin and GAP-DH were relatively quantified by reverse transcription real-time PCR (RT-rt-PCR) as reported previously [13].

In addition, it is well known that SLs exhibit a cytotoxic potential [14,15]. Since NF-κB also is involved in the regulation of cell death, e.g. by preventing apoptosis, it is not surprising that NF-κB inhibiting compounds like SLs might be cytotoxic [16,17]. Recently, it was shown that the bifunctional SL helenalin triggers apoptosis in Jurkat T cells [16]. To date, most studies have treated the anti-inflammatory and cytotoxic effects as more or less independent mechanisms, and no study has addressed both effects in the same experiment. Furthermore, most investigations about the cytotoxicity of SLs have been carried out with neoplastic cell lines and there are only few reports on the toxicity of SLs on primary cell cultures [18]. To differentiate between primary and neoplastic cells seems to be important because also cytoprotective effects of SLs have been described in primary cell cultures [19]. This prompted us to assess the cytotoxicity kinetics and the anti-inflammatory effects of the compounds in both PBMCs and Jurkat leukemia T cells. Due to the fact that an apoptotic effect has only been described for helenalin [16], we also determined whether the observed significant cytotoxicity of DHAc in Jurkat T cells was mediated by apoptosis, measuring caspase-8 and -3 induction, as well as by applying flow cytometry [20].

2. Materials and methods

2.1. Isolation of PBMCS

Fresh citrate (A^+) whole blood from healthy donors without medication (Blutspendedienst Zurich) was maintained at 4° and separated on an Axis Shield density gradient medium (Polymorphprep™), according to manufacturers protocol. The mononuclear cell fraction was isolated. To minimize variability the PBMCs of four different donors were pooled. The isolated PBMCs were washed twice in PBS and immediately suspended in RPMI 1640 medium as described below.

2.2. Cell culture

$CD4^+$ Jurkat human leukemia T cells (ATCC TIB-152) and PBMCs were maintained in RPMI 1640 medium (Gibco, Life Technologies), supplemented with 10% fetal bovine serum (FBS), 1 μg/mL fungizone (amphotericin B), 100 units/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine (all from Gibco, Life Technologies) at 37° and 5% CO_2 in 75 cm^2 culture flasks (TPP). 5×10^5 cells from a log-culture were suspended with 1 mL of fresh medium (in each well) in a 24 well plate and left to rest for 3 hr. The respective amount of SL (stock solution in $EtOH/H_2O$) was then added to the cells and left for 1 hr before stimulation. For mitogen activation 1 μg PMA (ICN) or 2.5 μg lipopolysaccharides (LPS, Sigma) was added to each well containing 1 mL cell suspension. The total solvent part never exceeded 0.5% and no effects were detected with a solvent control only. To asses whether the mitogen-induced expression of pro-inflammatory genes was influenced by the SLs, 10 μM of helenalin, DHAc, and chamissonolide were co-incubated for 2.5,

5, and 20 hr. 2.5 μM cyclosporin A (Fluka) was used as a positive reference compound [13]. To diminish variability and pipetting errors, three wells were finally pooled for RNA extraction as one experiment with 1.5×10^6 cells.

2.3. RNA extraction and reverse transcription

Cells were harvested and centrifuged. All pipette tips, plastic tubes and glassware were treated with diethyl pyrocarbonate (DEPC) to destroy ribonucleases. Total cytoplasmatic RNA was isolated from cells, using RLN buffer (Qiagen) and RNeasy® spin columns (Qiagen), according to manufacturers instructions, and dissolved in 50 μL deionized and DEPC-treated water. RNA was checked for DNA contamination on an ethidium bromide-stained 2.5% agarose gel and measured spectrophotometrically at 260 nm. Approximately 10 μg total RNA (equal volumes for all experiments) was incubated with random hexamers (Microsynth) and 30 units/mL ribonuclease inhibitor RNasin® (Promega) at 60° for 5 min and cooled stepwise to 20°. Final random hexamer concentration was 3 μM. 100 units/mL reverse transcriptase Omniscript® (Qiagen) and dNTPs (10 mM each) (Perkin Elmer) were added and reverse transcription took place at 37° for 60 min in a Gene Amp™ 2400 thermocycler (Perkin-Elmer). All samples were reverse transcribed under the same conditions and from the same reverse transcription master mix in order to minimise differences in the reaction efficiency.

2.4. TaqMan® real-time PCR and determination of relative mRNA amounts

Relative mRNA expression profiles were determined without normalizing to any specific gene because equal test cell populations showed very little variability in their mRNA levels. Relative mRNA levels could therefore be calculated between treated and non-treated Jurkat T cell populations. The house-keeping genes were used as controls to determine the degree of pharmacological specificity as described previously [13]. The TaqMan® technology employed for our RT-rt-PCR made use of labelled oligonucleotide probes and a 5′-nuclease PCR assay. The probe had a reporter dye FAM and a quencher dye TAMRA covalently attached to the 3′-end via a linker group. The reporter generated a fluorescent signal upon cleavage by the Taq polymerase. Quantification was based on the early, linear part of the reaction, and on determining the threshold cycle (C_T), at which fluorescence above background was first detected. 5 μL of each cDNA sample was mixed with Universal PCR Master Mix (PE Biosystems) and depending on the gene between 100 and 300 nM primers and probe were added to obtain a 25 μL reaction. Real time PCR was performed in 96-well reaction plates and optical caps (BioLabo). The amplification efficiency for each specific primer and probe template system was calculated on the ABI PRISM™ 7700 real time thermocycler (PE Biosystems) by measuring the increase in signal with each cycle. Negative controls with total RNA or no template were performed. Serial dilutions of the RNA were reverse transcribed to provide the standard curve. This was achieved by plotting the dilution factor against the C_T values as described previously [13]. At least four independent experiments and eight reverse transcriptions were performed. This gave a mean value for the mRNA expression level of each gene. To calculate the fold mRNA difference between the SL-treated cells and the untreated cell population, the C_T values derived from the untreated PMA-stimulated reactions were taken as reference. A theoretical zero amount of cDNA after cycle 34 was assumed, and C_T values >34 were not calculated. Data analysis was performed with Microsoft Excel.

2.5. Cell viability testing

The cytotoxicity of the pure compounds against Jurkat $CD4^+$ T cells (ATCC TIB-152), and peripheral blood mononuclear cells (PBMCs) was determined after 2.5, 20 and 72 hr in a WST-1 based cell viability assay as previously described [13]. All compounds were tested in a concentration range between 0.5 and 80 μM. Maximal standard deviation was 10% (absolute).

2.6. Measurement of NF-κB DNA-binding inhibition

5×10^5 cells were suspended with 1 mL of fresh medium (in each well) in a 24 well plate and left to rest for 3 hr. The respective amount of SL (stock solution in $EtOH/H_2O$) was then added to the cells and left for 1 hr before stimulation. For mitogen activation 1 μg PMA was added to each well containing 1 mL cell suspension. The total solvent part never exceeded 0.5% and no effects were detected with a solvent control only. To assess whether the mitogen-induced NF-κB DNA-binding was influenced by the SLs, 2, 5 and 20 μM of helenalin, DHAc, and chamissonolide were co-incubated. 5 μM gliotoxin was used as a positive control. Total stimulation time was 2 hr. To measure the NF-κB p65 binding in PBMCs we used an ELISA-based colorimetric Mercury™ Transfactor NF-κB p65 Kit, which has been shown to be 10-fold more sensitive than conventional electrophoretic mobility shift assays (EMSA) (2002 BD Clontech product catalogue). Nuclear extracts were prepared with the BD Clontech TransFactor Extraction Kit (PT3612). We followed the manufacturers instructions and added nuclear extracts to the plates precoated with the p65 DNA-binding consensus sequence. A primary antibody specific to p65 is added followed by a horseradish peroxidase-conjugated secondary antibody and substrate for the colour reaction. The Kit contained the positive control nuclear extracts. Signal was detected in a standard microtiter plate reader.

2.7. Measurement of apoptosis

Apoptosis was measured in Jurkat T cells by three distinct methods. (1) Optical analysis of cell death, (2) cell size and granularity analysis by flow cytometry, (3) activity in caspase-8 and -3 assays. (1) 2×10^6 cells/mL were incubated individually with each 10 μM of helenalin, DHAc, and chamissonolide for 20 hr. A negative (untreated) control was included. Cells were harvested and counted in a haemocytometer in the presence of 0.5% trypan blue. Cells counted as apoptotic included cells with characteristic nuclear chromatin condensation and fragmentation, as well as dead cells that had lost trypan blue exclusion capacity. (2) Another fraction of the cells

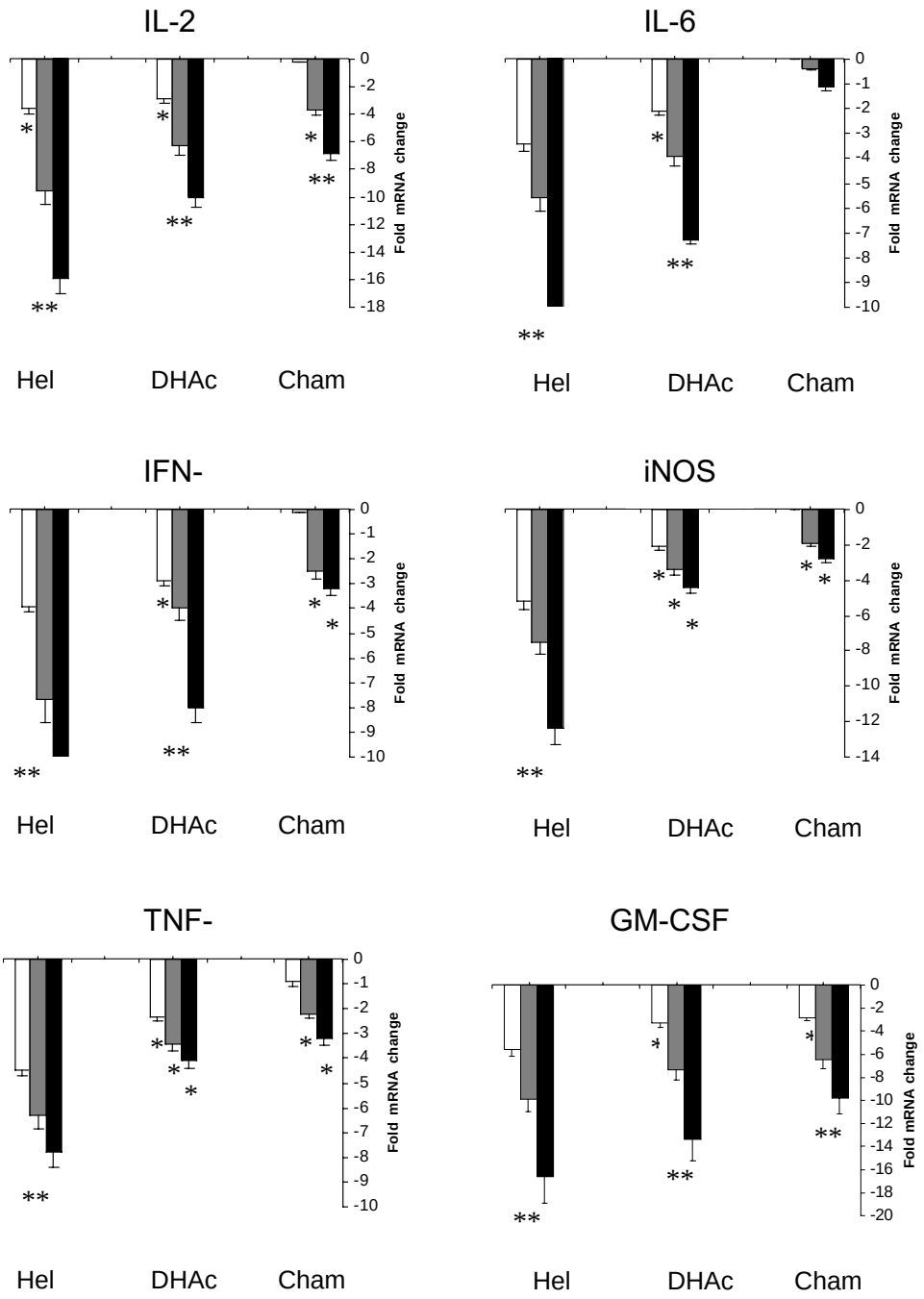

Fig. 2. Modulation of mRNA levels in PBMCs by helenalin (Hel), DHAc, and chamissonolide (Cham). Fold change (relative to control) of mRNA levels of cytokines: IL-2, IL-6, IFN-γ, TNF-α, GM-CSF; and iNOS, in PBMCs (1.5×10^6 cells) induced by 1 μg/mL PMA. Cells were treated with 10 μM of the respective SL for 2.5 hr (white bars), 5 hr (dark grey bars) and 20 hr (black bars). Total RNA was reverse transcribed and cDNAs measured with TaqMan® real time PCR. Data shown as mean values (no. of experiments = 5) ± SEM, $^{*}P < 0.05$, significantly different from controls. $^{**}P < 0.005$, significantly different from controls.

was washed in cold phosphate-buffered saline (PBS) and cell density was adjusted to 10^6 cells/mL and analysed for granularity by flow cytometry. Data show representative ungated data from four independent experiments. (3) For the caspase-8 and -3 assays (ApoAlert® Clontech) SL-treated (10 and 20 μM) and control cell populations (2×10^6/mL) were centrifuged and resuspended in 50 μL chilled lysis buffer and incubated for 10 min on ice. Centrifuged cytosol extract was diluted to a concentration of 100 μg protein per 50 μL of cell lysis buffer. For controls, positive samples were incubated with caspase inhibitors (DEVD-fmk) before adding substrates. Reaction buffer contained DTT. Colorimetric detection of protease activities was carried out according to user

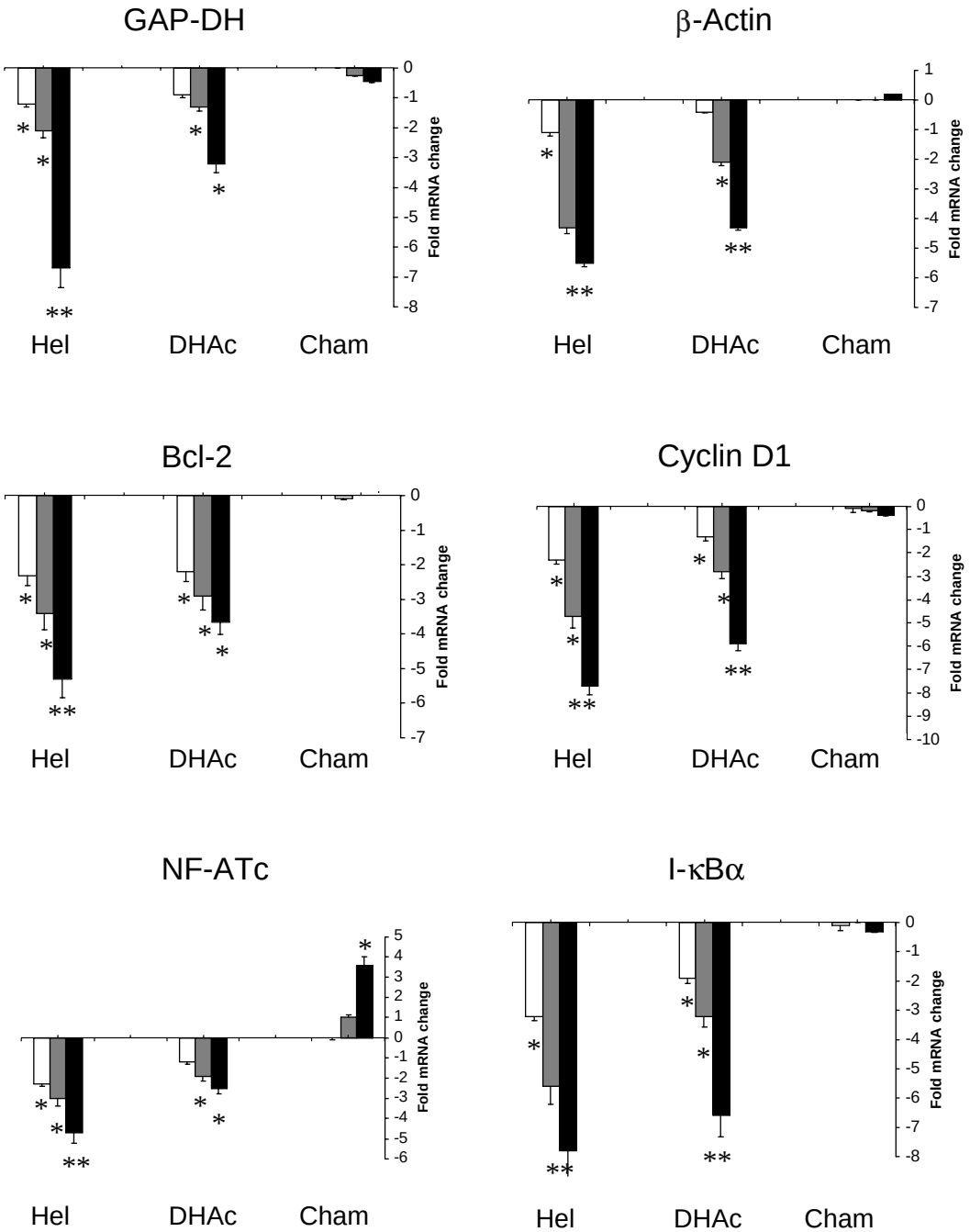

Fig. 3. Modulation of mRNA levels in PBMCs by helenalin (Hel), DHAc, and chamissonolide (Cham). Fold change (relative to control) of mRNA levels; house keeping genes: GAP-DH, β-actin; transcription factors: NF-ATc, IκBα; cell homeostasis factors: bcl-2, cyclin D1 in PBMCs (1.5×10^6 cells) induced by 1 μg/mL PMA. Cells were treated with 10 μM of the respective SL for 2.5 hr (white bars), 5 hr (dark grey bars) and 20 hr (black bars). Total RNA was reverse transcribed and cDNAs measured with TaqMan® real time PCR. Data shown as mean values (no. of experiments = 5) ± SEM, $^{*}P < 0.05$, significantly different from controls. $^{**}P < 0.005$, significantly different from controls.

manuals (Clontech K2027-1 and K2029-1) in a 96-well format at 405 nm. Maximal standard deviation was 15% (absolute).

2.8. Test compounds

Sesquiterpene lactones were isolated from *Arnica* species [for Lit. see [21]]. Gliotoxin was purchased from Fluka Biochemica. Purity of the SLs were checked by TLC and HPLC and was >99%.

2.9. Statistics

The Student's *t*-test was used to assess the statistical significance of differences. A confidence level of <0.05 was considered significant.

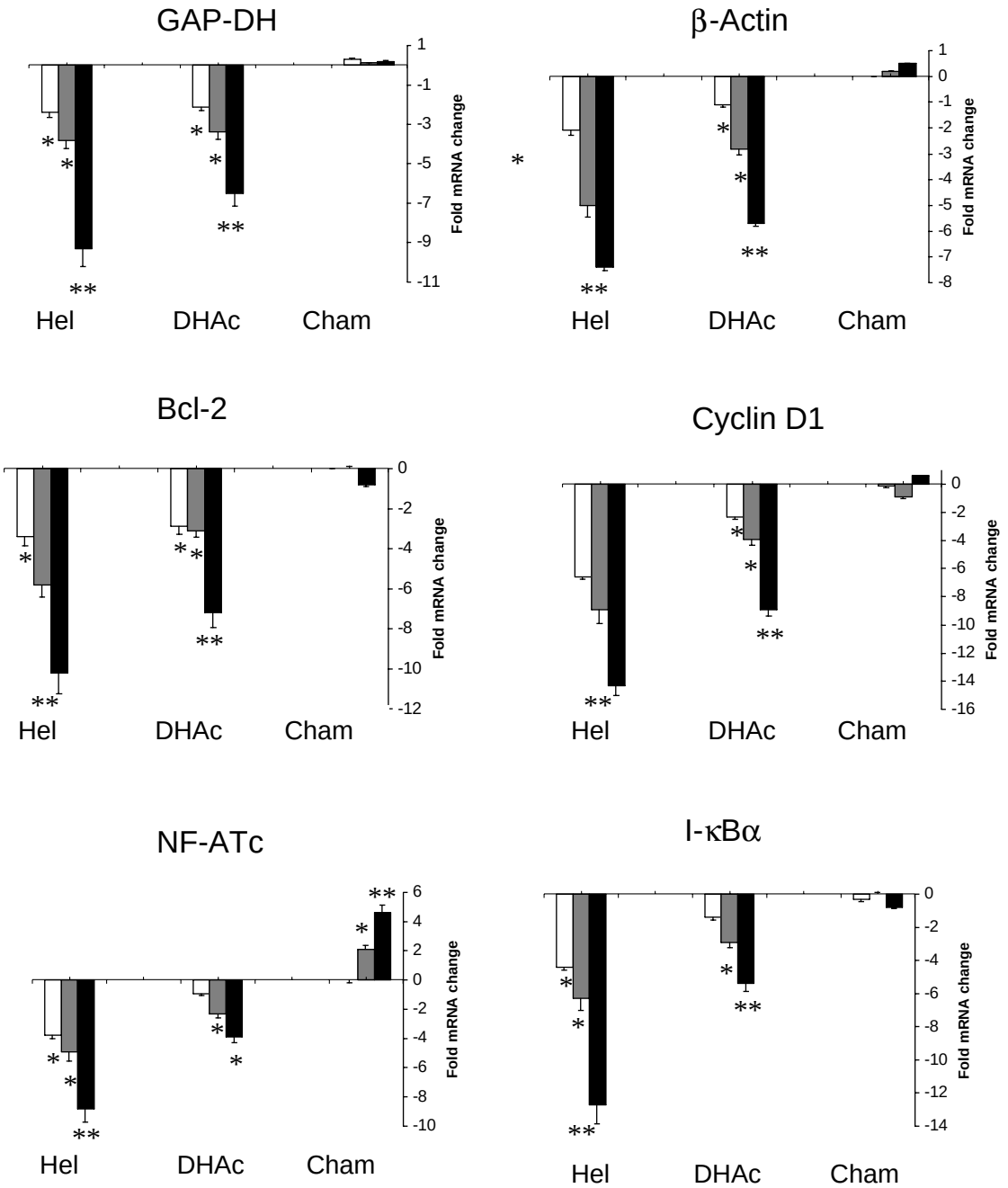

Fig. 4. Modulation of mRNA levels in Jurkat T cells by helenalin (Hel), DHAc, and chamissonolide (Cham). Fold change (relative to control) of mRNA levels; house keeping genes: GAP-DH, β-actin; transcription factors: NF-ATc, IκBα; cell homeostasis factors: bcl-2, cyclin D1 in Jurkat T cells (1.5×10^6 cells) induced by 1 μg/mL PMA. Cells were treated with 10 μM of the respective SL for 2.5 hr (white bars), 5 hr (dark grey bars) and 20 hr (black bars). Total RNA was reverse transcribed and cDNAs measured with TaqMan real time PCR. Data shown as mean values (no. of experiments = 5) ± SEM, $^{*}P < 0.05$, significantly different from controls. $^{**}P < 0.005$, significantly different from controls.

3. Results

3.1. Differential down-regulation of PMA-induced mRNA levels in Jurkat T cells and PMBCs by SLs

PMA treatment of Jurkat T- and PBM cells time-dependently increased cytokine and transcription factor steady-state mRNA levels as reported previously [13,22]. At 10 μM all three SLs induced a significant and time-dependent relative modulation of the cytokine mRNA levels in both cell types. Helenalin most potently inhibited the up-regulation of IL-2, IL-6, GM-CSF, TNF-α, IFN-γ, and iNOS mRNAs (Fig. 2, data from PBMCs) in a time-dependent manner. The strong relative down-regulation of the pro-inflammatory genes was connected to significant effects on the cell homeostasis factors cyclin D1, bcl-2, β-actin and GAP-DH in both cell types (Fig. 3, data from PBMCs), indicating a low degree of specificity. The effect of DHAc on the gene transcription profile was similar in quality, but significantly weaker when compared to helenalin (Figs. 2 and 3). At 10 μM, DHAc significantly down-regulated all transcripts investigated. The differential modulation of the mRNA levels by the investigated SLs was most obvious after short (2.5 hr) treatment with chamissonolide. Chamissonolide caused a down-regulation of the cytokine mRNA levels, but in contrast to helenalin and DHAc, had no significant effect on IL-6 and TNF-α mRNA (Fig. 2). Interestingly, chamissonolide did not influence the house-keeping genes β-actin and GAP-DH, cyclin D1 and I-κBα and bcl-2 at the transcriptome level. Furthermore, NF-ATc mRNA was significantly up-regulated by chamissonolide after 20 hr in Jurkat T cells and PBMCs (Figs. 3 and 4). Comparing the results obtained in Jurkat T cells and PBMCs revealed a close similarity for the down-regulation of the pro-inflammatory genes by all SLs (data not shown), but a significantly stronger down-regulation of the cell homeostasis factors cyclin D1, bcl-2, β-actin and GAP-DH in Jurkat T cells by helenalin and DHAc (Fig. 4). Using TNF-α and three different concentrations of each SL (5, 10, 20 μM) as an example it was shown that the influence on the transcription is also concentration dependent for all compounds (Fig. 5). The use of LPS instead of PMA as stimuli showed that the observed effects were comparable in quality to the results obtained after PMA stimulation in PBMCs, but LPS did not significantly induce pro-inflammatory gene expression in Jurkat T cells (data not shown).

3.2. NF-κB DNA-binding in PBMCs

Helenalin was found to be a strong inhibitor of PMA-induced NF-κB p65 DNA-binding in PBMCs after 2 hr stimulation, exhibiting an IC_{50} of <5 μM. At 2.5 μM helenalin already produced a significant inhibition (≈40%) of p65 binding. In contrast, DHAc and chamissonolide did not show any inhibition at 5 μM and only DHAc showed a significant inhibition at 20 μM (≈20%) (Fig. 9). The positive control gliotoxin potently inhibited NF-κB p65 DNA-binding at low micromolar concentrations ($IC_{50} < 2.5$ μM).

3.3. Lower cytotoxicity of helenanolide type SLs in PBMCs cultures

When we compared the cell viabilities of Jurkat T cells and PBMCs upon treatment with helenanolide-type SLs it became obvious that all three compounds were less cytotoxic against PBMCs (Fig. 6). A concentration of 10 μM helenalin rapidly killed residual (5–10%) erythrocytes in the PBMC culture, as judged by microscopic observation, but after 2.5 hr the cell viability of the PBMCs was not significantly impaired. Significant reduction of cell viability by 10 μM helenalin was first observed after 10 hr (data

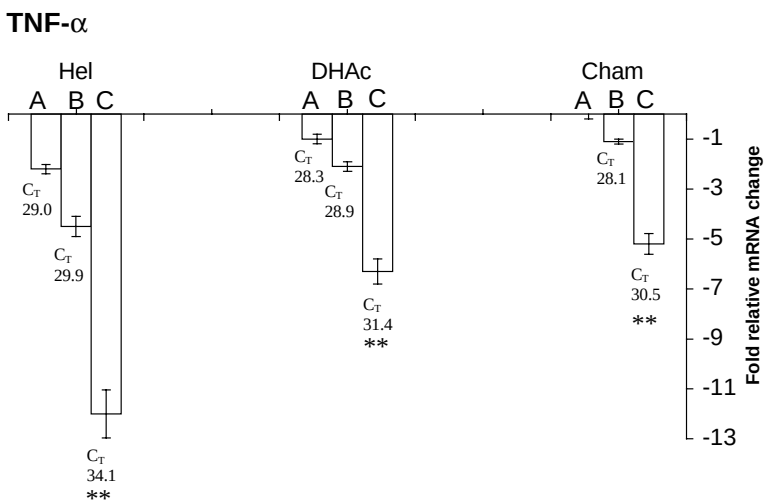

Fig. 5. Concentration-dependent down-regulation of PMA-induced TNF-α mRNA in PBMCs. (A) 5 μM, (B) 10 μM, (C) 20 μM of sesquiterpene lactones. Total RNA was reverse transcribed and cDNAs measured with TaqMan® real time PCR. Data shown as mean values (no. of experiments = 4) ± SEM. Mean C_T values indicate relative mRNA levels. $^{*}P < 0.05$, significantly different from controls. $^{**}P < 0.005$, significantly different from controls.

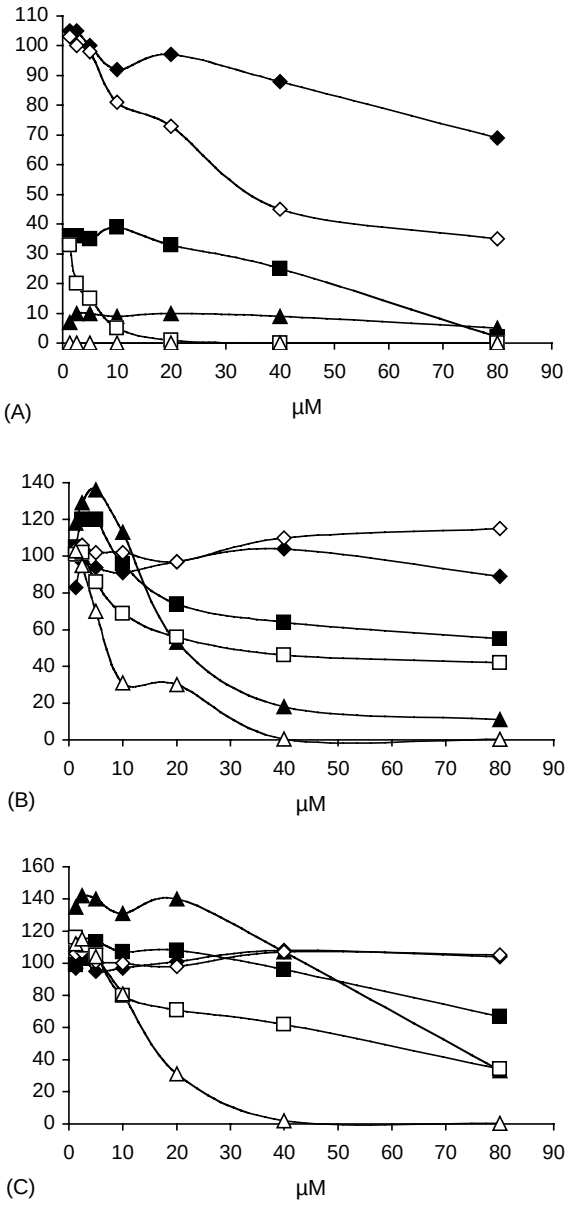

Fig. 6. Time-dependent cytotoxicity of helenalin (A), DHAc (B), and chamissonolide (C) in Jurkat T and PBM cells. 1.5×10^5 cells were seeded in each well and incubated with SLs in a concentration range of 0.5 and 80 μM. For quantification of the cell viability, 1, 18.5 and 70.5 hr after SL addition, 10 μL of WST-1 (4-(3-[4-iodophenyl]-2-[4-nitrophenyl]-2H-5-tetrazolio)-1,3-benzene disulfonate) solution (Roche) was added to each well and incubated for further 1.5 hr. Absorption was measured at a wavelength of 450 nm and a reference wavelength of 690 nm (N = 6). Open symbols: cytotoxicity in Jurkat T cells. Filled symbols: cytotoxicity in PBMCs. Rhombus: measurement after 2.5 hr; square, measurement after 20 hr; triangle, measurement after 72 hr.

not shown). Compared to helenalin, DHAc showed a later onset of the cytotoxicity (20 hr) and was less active (Fig. 6). Chamissonolide was not significantly cytotoxic against PBMCs up to 40 μM at all time-points measured. The onset of cytotoxicity against Jurkat T cells at higher concentrations of chamissonolide was observed between 20 and 72 hr, and thus at a later stage when compared to helenalin and DHAc (Fig. 6).

3.4. Induction of apoptosis by helenalin and DHAc in Jurkat T cells; anti-apoptotic effects of chamissonolide

The potent reduction of cell viability in Jurkat T cells by helenalin and DHAc (Fig. 6) was found to correlate with cell death. To assess whether the observed cell death was the result of apoptosis we carried out FACS granularity and cell size measurements, as well as caspase-3 and -8 assays. For FACS, 10 μM of each SL was incubated with Jurkat T cells for 20 hr along with an untreated control population. The control populations showed a great number of normal cells and a small number of apoptotic cells. Helenalin strongly induced apoptosis in Jurkat T cells (Fig. 7). DHAc also induced apoptosis but to a lesser degree. On the other hand, chamissonolide (10 μM) reduced the apoptotic cell populations (Fig. 7). The cell morphology of chamissonolide-treated Jurkat T cells was identical to untreated control populations. The caspase-3 and -8 assays showed a strong induction of caspase activity (43-fold induction of caspase-3 and 57-fold induction of caspase-8 at 10 μM, Fig. 10) by helenalin, and a moderate induction (18-fold induction of caspase-3 and 25-fold induction of caspase-8 at 10 μM, Fig. 10) by DHAc. At 20 μM the caspase induction was even more pronounced. Chamissonolide did not induce caspase-8 and -3 activity up to 20 μM (Fig. 10).

3.5. Different modulation of mRNA levels in PBMCs by helenalin and gliotoxin

In PBMCs, 10 μM of gliotoxin strongly inhibited PMA-induced mRNA up-regulation of all cytokines, and also potently down-regulated the house-keeping genes, as well as IκBα, NF-ATc and bcl-2. Interestingly, p65 was slightly up-regulated and cyclin D1 was not influenced. The up-regulated p65 mRNA level contrasts the down-regulation of IκBα and NF-ATc, and might therefore be a response to NF-κB inhibition (Fig. 8). However, this effect was not seen with helenalin. Contrary to gliotoxin, helenalin strongly inhibited PMA-induced cyclin D1 up-regulation in PBMCs.

4. Discussion

SLs represent the active principle of many anti-inflammatory remedies used in traditional medicine. The anti-inflammatory effects of extracts of *Arnica* flowerheads (externally used to treat haematomas, dislocations, rheumatic muscle complaints and other ailments related to inflammation [23]) are most probably mediated by helenalin, DHAc and chamissonolide derivatives and their corresponding esters. There are several studies addressing the anti-inflammatory effects caused by these compounds. It was shown that these compounds interfere with distinct cellular processes, including oxidative phosphorylation, platelet aggregation, histamine and serotonin release [23,24].

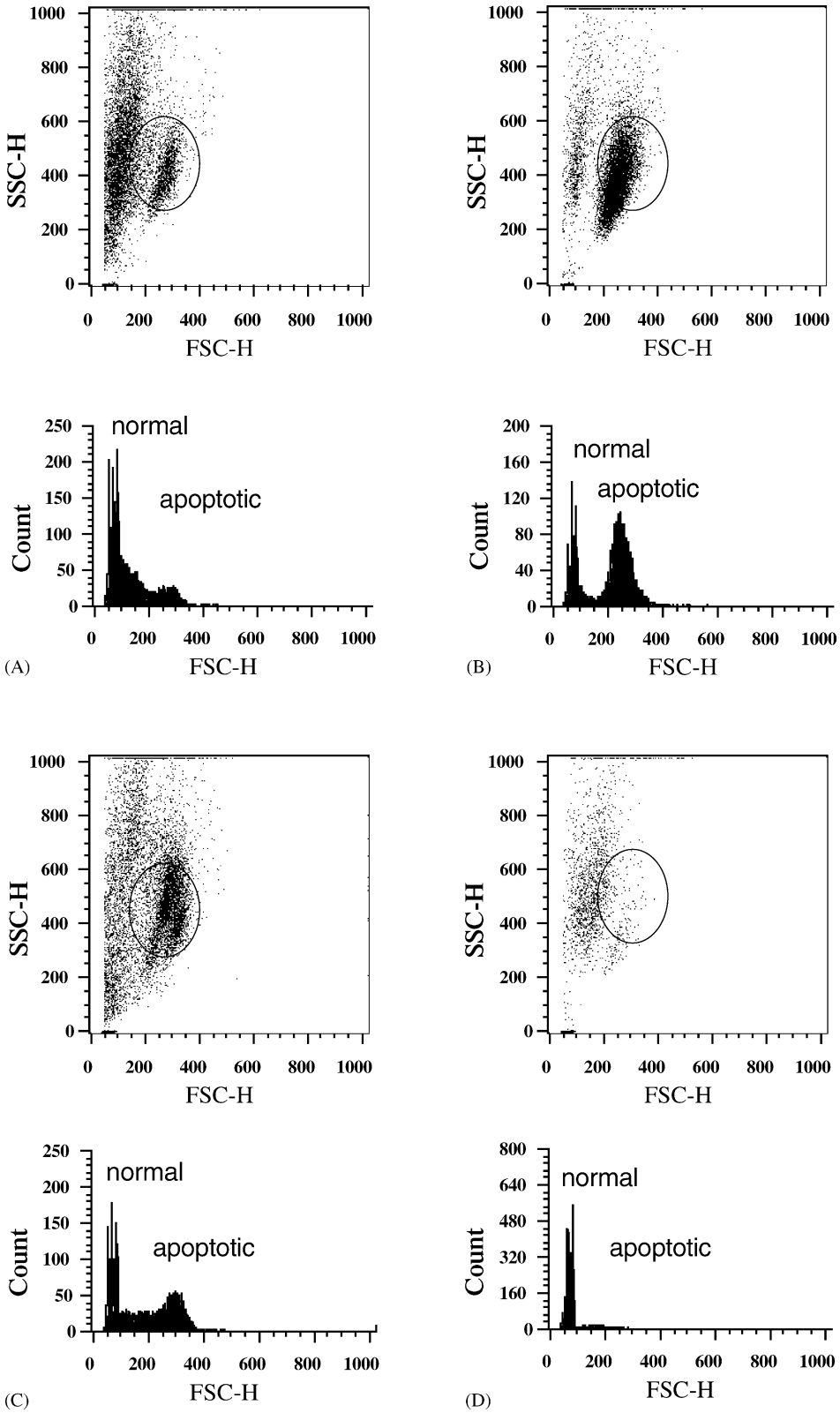

Fig. 7. Apoptotic and anti-apoptotic effects of the SLs in Jurkat T cells. Helenalin (B) and DHAc (C) (10 μM each) treatment for 20 hr strongly induces apoptosis in a 72 hr culture of Jurkat T cells as assessed by cell granularity and size. Chamissonolide (10 μM) treatment causes cytoprotective effects (D) when compared to an untreated control population (A). Representative population characteristics of 10,000 Jurkat T cells treated with SLs (B–D) for 20 hr, were analyzed on FACS. Circled population represent the amount of apoptotic cells, and the remaining cell population represents normal cell size. Data show ungated cell size (SSC-H) vs. granularity (FSC-H) dot plots with corresponding histograms (FCS Press 1.3 software).

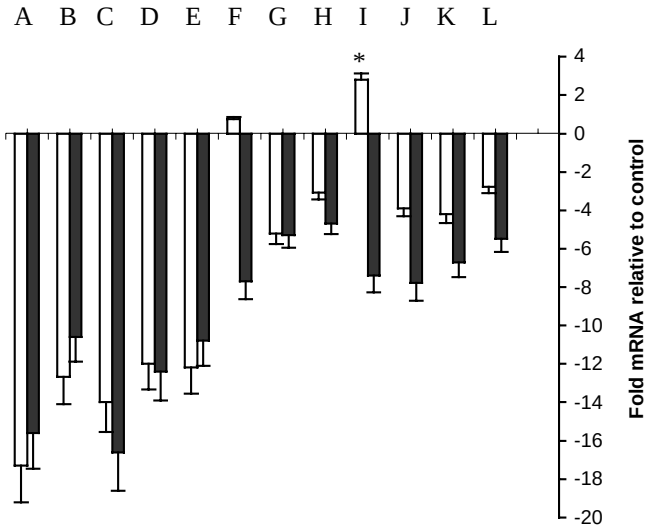

Fig. 8. The effects of helenalin and gliotoxin on the gene transcription profile in PBMCs. Fold change (relative to control) of mRNA levels in PBMCs (1.5×10^6 cells). Cells were treated with 10 μM of helenalin (black bars) and gliotoxin (white bars) and stimulated with 1 μg/mL PMA for 20 hr. Total RNA was reverse transcribed and cDNAs measured with TaqMan® real time PCR. Data shown as mean values (no. of experiments = 5) ± SEM, P=0.05. Lane A: IL-2, B: IL-6; C: GM-CSF; D: iNOS; E: TNF-α; F: cyclin D1; G: bcl-2; H: NF-ATc; I: p65; J: IκBα; K: GAP-DH; L: β-actin.

The inhibition of DNA-binding activity of the transcription factor NF-κB has been postulated repeatedly to be the underlying molecular mechanism for the anti-inflammatory activity of SLs. The fact that some monofunctional SLs of the pseudoguaianolide-type, like DHAc, also showed anti-inflammatory activity *in vivo*, but only weak inhibition of NF-κB *in vitro*, prompted us to profile their effect on specific mRNAs in Jurkat T-cells and human PBMC order to assess their anti-inflammatory potential. In parallel, the NF-κB binding inhibition in PBMCs was also determined. Up to now, the question whether the observed inhibition of NF-κB actually modulates the expression of NF-κB-driven genes is mostly discussed on the basis of results obtained in luciferase reporter gene assays with IL-6, VCAM-1, and inducible nitric oxide synthase (iNOS) promoters [4,25,26], but there are no comparative studies on the effects of SLs on the native transcriptome.

The investigation of mRNA levels of pro-inflammatory genes (IL-2, IL-6, IFN-γ, iNOS), transcription factor-related genes (NF-ATc, p65, I-κBα) and cell homeostasis factors (cyclin D1, bcl-2, β-actin and GAP-DH) provided the basis for a comparative analysis of SL-induced changes in mRNA levels (Figs. 2–4). Helenalin showed an overall down-regulation of mRNA levels, but with a significant specificity for the pro-inflammatory genes. Whereas DHAc showed a qualitatively very similar modulation of mRNAs compared to helenalin, chamissonolide exhibited a different effect and a more pronounced differential modulation of the mRNA levels. Especially, the lacking down-regulation of the house-keeping genes GAP-DH, and β-actin, as well as insignificant modulation of IL-6 mRNA and the up-regulation of NF-ATc mRNA are worth mentioning. The up-regulated NF-ATc mRNA level after 20 hr incubation with 10 μM chamissonolide might be a sign of secondary regulation due to possible inhibition of NF-AT DNA-binding or related to the lack of IL-6 inhibition, which is a newly discovered inducer of NF-ATc gene expression [27] and a promotor of the TH2 response. Further investigations would have to focus on the TH1/TH2 differentiation after chamissonolide treatment. It was shown for TNF-α mRNA levels that the influence on the transcription is concentration dependent for all three SLs. The inhibition of pro-inflammatory mRNA expression in PBMCs by the investigated SLs does not appear to depend on the mitogen used, due to the fact that it was comparable in quality in PMA and LPS stimulated PBMCs.

Helenalin is known as a potent NF-κB inhibitor, which has previously been shown to completely inhibit NF-κB DNA-binding at 10 μM in Jurkat T cells [28]. Thus, the observed strong down-regulation of NF-κB controlled genes by 10 μM of helenalin in Jurkat-T cells could be expected. Our results obtained with the PBMCs were in the same line, showing a strong inhibition of DNA-binding, with a complete inhibition of <20 μM, as well as a strong down-regulation of NF-κB controlled genes. 11α,13-Dihydrohelenalin acetate (DHAc) and chamissonolide (all Fig. 1) were reported to be insignificant inhibitors of NF-κB binding (complete inhibition = 200 μM [28]) in Jurkat T cells. In our experiments only DHAc weakly inhibited p65 DNA-binding. It therefore is of great interest that DHAc and chamissonolide also significantly down-regulated NF-κB-controlled pro-inflammatory genes in Jurkat T cells and PBMCs at a concentration of 10 μM without significant inhibition of p65 NF-κB DNA-binding. Determination of NF-κB (p65) binding inhibition in PBMCs revealed IC_{50} values of >20 μM for DHAc and chamissonolide, whereas the latter does not show any inhibition at all at 20 μM treatment (Fig. 9). These observations suggest that the anti-inflammatory potency of SLs (like chamissonolide and DHAc), might not only necessarily be coupled to NF-κB binding inhibition, but can also be mediated at the transcriptome level by other mechanisms, such as e.g. inhibition of NF-ATc DNA-binding, as proposed in a recent study, where it was shown that *Arnica* tinctures inhibited NF-AT more strongly than NF-κB [12]. Future studies need to address the possible effect of chamissonolide on NF-AT DNA-binding. The potent down-regulation of TNF-α mRNA by helenalin, DHAc, and chamissonolide is in accordance with a recent report on the inhibition of TNF-α protein release from PBMCs by *Arnica* tinctures [12].

The obtained cytotoxicity data in the WST-1 based cell viability assay correlate with the results obtained in the RT-rt-PCR analysis. The overall down-regulation of mRNA levels observed at 10 μM helenalin treatment for 20 hr in Jurkat T cells and PBMCs is indicative of a relatively early

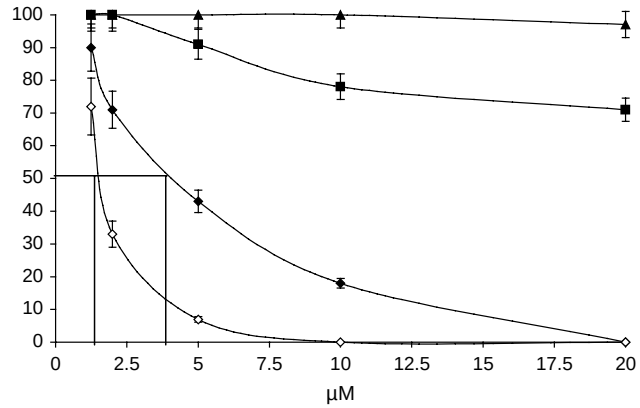

Fig. 9. NF-κB (p65) DNA-binding as (%) relative to PMA-stimulated control. PBM cells treated with helenalin (black rhombus), DHAc (black rectangle), and chamissonolide (black triangle). 5×10^5 cells suspended with 1 mL of fresh medium and preconditioned with SLs for 1 hr before PMA (1 μg/mL) stimulation for 2 hr. Gliotoxin (open rhombus) was used as a positive reference compound. NF-κB p65 DNA-binding was measured with Mercury™ Transfactor Kit. Data represent three independent experiments ± SEM.

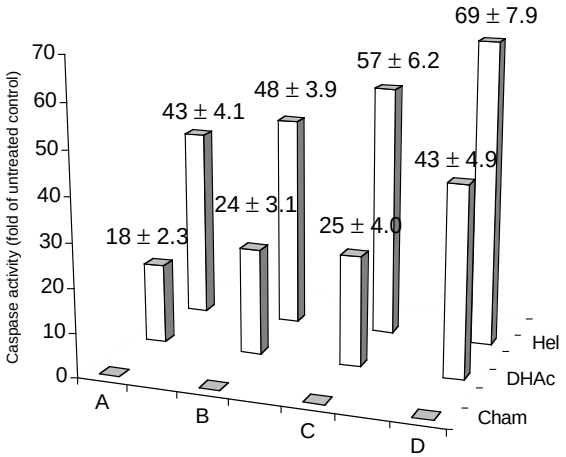

Fig. 10. Helenalin and DHAc induce caspase-8 and -3 (% of control). 2×10^6 Jurkat T-cells were treated with 10 and 20 μM of SLs and left incubated for 20 hr. (A) Fold caspase-3 induction upon 10 μM SL treatment. (B) Fold caspase-3 induction upon 20 μM SL treatment. (C) Fold caspase-8 induction upon 10 μM SL treatment. (D) Fold caspase-8 induction upon 20 μM SL treatment Data represent three independent experiments. Maximal standard deviation was 15% (absolute).

potent cytotoxic effect. Reduction of cell viability by DHAc is less potent and showed a delayed onset. Cytotoxic phenomena, namely apoptosis and necrosis are visible at the gene expression level and a strong and simultaneous down-regulation of a plethora of mRNAs (including housekeeping genes, transcription factors and cyclin kinases) might possibly be an indication for the cytotoxic activity of the investigated compound. Transcription profiles therefore encode valuable information about the physical state of a treated cell population, especially when the data can be linked to results obtained in a classical cell viability test [13]. The lack of GAP-DH and β-actin down-regulation after chamissonolide treatment match the unimpaired cell viability of both cell types. A concentration of 10 μM chamissonolide was not significantly cytotoxic against Jurkat T cells or PBMC over a time-span of 72 hr. The different sensitivity of Jurkat T cells and PBMCs against SL caused cytotoxicity is most obvious after 72 hr treatment with chamissonolide. Against PBMCs chamissonolide is not cytotoxic at concentrations up to 40 μM, whereas the IC_{50} against Jurkat T cells was determined to be 13 μM. These results suggest that different biological effects of sesquiterpene lactones on the transcriptome and cellular level have different structural requirements in neoplastic and primary non-neoplastic cells.

The induction of apoptosis by helenalin has recently been reported and it was shown, that helenalin-triggered apoptosis requires the activation of caspases [16]. Our results obtained by FACS flow cytometry analyses and caspase-3 and -8 assays are on the same line and confirm the potent induction of apoptosis by helenalin in Jurkat T cells after 20 hr. The induced caspase-3 and -8 activation (Fig. 10) is correlated with the down-regulation of bcl-2 and cyclin D1 mRNA levels. For the first time, we show that also DHAc triggers apoptosis through the activation of caspases-8 and -3 (Fig. 10), which also seems to be correlated with a strong down-regulation of bcl-2 and cyclin D1 mRNA. Both bcl-2 and cyclin D1 are under the control of NF-κB. The induction of apoptosis by DHAc is surprising because DHAc has previously been reported as a weak NF-κB binding inhibitor [28] and in our assay it only weakly inhibited p65 DNA-binding. Somewhat unexpectedly, we observed that chamissonolide did not induce caspases at concentrations up to 20 μM but inhibited naturally occurring apoptosis in Jurkat T cell populations at 10 μM during the first 20 hr. This was concluded from the increased number of unimpaired cultured cells compared to the control population in the FACS experiment. This cytoprotective effect in Jurkat T cells cannot be explained by the observed mRNA levels, due to the fact that the investigated mRNA levels were slightly down-regulated or not influenced (e.g. expression of the anti-apoptotic bcl-2 steady state mRNA was not influenced by chamissonolide). The cytoprotective effects of 10 μM chamissonolide were also seen in primary blood cells as reflected by the increased WST-1 cleavage and hence improved cell viability of PBMCs after 72 hr incubation (Fig. 6). We are currently investigating whether the anti-apoptotic effect can be correlated to a specific modulation of the transcriptome with respect to other genes. On the basis that chamissonolide exerts significant cytotoxicity against Jurkat T cells in a later phase of the cell viability test, the results underline the importance of the time factor in differential, and thus selectively cytotoxic or anti-inflammatory effects caused by SLs.

Because helenalin is a potent NF-κB DNA-binding inhibitor we postulated that its effect on PMA-induced and NF-κB dependent mRNAs might possibly be similar

in quality to other NF-κB DNA-binding inhibitors. We therefore determined the effects of the potent NF-κB inhibitor gliotoxin on the transcriptome of mitogen-activated PBMCs. Gliotoxin from *Gliocladium fimbriatum* and *Aspergillus fumigatus* has been described as a non-competitive inhibitor of the chymotrypsin-like activity of the 20S proteasome *in vitro*. In intact cells, gliotoxin inhibits NF-κB induction through inhibition of proteasome-mediated degradation of I-κBα [29]. The relatively high cytotoxicity of gliotoxin has been reported previously [30], and correlates well with the strong down-regulation of the house-keeping genes, as well as IκBα, NF-ATc and bcl-2. However, in contrast to helenalin treatment, the mRNA level of p65 was up-regulated and the PMA-induced up-regulation of cyclin D1 in PBMCs was not influenced. These results might indicate that either a distinct molecular mechanism of NF-κB inhibition causes a different modulation of the transcriptome or that the postulated selectivity of the NF-κB inhibitors gliotoxin and helenalin need to be questioned [5,29]. Moreover, the opinion that SLs specifically inhibit NF-κB DNA was recently also challenged by an observed NF-AT inhibition [12]. In conclusion, it is likely that SLs exert their anti-inflammatory action via different mechanisms mediated by distinct structural elements.

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