Available online at www.sciencedirect.com
ScienceDirect
Life Sciences 80 (2007) 1608–1618
Life Sciences
www.elsevier.com/locate/lifescie

Microarray analysis reveals influence of the sesquiterpene lactone parthenolide on gene transcription profiles in human epithelial cells

M.T. Lindenmeyer [a], C. Kern [a], T. Sparna [a], J. Donauer [b], J. Wilpert [b], J. Schwager [c], D. Porath [c], C. Kreutz [d], J. Timmer [d], I. Merfort [a,*]

[a] Institute for Pharmaceutical Sciences, Department of Pharmaceutical Biology and Biotechnology, Albert-Ludwigs-University, Freiburg, Germany
[b] Renal Division, Department of Medicine, University Hospital Freiburg, Germany
[c] DSM Nutritional Products 4303 Kaiseraugst, Switzerland
[d] Center for Data Analysis and Modeling, Albert-Ludwigs-University, Freiburg, 79104 Freiburg, Germany

Received 13 August 2006; accepted 20 January 2007

Abstract

Sesquiterpene lactones are known for their anti-inflammatory activity which has been proven in various assays on DNA, mRNA and protein level. Here we report on the change in the gene expression profile in TNF-α stimulated human 293 cells after treatment with parthenolide using a cDNA microarray analysis. Twenty-one of 7028 genes were found to be up- and 18 down-regulated. They encode for chemoattractants, immune system proteins, glycoproteins, metabolism, serine proteinases, and transcription factors. Confirmatory analyses were carried out using quantitative real-time RT-PCR (TaqMan®). Additional studies with selected genes revealed the concentration-dependent influence of parthenolide on the expression of these genes.

Keywords: Sesquiterpene lactones; Parthenolide; Gene transcription; cDNA microarray; Real-time quantitative PCR

Introduction

Sesquiterpene lactones (SLs) are the active constituents of many medicinal plants from the Asteraceae family and possess a variety of biological effects (Schmidt, 1999). In particular, their potent anti-inflammatory and cytotoxic properties have received considerable attention. Their anti-inflammatory activity has been corroborated using various assays and it has been established that one of the main targets inhibited by SLs is the transcription factor NF-κB (Bork et al., 1997; Garcia-Pineres et al., 2001; Hehner et al., 1999; Lyss et al., 1997). This protein plays a pivotal role in controlling the expression of multiple proinflammatory, inflammatory and immune genes involved in diseases, such as asthma, rheumatoid arthritis or cancer (Aggarwal et al., 2004; Bacher and Schmitz, 2004). NF-κB is a dimeric transcription factor formed by the hetero- or homodimerization of proteins of the rel-family (Bonizzi and Karin, 2004). In most cell types, NF-κB dimers are cytoplasmic and retained in an inactive complex by binding to IκB, its inhibitory subunit (Cramer and Muller, 1999; Hoffmann et al., 2002a). Translocation to the nucleus takes place in response to a variety of proinflammatory stimuli. Two major pathways account for translocation of NF-κB dimers from the cytoplasm to the nucleus where they activate target genes by binding with high affinity to κB elements in their promotors (Bonizzi and Karin, 2004; Hoffmann and Baltimore, 2006).

It has been shown that SLs exert most of their biological effects, e.g., cytotoxicity and anti-inflammatory activity, by alkylating biological nucleophiles. The α,β-unsaturated carbonyl structures of SLs, such as α-methylene-γ-lactone and α,β-en-one groups, react with thiol groups of enzymes and proteins via a Michael type addition thus interfering with their functions (Picman et al., 1979; Schmidt, 1999). Recently, we could demonstrate that these structural elements are also involved in inhibiting NF-κB DNA binding, probably by alkylating the p65 subunit (Garcia-Pineres et al., 2001).

* Corresponding author. Tel.: +49 761 203 8373; fax: +49 761 203 8383.
E-mail address: irmgard.merfort@pharmazie.uni-freiburg.de (I. Merfort).

0024-3205/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2007.01.036

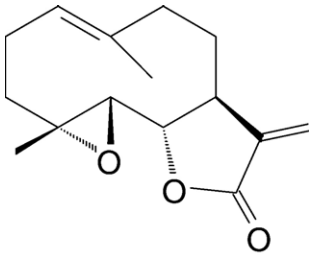

Fig. 1. Structure of parthenolide.

Inhibition of IκB kinase also occurs (Hehner et al., 1999), but this might not be the decisive step in NF-κB inhibition (Garcia-Pineres et al., 2001).

SLs do not only inhibit the DNA binding activity of NF-κB but also influence the NF-κB dependent gene expression (Bork et al., 1997; Li-Weber et al., 2002a; Mazor et al., 2000) and consequently mRNA levels of NF-κB regulated genes, (Li-Weber et al., 2002a; Mazor et al., 2000; Lindenmeyer et al., 2006; Gertsch et al., 2003) as well as the respective protein (Li et al., 2006). Recently, a comprehensive analysis of the effects of the SL parthenolide on gene expression in keratinocytes has been carried out using cDNA microarray technology (Banno et al., 2005). This technique allows the monitoring of the expression of thousands of individual genes including those regulated by NF-κB (Zhou et al., 2003).

Using a 7 K cDNA microarray, we here analyzed gene expression changes in cultures of human 293 cells, a permanent line of primary human embryonal kidney cells, after treatment with the SL parthenolide (for structure see Fig. 1) and stimulation with TNF-α. Some of the results were confirmed using quantitative real-time RT-PCR (TaqMan® analysis). We found distinct alterations in the expression pattern of genes associated with the immune response and with a variety of cellular pathways. This study represents the second global analysis of the transcriptional response of cells to SLs using parthenolide as an example. As SLs may serve as lead structures for the development of therapeutically and cytokine supressing remedies valuable for the treatment of various inflammatory diseases (Wagner et al., 2006), knowledge concerning their effects on genes stimulated by TNF-α and regulated by NF-κB and other transcription factors is important for estimating possible side effects.

Materials and methods

Reagents

Parthenolide was purchased from Sigma-Aldrich, TNF-α from Roche Molecular Biochemicals.

Cell culture

Human 293 cells were maintained in Dulbecco's modified Eagle Medium and supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (all Gibco-BRL).

Microarrays

cDNA microarrays were produced and processed essentially according to the Standford protocol described recently (Eisen and Brown, 2004). In short: approximately 7000 annotated genes from the RZPD (Resource Center and Primary Database, Berlin, Germany) were obtained as bacterial stocks. Plasmids were purified using the Qiagen 96-well Turbo Kit (Qiagen, Hilden, Germany). Inserts were purified by polymerase chain reaction (PCR) using vector primers flanking the individual inserts (5′-CTG CAA GGC GAT TAA GTT GGG TAA C-3′ and 5′-GTG AGC GGA TAA CAA TTT CAC ACA GGA AAC AGC-3′). PCR products were purified by ethanol precipitation and dissolved in H_2O. Aliquots were transferred into 384-well plates, dried and dissolved in 3× standard saline citrate to a final concentration of approximately 40 ng/μl. Printing was performed on aminosilane-coated slides (CMT-GAP II slides, Corning, NY), using an arrayer that was assembled according to specifications by the Stanford group with software provided by J. de Risi (http://cmgm.stanford.edu/pbrown).

Cell treatment and RNA preparation for microarray analysis

293 cells were maintained in Dulbecco's modified eagle medium (Gibco), supplemented with 10% fetal bovine serum (Sigma), 100 IU/ml penicillin and 100 μg/ml streptomycin (Roche Diagnostics). TNF-α was purchased from Roche Diagnostics.

293 cells were plated 48 h prior to treatment with parthenolide (20 μM) at a density of 1.5×10^6 cells per 10 cm-dish in 10 ml medium. Cells were preincubated with parthenolide for 1 h and subsequently stimulated with TNF-α (2 ng/ml) for 1 h. Untreated cells were used as negative control; cells stimulated only with TNF-α as positive control. Cells from four 10 cm-dishes were pooled and homogenized in 4 M guanidinium isothiocyanate with 0.72% β-mercaptoethanol. Total RNA was purified on a caesium chloride gradient. After ethanol precipitation, RNA was dissolved in water and stored at −80 °C.

Hybridization

All 12 hybridizations were performed in the presence of an equal amount of human reference RNA (Stratagene, La Jolla, CA) as recently described (Boldrick et al., 2002). Twelve micrograms of total RNA from 293 cells or reference RNA were transcribed into cDNA in the presence of Cy3-and Cy5-labeled dUTP, respectively, using Superscript II reverse transcriptase (RT) (Invitrogen, Carlsbad, CA). All other steps were performed according to the protocol published by P. Brown et al. (http://cmgm.stanford.edu/pbrown). A cDNA purification kit (Qiagen, Hilden, Germany) was used for cDNA purification after dye labeling.

Data analysis

Signal intensities were measured by an Axon 4000A scanner using GenePix 4.1 software (Axon Instruments Inc., Union City, CA). The experimental design included a color-reversal experiment for every sample to correct for dye-specific effects. The log

Table 1
Sequences of primers and probes for the TaqMan® analysis

Gene	Abbreviation	Primer/probe name	Sequence
Human complement factor B	HCFB	F hCFb	TGCTGACCCCAATACTTGCA
		R hCFb	GAATGAAACGACTTCTCTTGTGAACT
		P hCFb	AGGTGATTCTGGCGGCCCCTTG
Human cyclooxygenase-2	hCOX-2	F hCOX-2	GCCCTTCCTCCTGTGCC
		R hCOX-2	AATCAGGAAGCTGCTTTTTACCTTT
		P hCOX-2	ATGATTGCCCGACTCCCTTGGGTGT
Human cytosolic epoxide hydrolase	HCEpoxH	F hCEPOXH	GTAAAGCCCAGGGTCCGTC
		R hCEPOXH	GAAATCCATGGCAGAGGCA
		P hCEPOXH	CATTTTGTGGAGCTGGGCTGGCC
Human epithelial-cell derived neutrophil activating protein-78	hENA-78	F hENA-78	CAGGCCCATCGCCA
		R hENA-78	TCTGTAAACAAACGCAACGCA
		P hENA-78	TCCTGCCGCTGCTGTGTTGAGAGAG
Human Fos-related antigene	hFra-1	F hFRA-1	CACGACCCATCTGCAAAA
		R hFRA-1	GCCACTGGTACTGCCTGTGTC
		P hFRA-1	CCCGGAAGGAGCCAAGGAGGG
Human growth related oncogene-α	hGRO-α	F hMGSA-a	AAGTCCCCCGGACCCC
	hMGSA-α	R hMGSA-a	TTCCGCCCATTCTTGAGTGT
		P hMGSA-a	CTGCGCCCAAACCGAAGTCATAGC
Human growth related oncogene-β	hGRO-β,	F hMIP-2a	AGTCCCCCGGACCCC
	hMIP-2α,	R hMIP-2a	GCCCATTCTTGAGTGTGGC
	hMGSA-β	P hMIP-2a	CTGCGCCCAAACCGAAGTCAT
Human growth related oncogene-γ	hGRO-γ,	hGSAg-F186	GTCCCCCGGACCCCA
	hMIP-2β,	hGSAg-R252	ACAAGCTTTCTTCCCATTCTTGAG
	hMGSA-γ	hGSAg-P204	CGCCCAAACCGAAGTCATAGCCAC
Human IEX-IL CDS	hIEX-IL	h-IEX-F196	TCTCTACCCTCGAGTGGTGAGTATC
		hIEX-R258	ACTCCAGGGCAGCGCA
		hIEX-P222	CCGAAGTGGGCATTCGCGGT
Human interleukin-1β	hIL-1β	F hIL-1b	GGGCTCAAGGAAAAGAATCTGTAC
		R hIL-1b	GGGATCTACACTCTCCAGCTGTAGA
		P hIL-1b	TCCTGCGTGTTGAAAGATGATAAGCCCA
Human interleukin-8	hIL-8	F hIL-8	ACTGACATCTAAGTTCTTTAGCACTCC
		R hIL-8	GCCTTCCTGATTTCTGCAGC
		P hIL-8	TGGCAAAACTGCACCTTCACACAG
Human macrophage inflammatory protein-3α	hMIP-3α,	F hMIP-3a	TGTCAGTGCTGCTACTCCACCT
	LARC	R hMIP-3a	CCAAGACAGCAGTCAAAGTTGC
		P hMIP-3a	TGCGGCGAATCAGAAGCAGCA
Human matrix metalloproteinase-2	hMMP-2	F human MMP-2	CCCTCGCAAGCCCAA
		R human MMP-2	CAGATCAGGTGTGTAGCCAATG
		P human MMP-2	TGGGACAAGAACCAGATCACATACAGGA
Human matrix metalloproteinase-7	hMMP-7,	F human MMP-7	CGGAGGAGATGCTCACTTCG
	hPUMP-1	R human MMP-7	CAGCATACAGGAAGTTAATCCCTAGA
		P human MMP-7	GCTACCATCCGTCCAGCGTTCATCCT
Human matrix metalloproteinase-9	hMMP-9	F h MMP-9 (1/2)	CCTGAGAACCAATCTCACCGA
		R h MMP-9 (1/2)	GCCACCCGAGTGTAACCATAG
		P h MMP-9 (1/2)	AGGCAGCTGGCAGAGGAATACCTGTACC
Human peroxisome proliferator activated receptor-α	hPPAR-a	F h PPAR-alpha	CAAGTGCCTTTCTGTCGGGA
		R h PPAR-alpha	GTTTTGCTTTCTCAGATCTT GGC
		P h PPAR-alpha	CACACAACGCGATTC GTTTTGGACG
Human receptor activator of nuclear factor kappa B exon 3/4	HRANK	F human RANK	GCTTGCTGCATAAAGTTTGTGA
		R human RANK	TGTTGCCGGCGACCA
		P human RANK	AGGCAAGGCCCTGGTGGCC
Human transforming growth factor-β1	hTGF-b1	F h TGF-beta1	AAGGGCTACCATGCCAACTTC
		R h TGF-beta1	TGCGTGTCCAGGCTCCA
		P h TGF-beta1	CCTCGGGCCCTGCCCCTACA
Human tumor necrosis factor-α	hTNF-α	F hTNF-a	TCGAACCCCGAGTGACAA
		R hTNF-a	AGCTGCCCCTCAGCTTG
		P hTNF-a	CCTGTAGCCCATGTTGTAGCAAACCC
Human 18S rRNA	h18S rRNA	F h 18S rRNA	CGGCTACCACATCCAAGG
		R h 18S rRNA	CGGGTCGGGAGTGGGT
		P h 18S rRNA	TTGCGCGCCTGCTGCCT

Table 2

Biological process	ID	Gene name	Symbol	$F_{parthenolide/TNF-\alpha}$	$F_{parthenolide}$	NF-κB[a]
Immune response	X06026	CD3-γ	CD3G	↑ 6.83	1.21	+
	L15702	**Complement factor B**	CFB	↑ 4.45	1.18	+
	X01683	Alpha-1-antitrypsin	SERPINA1	↑ 2.94	0.85	
	X06341	Lymphocte activation marker blast-1	CD48	↑ 2.59	0.98	+
	M65292	Factor H homologue	CFHR1	↑ 2.55	1.04	
	X54489	**GRO-α**	CXCL1	↓ 0.39	0.98	+
	M36820	**GRO-β**	CXCL2	↓ 0.37	0.98	+
	M36821	**GRO-γ**	CXLC3	↓ 0.23	1.12	+
	D86955	**CC chemokine LARC precursor (MIP-3α)**	CCL20	↓ 0.29	1.22	+
	Y14737	Immunoglobulin lambda heavy chain	IGHG1	↓ 0.37	0.87	
Signalling	M75154	Myeloblastin (MBN)	PRTN3	↑ 5.46	0.94	
	U68186	Extracellular matrix protein 1	ECM1	↑ 3.62	1.34	
	X16323	Hepatocyte growth factor (HGF)	HFG	↑ 3.45	↑1.66	
	X81006	HCG I	TRIM31	↑ 2.77	1.23	
	M29893	Low molecular mass GTP-binding protein (ral)	RALA	↑ 2.72	1.06	
	U01134	Soluble vascular endothelial cell growth factor receptor	FLT1	↑ 2.64	1.06	
	AF018253	**RANK, receptor activator of NF-κB**	TNFRSF11A	↓ 0.80	1.08	+
	M69043	MAD-3, encoding IkB-like activity	NFKBIA	↓ 0.38	1.10	+
	X02910	**Tumor necrosis factor (TNF-α)**	TNF	↓ 0.34	1.09	+
	AF036271	EEN-B2-L3	SH3GL3	↓ 0.27	1.00	
	U79457	WW domain binding protein-1	WBP1	↓ 0.26	1.15	
Protein metabolism	AB007948	Nicotinamide nucleotide adenylyltransferase	NMNAT2	↑ 3.74	1.12	
	AB014523	KIAA0623	ULK2	↑ 2.96	0.23	
	J00146	Dihydrofolate reductase pseudogene	DHFRP1	↓ 0.32	0.91	
Cytoskeleton	U58331	Placental delta sarcoglycan	SGCD	↑ 2.85	1.05	
	M12125	Fibroblast muscle-type tropomyosin	TPM2	↑ 2.55	0.99	
	D49958	Membrane glycoprotein M6	GPM6A	↓ 0.34	1.06	
Transcription	X16707	**Fra-1**	FOSL1	↑ 4.04	1.00	+
	M24898	Triiodothyronine recptor (THRA1, ear1)	NR1D1	↑ 3.51	1.00	
	V01512	Cellular oncogene c-Fos	FOS	↑ 2.89	1.20	+
	U72621	LOT1	PLAGL1	↓ 0.33	1.27	
Apoptosis	AF071596	**Apoptosis inhibitor (IEX-1L)**	IER3	↓ 0.39	1.11	+
	X76105	DAP-1	DAP	↓ 0.34	↑3.63	
Coagulation	AF093119	UPH1	CFL1	↑ 2.64	1.07	
	M68516	Protein C inhibitor	SERPINA5	↓ 0.38	1.09	
Metabolism	U56417	Lysophosphatidic acid acyltransferase-a	AGPAT1	↑ 4.73	1.02	
	L05779	**Cytosolic epoxide hydrolase (CepoxH)**	EPHX2	↑ 3.21	↑3.49	
Adhesion	D79206	Ryudocan core protein (Syndecan-4)	SDC4	↓ 0.32	1.05	+
Energy metabolism	AF047435	CI-KFYI protein	NDUFC1	↓ 0.37	1.12	
No category	AB002366	KIAA0368	KIAA0368	↓ 0.28	1.11	

Gene expression profiling in TNF-α stimulated (1 h) and parthenolide (20 μM) treated (2 h) 293 cells analyzed by cDNA microarray analysis. $F_{parthenolide/TNF-\alpha}$ represents the ratio of medians of parthenolide stimulated cells versus TNF-α stimulated cells, generated in four microarray experiments per group (two independent microarrays and two color reversals). All genes with at least 2.5-fold up- or 2.5 fold down-regulation by parthenolide pretreatment in comparison to the positive control are listed. Annotation is based on Source database (http://genome-www5.stanford.edu/cgi-bin/source/). Genes written in bold letters were confirmed by TaqMan®analysis.

[a] known as NF-κB regulated (Gilmore, 2006; Li et al., 2001).

ratio of measured Cy3 and Cy5 values obtained from the image analysis software was computed. Global normalization of expression values was performed by adjusting the data to zero median and unit variance to obtain an identical distribution of overall gene expression. Taking the mean of the expression values of the dye-swap experiments allows correction for dye-specific effects. A smooth nonlinear least squares fit was computed to correct for an intensity-dependent bias (Donauer et al., 2003), following the approach that the computed expression ratios should not depend on the intensity of these spots.

Cell treatment and RNA-isolation for TaqMan® analysis

293 cells were plated 48 h prior to treatment with parthenolide at a density of 7.5×10^5 cells per 60 mm-dish in 5 ml medium. Cells were preincubated with different concentrations of parthenolide for 1 h and subsequently stimulated with TNF-α (2 ng/ml) for 3 h. Untreated cells were used as negative control, cells stimulated only with TNF-α as positive control. Total RNA isolation was performed using RNeasy Mini Kit (Qiagen) according to the manufacturers' recommendations. RNA was dissolved in RNase-free water and stored at −80 °C.

cDNA-synthesis and TaqMan® analysis

cDNA was prepared using random primers and Superscript II (all from Invitrogen). In a 50 μl PCR reaction, 3 μl cDNA (corresponding to 30 to 50 ng of total RNA input) was amplified in a 7700 Sequence Detector, using the 2× Universal Master Mix,

Table 3

Gene	Microarray	TaqMan®	
	Parthenolide/ TNF-α-Ratio	Parthenolide/ TNF-α-Ratio	s.d.
hGRO-α	↓ 0.39	↓ 0.35	0.008
hGRO-β	↓ 0.37	↓ 0.51	0.024
hGRO-γ	↓ 0.23	↓ 0.47	0.090
hMIP-3α (LARC)	↓ 0.29	↓ 0.37	0.025
hCFB	↑ 4.45	↑ 2.00	0.077
hTNF-α	↓ 0.34	↓ 0.45	0.051
hRANK	↓ 0.80	↑ 1.01	0.012
hIEX-1L	↓ 0.39	↓ 0.50	0.057
hFRA-1	↑ 4.04	↑ 1.72	0.136
hCepoxH	↑ 3.21	↑ 1.75	0.159

Comparison of the results from microarray and TaqMan® analysis.The mRNA expression of 10 different genes was analyzed with both real-time quantitative RT-PCR and microarray analysis. The expression ratios by TaqMan® were generated by comparing the mRNA level for a given gene in parthenolide treated cells relative to the amount of 18S rRNA. The expression ratios by microarray analysis represent the ratio of medians of parthenolide stimulated cells versus TNF-α stimulated cells.

50 nM primers and 100 nM probe (VIC-TAMRA labeled) for the 18S rRNA internal control and 300 nM primers and 100 nM probe (FAM-TAMRA labeled) for the gene of interest. Each PCR was carried out in duplicate. For an optimal activity of the Uracil-*N*-glycosylase AmpErase the PCR mix was heated for 2 min at 50 °C. The PCR reaction was initiated by a 10 min step at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C in the ABI Prism 7700 sequence Detection System. Primers and probe sets were designed with the Primer Express™ software.

The cycle number at which the fluorescence exceeded the threshold of detection (C_T) for 18S rRNA was subtracted from that of the target genes for each well (ΔC_T). Messenger RNA levels were then indicated as $2^{-\Delta\Delta C}{}_T$ where $\Delta\Delta C_T$ is the ΔC_T of unstimulated minus treated cells. The primers and probes used for PCR analysis are listed in Table 1.

Viability test

The viability of cells which were treated with parthenolide for 4 h was determined by Trypan blue exclusion as described by Ausubel et al. (1998).

Statistical analysis

Statistical analysis was performed by using the Origin 7.0 software. Data are reported as means ± SD and analyzed using an independent t-test (2 groups). A P-value < 0.05 is considered statistically significant. $^*P<0.05$, $^{**}P<0.005$ versus positive control.

Results

Microarray analysis of gene expression after treatment with parthenolide

To investigate systematically genes that are modulated by parthenolide, a 7 K cDNA microarray (7028 genes) was used. 293 cells were preincubated with parthenolide (20 μM) for 1 h and subsequently stimulated with TNF-α (2 ng/ml) for another hour. These conditions have recently been used to show the inhibitory effect of parthenolide on NF-κB DNA binding (Garcia-Pineres et al., 2004). Untreated cells were used as a negative control, cells treated only with TNF-α for 1 h as a positive control. Cells were harvested and total RNA extracted and reverse-transcribed into cDNA using Cy5- and Cy3-labeled dUTP for two-color microarray analysis. Two samples incubated with parthenolide and stimulated with TNF-α, two

Table 4

Gene accession number (ID)	Gene name	Acronym	Function
U04636	Cyclooxygenase-2	COX-2	Proinflammatory process
BC008678	Interleukin-1β	IL-1β	Cytokine, proinflammatory process
E03028	Transforming growth factor-β1	TGF-β1	Cytokine, cell growth, differentiation, function
X02910	Tumor necrosis factor-α	TNF-α	Cytokine, proinflammatory process
M28130	Interleukin-8	IL-8	Chemokine, proinflammatory process
X54489	Growth related oncogene-α	GRO-α	Chemokine, proinflammatory process
M36820	Growth related oncogene-β	GRO-β	Chemokine, proinflammatory process
M36821	Growth related oncogene-γ	GRO-γ	Chemokine, proinflammatory process
L37036	Epithelial neutrophil-activating peptide-78	ENA-78	Chemokine, proinflammatory process
D86955	Macrophage inflammatory protein-3α	MIP-3α	Chemokine, proinflammatory process
BC002576	Matrix metalloproteinase-2	MMP-2	Degradation of collagen and gelatine
S41832	Matrix metalloproteinase-7	MMP-7	Degradation of extracellular matrix macromolecules and surface molecules
M68343	Matrix metalloproteinase-9	MMP-9	Degradation of collagene and gelatine
AF071596	Apoptosis inhibitor	IEX-1L	Apoptosis inhibitor
L02732	Peroxisome proliferator-activated receptor-α	PPAR-α	Lipid metabolism
AF018253	Receptor activator of NF-κB	RANK	Regulation of bone and immune system
L15702	Complement factor B	CFB	Alternative complement pathway
X16707	Human fos related gene	Fra-1	Transcription factor
L05779	Cytosolic epoxide hydrolase	CEpoxH	Oxygen and radical metabolism

Genes studied by quantitative real-time RT-PCR (TaqMan® analysis).

positive controls stimulated with TNF-α, two samples incubated with parthenolide and two negative controls, all generated in independent assays, were hybridized in separate color-reversal microarray experiments, making a total of 16 different hybridizations, four in each group.

We limited our analysis to genes that were at least 2.5-fold up- or 2.5-fold down-regulated after treatment of parthenolide in comparison to the positive control. A list of 39 genes was obtained which fulfilled these criteria (see Table 2). Parthenolide alone had no effect on these genes in unstimulated

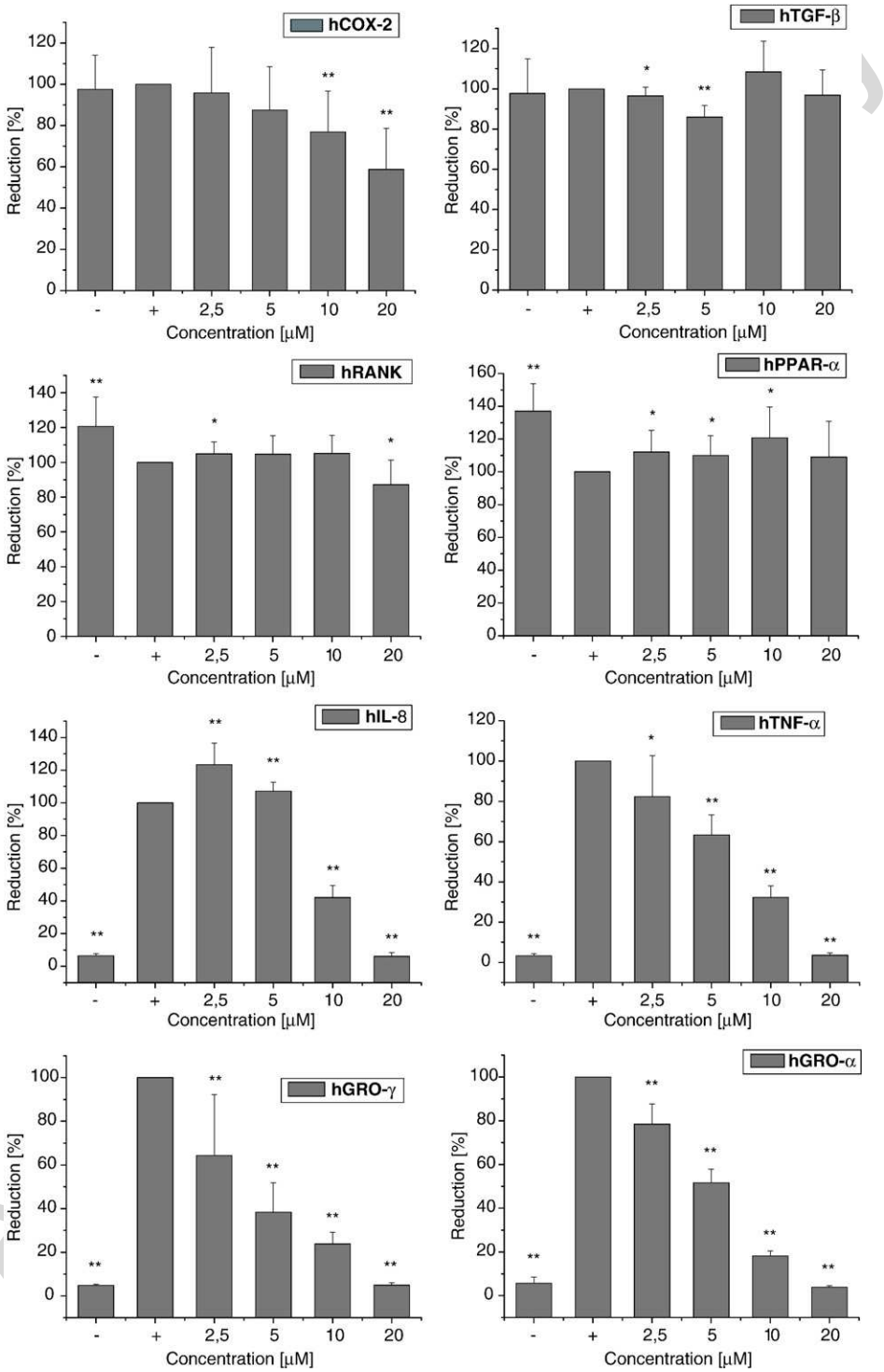

Fig. 2. TaqMan® analyses of 13 different genes. Given are the mRNA levels in %. The percentage change, relative to the stimulated cells, was defined as ($2^{\Delta\Delta CT} \times 100$). 293 cells were preincubated with different concentrations of parthenolide for 1 h and subsequently stimulated with TNF-α (2 ng/ml) for 3 h. Total RNA isolation was performed using RNeasy Mini Kit (Qiagen), reverse-transcribed in cDNA and analyzed via TaqMan®–RT-PCR. Untreated cells were used as negative control, cells stimulated only with TNF-α as positive control. Data represent the mean ± SEM of at least three separate experiments with each condition carried out in duplicate. A *P*-value < 0.05 is considered statistically significant. **P* < 0.05, ***P* < 0.005 versus positive control.

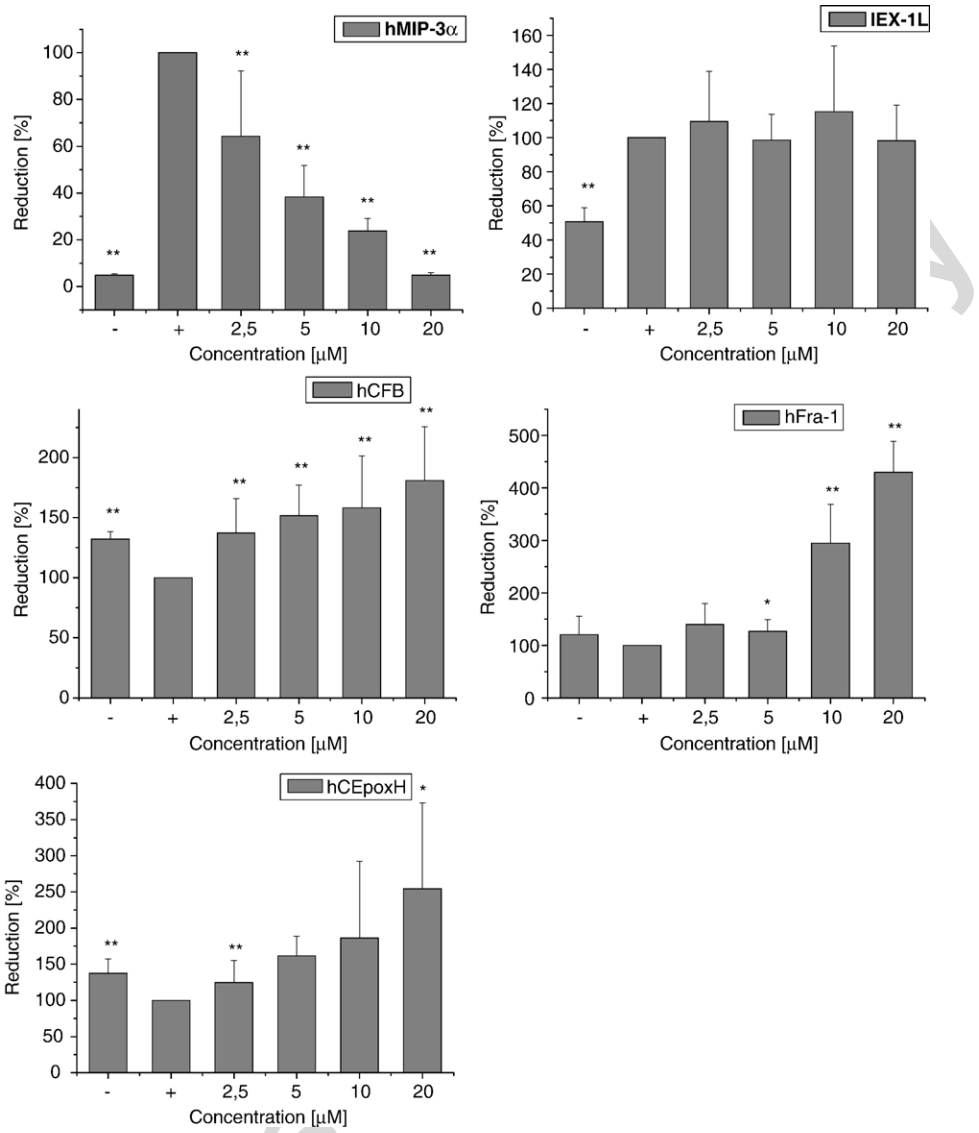

Fig. 2 (*continued*).

cells except for DAP-1 and CepoxH using the same criteria. The 39 influenced genes belong to different groups including chemoattractants (e.g., GRO-α, -β, -γ, MIP-3α), cytokines (e.g., TNF-α, HGF), immune system proteins (e.g., CD3-γ, CFB), glycoproteins (e.g., δ-sarcoglycan, syndecan-4, protein-C inhibitor), metabolism (e.g., cytosolic epoxide hydrolase, lysophosphatidic acid acyltransferase-α), serine proteinases (e.g., myeloblastin), and transcription factors (e.g., Fra-1, c-Fos). A number of genes (marked in bold letters) were chosen for confirmatory studies and further analysis. Access to the microarray data can be obtained upon request to the authors.

Confirmatory studies with differentially expressed genes by TaqMan® real-time PCR

Quantitative real-time RT-PCR was performed for 9 regulated genes and one unaffected gene (RANK) as control. One microgram of total RNA from the same preparation used for microarray analysis was reverse-transcribed and amplified in the ABI Prism 7700 sequence Detection System for TaqMan® analysis. The results obtained by quantitative real-time RT-PCR showed comparable tendencies to the microarray data (see Table 3), though the fold increase of the up-regulated genes was lower in the Taqman® analyses.

Concentration-dependent influence of parthenolide on gene expression

293 cells were preincubated with different concentrations of parthenolide (2.5; 5; 10; 20 μM), for 1 h and subsequently stimulated with TNF-α (2 ng/ml) for 3 h. The time of TNF-α stimulation was increased from 1 h (microarray) to 3 h to get different levels of mRNA. Total RNA was isolated, reverse-transcribed and 19 different genes (for genes see Table 4) were analyzed via the TaqMan® method. Some genes such as COX-2, TGF-β1, PPAR-α, and RANK were constitutively expressed and only COX-2 was significantly inhibited by parthenolide at the highest concentration (see Figs. 2 and 3). mRNA of Fra-1,

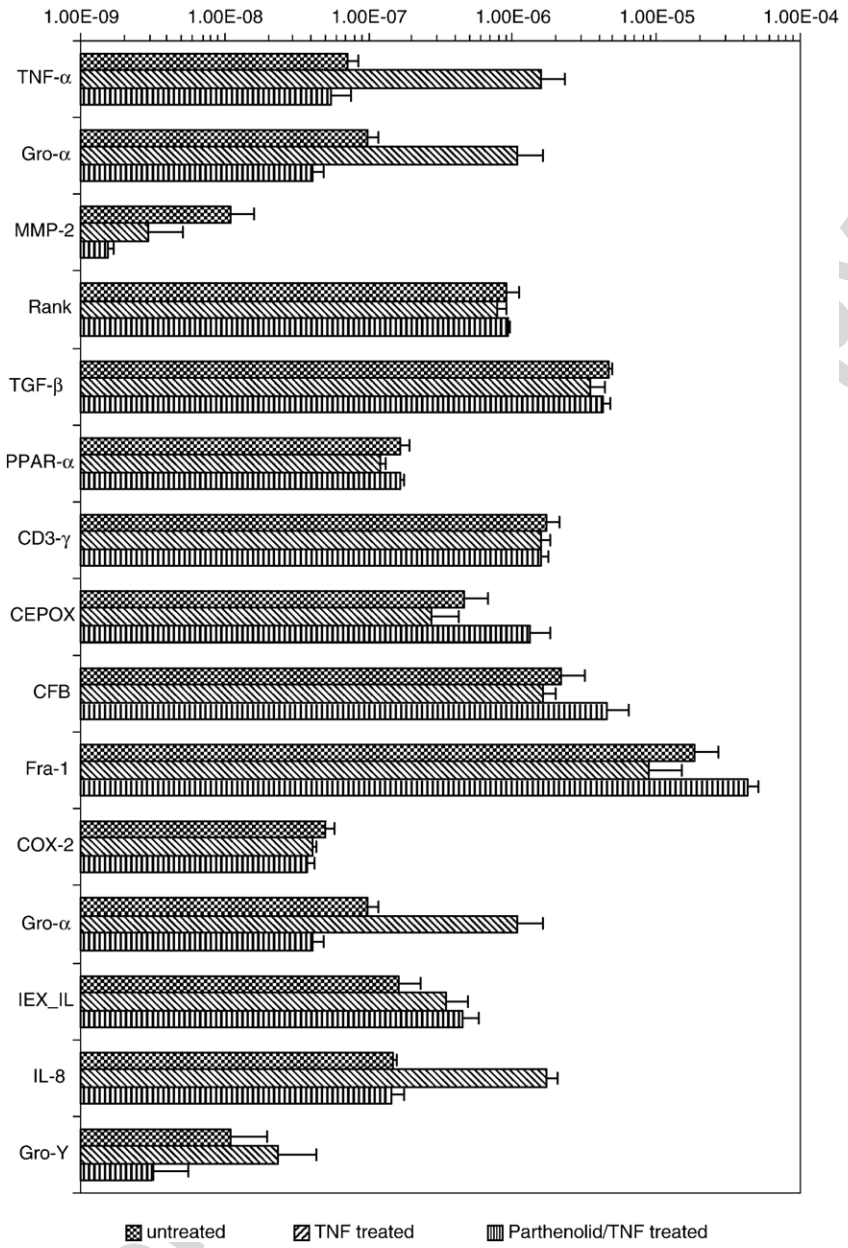

Fig. 3. Expression levels of selected genes in 293 cells studied by real-time RT-PCR after 1 h pretreatment with parthenolide (20 μM) and subsequent 3 h stimulation with TNF-α. Values are expressed relative to 18S rRNA and are indicated as means±S.D. of at least three experiments.

complement factor B and CEpoxyH increased after pretreatment with parthenolide and TNF-α stimulation, with Fra-1 showing the most pronounced effect (see Fig. 2). mRNA of IL-1β, GRO-β, ENA-78, MMP-2, MMP-7 and MMP-9 were not expressed at significant amounts after 3 h TNF-α stimulation (data not shown). However, levels of TNF-α, IL-8, GRO-α, GRO-γ, MIP-3α, and IEX-1L increased after TNF-α-stimulation (see Fig. 3), and decreased after pretreatment with parthenolide in a concentration-dependent manner, except for IEX-1L (Figs. 2 and 3).

Discussion

The biologically active SLs belong to those natural products which have been tested in different assays for their influence on DNA binding and gene expression of different transcription factors and genes (Bork et al., 1997; Garcia-Pineres et al., 2001, 2004; Gertsch et al., 2003; Hehner et al., 1999; Li-Weber et al., 2002a; Lindenmeyer et al., 2004; Mazor et al., 2000; Lyss et al., 1997). Especially, parthenolide, the active principle in the herb of *Tanacetum parthenium*, (Knight, 1995) is one of the SLs, which has attracted much attention. In numerous studies, its inhibition of the transcription factors NF-κB (Garcia-Pineres et al., 2004; Hehner et al., 1999), AP-1 (Lindenmeyer et al., 2006) and STAT3 (Sobota et al., 2000), cytokine suppressing (Li-Weber et al., 2002a; Li et al., 2006) and apoptotic activities (Li-Weber et al., 2002b) as well as its inihibitory activity on the p38 MAPK and p42/44 MAP kinase pathways (Uchi et al., 2002; Fiebich

et al., 2002) have been proven. In this study we used a cDNA microarray containing ~7000 human genes to analyze the effect of parthenolide on gene expression in TNF-α activated 293 epithelial cells.

Our results show that in 293 cells stimulated for a period of 1 h by TNF-α a set of 39 genes could be identified from which 21 genes were up- and 18 down-regulated by parthenolide, 14 genes related to the transcription factor NF-κB (see Table 2). According to the experimental design these genes belong to the "early group". These results should be considered as a reliable basis for designing further experiments. Therefore, we do not discuss all influenced genes, but the most interesting ones were depicted in respect to the biological activities of SLs.

The genes for the transcription factors Fra-1 and c-Fos, both members of the Fos-family and constituents of the AP-1 proteins, were up-regulated by parthenolide. As DNA binding of the transcription factor AP-1 itself is inhibited by parthenolide in Jurkat T-cells by direct alkylation (Lindenmeyer et al., 2006), up-regulation of members of the AP-1 family could be due to a negative feedback loop. The transcription factor AP-1 also plays a central role in the inflammatory response. It contributes to the induction of matrix metalloproteinases, COX-2 and IL-8, among other genes. Further experiments must show to what extent the activating effect on Fra-1 and c-Fos gene expression may influence the inhibiting effect on AP-1 DNA binding.

Furthermore, gene expression for the complement factor B (CFB) is up-regulated after incubation with parthenolide and subsequent TNF-α stimulation. CFB plays an important role in activating the alternative complement pathway, which is associated with inflammation and immune regulation. Additionally, it is involved in bacterial defense by enhancing bacterial phagocytosis by macrophages (Huang et al., 2002). Whether up-regulation of CFB finally may have positive effects on the host's defense against bacteria and contributes to the known antibacterial activity of parthenolide has to be clarified in future experiments (Picman and Towers, 1983). This is the same with the induced up-regulation of factor H homologue, which plays an important role in regulation of complement activation and possesses anti-inflammatory activities (Zipfel et al., 2002). Interestingly, it has multiple binding sites for microbial surface proteins and may thus also participate in the antibacterial activity of SLs such as parthenolide.

The level of α-1-antitrypsin is elevated, which may also have beneficial effects in inflammation. α-1-antitrypsin is a physiological inhibitor of the serin protease elastase (Doering, 1994). During inflammation processes elastase levels increase and those of α-1-antitrypsin decrease. This imbalance causes tissue erosion. Thus, increasing amounts of the physiological inhibitor can neutralize elastase. This effect of SLs on α-1-antitrypsin strengthens the importance of SLs as potent inhibitors of elastase release from human neutrophils (Siedle et al., 2003).

It is not surprising that parthenolide, which has an epoxide group, slightly stimulates the expression of cytosolic epoxide hydrolase, either in TNF-α stimulated or unstimulated cells. This enzyme is the first line of chemical defense against xenobiotic-derived epoxides. It hydrolyzes epoxides to form vicinal 1,2-diols which are normally less reactive and mutagenic (Argiriadi et al., 1999).

Considering that gene regulation is embedded into a complex network, several reasons may be responsible for the observed up-regulation of genes. Besides NF-κB several transcription factors have been revealed to be involved in regulation of differential gene expression upon TNF-α stimulation (Kel et al., 2006). Parthenolide may differently impair promoter binding of these transcription factors resulting finally in up- or down-regulation of their target genes. Signals coming from one pathway may activate other signalling pathways. Moreover, negative feedback regulatory mechanism as discussed for c-Fos and Fra-1 can also occur.

The group of down-regulated genes encompasses proteins that are regulated by the transcription factor NF-κB such as the chemokines GRO-α and GRO-γ, IL-8 and MIP-3α as well as the cytokine TNF-α. These genes were also down-regulated by parthenolide in keratinocytes after a stimulation time of 1 h (Banno et al., 2005). Only a minor effect could be observed on mRNA of GRO-β and ENA-78 in our study. GRO-α, GRO-β, GRO-γ and IL-8 belong to the CXC-chemokines which possess a wide range of potent stimulatory effects on neutrophils in vitro, including chemotaxis, degranulation, formation of bioactive lipids, integrin up-regulation, expression of adhesion molecules on neutrophils, transendothelial migration and cytoplasmatic Ca^{2+} elevation (Geiser et al., 1993; Wuyts et al., 1998). MIP-3α is a CC-chemokine and a chemoattractant factor for dendritic cells as well as T-cells and plays a crucial role in the initiation of immune response (Sugita et al., 2002). TNF-α is an inflammatory cytokine with cytotoxic properties and involved in cell cycle proliferation and apoptosis (Wuyts et al., 1998).

Comparison of the promoter regions of all these down-regulated genes showed that at least one NF-κB binding site is present, but that also other transcription factors seem to be relevant and responsible for the different expression levels. Thus, MIP-3α possesses binding sites for NF-κB, AP-1, Ets, Sp1 and C/EBP, whereas the promoter of IL-8 obtains binding sites for NF-κB, AP-1, C/EBP and Oct-1 (Hoffmann et al., 2002b; Kwon et al., 2003). Therefore, different compositions of the promoters could contribute to the various strengths and time point of gene induction, resulting finally in different kinetics which enables the chemokines to take over different roles in the inflammatory response.

Moreover, down-regulation of COX-2 gene expression observed by treatment with parthenolide has to be emphasized, too. This inhibitory effect by the SL was also demonstrated in macrophages (Hwang et al., 1996), in which COX-2 belongs to the inducible genes, in contrast to cells of the kidney, in which COX-2 mRNA are constitutively expressed (Parente and Perretti, 2003). COX-2 is involved in prostaglandin production and contributes to inflammation and tumorigenesis. The inhibitory effect of parthenolide on COX-2 gene expression was recently correlated to suppression of protein tyrosine phosphorylation (Hwang et al., 1996), but inhibition of NF-κB DNA binding should also be considered, because the COX-2 promoter contains two binding sites for NF-κB, besides one for NF-IL6/EBF and CREB (Nie et al., 2003).

Interestingly, in unstimulated cells parthenolide increased expression of DAP-1, which interacts with the cytoplasmic death domain of TNF-R1 and induces apoptosis (Liou and Liou, 1999), whereas gene expression is reduced after TNF-α stimulation. Further experiments have to show whether this effect may be of relevance in the apoptotic process influenced by parthenolide besides the already known down-regulating effect of the CD95 system (Li et al., 2003).

Altogether, we provided evidence that parthenolide alters the expression of a variety of genes in 293 cells. In the present study yet unknown genes influenced by parthenolide were identified using microarray analysis. We confirmed that NF-κB is an important target of SLs, because many genes which are regulated by NF-κB were modulated in SL-treated cells. Although, in comparison to quantitative real-time RT-PCR, cDNA microarrays are less discriminatory for the detection of changes in gene expression, the ability of microarrays to globally identify genes affected by parthenolide or other compounds makes them an important tool to understand the molecular mechanism of biologically active compounds.

Acknowledgements

We gratefully acknowledge the financial support of the Volkswagen-Stiftung. MTL is grateful for a scholarship of the Landesgraduiertenförderung Baden-Württemberg. We thank Marinella Klein (Renal Division, Department of Medicine, University Hospital Freiburg, Germany) for technical assistance with the microarray analysis.

References

Aggarwal, B.B., Takada, Y., Shishodia, S., Gutierrez, A.M., Oommen, O.V., Ichikawa, H., Baba, Y., Kumar, A., 2004. Nuclear transcription factor NF-κB: role in biology and medicine. Indian Journal of Experimental Biology 42, 341–353.

Argiriadi, M.A., Morisseau, C., Hammock, B.D., Christianson, D.W., 1999. Detoxification of environmental mutagens and carcinogens: structure, mechanism, and evolution of liver epoxide hydrolase. Proceedings of the National Academy of Sciences of the United States of America 96, 10637–10642.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidmann, J.G., Smith, J.A., Struhl, K., 1998. Current Protocols in Molecular Biology. John Wiley & Sons. Chapter 11.5.1.

Bacher, S., Schmitz, M.L., 2004. The NF-kappaB pathway as a potential target for autoimmune disease therapy. Current Pharmaceutical Design 10, 2827–2837.

Banno, T., Gazel, A., Blumenberg, M., 2005. Pathway-specific profiling identifies the NF-kappa B-dependent tumor necrosis factor alpha-regulated genes in epidermal keratinocytes. Journal of Biological Chemistry 280, 18973–18980.

Boldrick, J.C., Alizadeh, A.A., Diehn, M., Dudoit, S., Liu, C.L., Belcher, C.E., Botstein, D., Staudt, L.M., Brown, P.O., Relman, D.A., 2002. Stereotyped and specific gene expression programs in human innate immune responses to bacteria. Proceedings of the National Academy of Sciences of the United States of America 99, 972–977.

Bonizzi, G., Karin, M., 2004. The two NFkappaB activation pathways and their role in innate and adaptive immunity. Trends in Immunology 25, 280–288.

Bork, P.M., Schmitz, M.L., Kuhnt, M., Escher, C., Heinrich, M., 1997. Sesquiterpene lactone containing Mexican Indian medicinal plants and pure sesquiterpene lactones as potent inhibitors of transcription factor NF-kappaB. FEBS Letters 402, 85–90.

Cramer, P., Muller, C.W., 1999. A firm hand on NFkappaB: structures of the IkappaBalpha–NFkappaB complex. Structure with Folding & Design 7, R1–R6.

Doering, G., 1994. The role of neutrophil elastase in chronic inflammation. American Journal of Respiratory and Critical Care Medicine 150, S114–S117.

Donauer, J., Rumberger, B., Klein, M., Faller, D., Wilpert, J., Sparna, T., Schieren, G., Rohrbach, R., Dern, P., Timmer, J., Pisarski, P., Kirste, G., Walz, G., 2003. Expression profiling on chronically rejected transplant kidneys. Transplantation 76, 539–547.

Eisen, M.B., Brown, P.O., 2004. DNA arrays for analysis of gene expression. Methods in Enzymology 303, 179–205.

Fiebich, B.L., Lieb, K., Engels, S., Heinrich, M., 2002. Inhibition of LPS-induced p42/44 MAP kinase activation and iNOS/NO synthesis by parthenolide in rat primary microglial cells. Journal of Neuroimmunology 132, 18–24.

Garcia-Pineres, A.J., Castro, V., Mora, G., Schmidt, T., Strunck, E., Pahl, H.L., Merfort, I., 2001. Cysteine 38 in p65/NF-kappaB plays a crucial role in DNA binding inhibition by sesquiterpene lactones. Journal of Biological Chemistry 276, 39713–39720.

Garcia-Pineres, A.J., Lindenmeyer, M.T., Merfort, I., 2004. Role of cysteine residues of p65/NFkappaB on the inhibition by the sesquiterpene lactone parthenolide and *N*-ethyl maleimide, and on its transactivating potential. Life Sciences 75, 841–856.

Geiser, T., Dewald, B., Ehrengruber, M.U., Clark-Lewis, I., Baggiolini, M., 1993. The interleukin-8-related chemotactic cytokines GRO alpha, GRO beta, and GRO gamma activate human neutrophil and basophil leukocytes. Journal of Biological Chemistry 268, 15419–15424.

Gertsch, J., Sticher, O., Schmidt, T., Heilmann, J., 2003. Influence of helenanolide-type sesquiterpene lactones on gene transcription profiles in Jurkat T cells and human peripheral blood cells: anti-inflammatory and cytotoxic effects. Biochemical Pharmacology 66, 2141–2153.

Gilmore, T.D., 2006. http://people.bu.edu/gilmore/nf-kb/Boston University's Rel/NF-kB.

Hehner, S.P., Hofmann, T.G., Droge, W., Schmitz, M.L., 1999. The antiinflammatory sesquiterpene lactone parthenolide inhibits NF-kappa B by targeting the I kappa B kinase complex. Journal of Immunology 163, 5617–5623.

Hoffmann, A., Baltimore, D., 2006. Circuitry of nuclear factor kappaB signaling. Immunology Reviews 210, 171–186.

Hoffmann, A., Levchenko, A., Scott, M.L., Baltimore, D., 2002a. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. Science 298, 1241–1245.

Hoffmann, E., Dittrich-Breiholz, O., Holtmann, H., Kracht, M., 2002b. Multiple control of interleukin-8 gene expression. Journal of Leukocyte Biology 72, 847–855.

Huang, Y., Krein, P.M., Muruve, D.A., Winston, B.W., 2002. Complement factor B gene regulation: synergistic effects of TNF-alpha and IFN-gamma in macrophages. Journal of Immunology 169, 2627–2635.

Hwang, D., Fischer, N.H., Jang, B.C., Tak, H., Kim, J.K., Lee, W., 1996. Inhibition of the expression of inducible cyclooxygenase and proinflammatory cytokines by sesquiterpene lactones in macrophages correlates with the inhibition of MAP kinases. Biochemical and Biophysical Research Communications 226, 810–818.

Kel, A., Voss, N., Jauregui, R., Kel-Margoulis, O., Wingender, E., 2006. Beyond microarrays: finding key transcription factors controlling signal transduction pathways. BMC Bioinformatics 7, 1–13.

Knight, D.W., 1995. Feverfew: chemistry and biological activity. Natural Product Reports 12, 271–276.

Kwon, J.H., Keates, S., Simeonidis, S., Grall, F., Libermann, T.A., Keates, A.C., 2003. ESE-1, an enterocyte-specific Ets transcription factor, regulates MIP-3alpha gene expression in Caco-2 human colonic epithelial cells. Journal of Biological Chemistry 278, 875–884.

Li, J., Peet, G.W., Balzarano, D., Li, X., Massa, P., Barton, R.W., Marcu, K.B., 2001. Novel NEMO/IkappaB kinase and NF-kappa B target genes at the pre-B to immature B cell transition. Journal of Biological Chemistry 276, 18579–18590.

Li, A., Dubey, S., Varney, M.L., Dave, B.J., Singh, R.K., 2003. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. Journal of Immunology 170, 3369–3376.

Li, X., Cui, X., Li, Y., Fitz, Y., Hsu, L., Eichacker, P.Q., 2006. Parthenolide has limited effects on nuclear factor-kappabeta increases and worsens survival in lipopolysaccharide-challenged C57BL/6J mice. Cytokine 33, 299–308.

Li-Weber, M., Giaisi, M., Treiber, M.K., Krammer, P.H., 2002a. The anti-inflammatory sesquiterpene lactone parthenolide suppresses IL-4 gene expression in peripheral blood T. European Journal of Immunology 32, 3587–3597.

Li-Weber, M., Giasi, M., Baumann, S., Treiber, M.K., Krammer, P.H., 2002b. The anti-inflammatory sesquiterpene lactone parthenolide suppresses CD95-mediated activation-induced-cell-death in T-cells. Cell Death and Differentiation 9, 1256–1265.

Lindenmeyer, M.T., Garcia-Pineres, A.J., Castro, V., Merfort, I., 2004. Sesquiterpene lactones inhibit luciferase but not beta-galactosidase activity in vitro and ex vivo. Analytical Biochemistry 328, 147–154.

Lindenmeyer, M.T., Hrenn, A., Kern, C., Castro, V., Murillo, R., Muller, S., Laufer, S., Schulte-Monting, J., Siedle, B., Merfort, I., 2006. Sesquiterpene lactones as inhibitors of IL-8 expression in HeLa cells. Bioorganic and Medicinal Chemistry 14, 2487–2497.

Liou, M.L., Liou, H.C., 1999. The ubiquitin-homology protein, DAP-1, associates with tumor necrosis factor receptor (p60) death domain and induces apoptosis. Journal of Biological Chemistry 274, 10145–10153.

Lyss, G., Schmidt, T.J., Merfort, I., Pahl, H.L., 1997. Helenalin, an anti-inflammatory sesquiterpene lactone from Arnica, selectively inhibits transcription factor NF-kappaB. Biological Chemistry 378, 951–961.

Mazor, R.L., Menendez, I.Y., Ryan, M.A., Fiedler, M.A., Wong, H.R., 2000. Sesquiterpene lactones are potent inhibitors of interleukin 8 gene expression in cultured human respiratory epithelium. Cytokine 12, 239–245.

Nie, M., Pang, L., Inoue, H., Knox, A.J., 2003. Transcriptional regulation of cyclooxygenase 2 by bradykinin and interleukin-1beta in human airway smooth muscle cells: involvement of different promoter elements, transcription factors, and histone h4 acetylation. Molecular and Cellular Biology 23, 9233–9244.

Parente, L., Perretti, M., 2003. Advances in the pathophysiology of constitutive and inducible cyclooxygenases: two enzymes in the spotlight. Biochemical Pharmacology 65, 153–159.

Picman, A.K., Towers, G.H., 1983. Antibacterial activity of sesquiterpene lactones. Biochemical Systematics and Ecology 11, 321–327.

Picman, A.K., Rodriguez, E., Towers, G.H., 1979. Formation of adducts of parthenin and related sesquiterpene lactones with cysteine and glutathione. Chemical and Biological Interaction 28, 83–89.

Schmidt, T.J., 1999. Toxic activities of sesquiterpene lactones: structural and biochemical aspects. Current Organic Chemistry 3, 577–608.

Siedle, B., Gustavsson, L., Johansson, S., Murillo, R., Castro, V., Bohlin, L., Merfort, I., 2003. The effect of sesquiterpene lactones on the release of human neutrophil elastase. Biochemical Pharmacology 65, 897–903.

Sobota, R., Szwed, M., Kasza, A., Bugno, M., Kordula, T., 2000. Parthenolide inhibits activation of signal transducers and activators of transcription (STATs) induced by cytokines of the IL-6 family. Biochemical and Biophysical Research Communications 267, 329–333.

Sugita, S., Kohno, T., Yamamoto, K., Imaizumi, Y., Nakajima, H., Ishimaru, T., Matsuyama, T., 2002. Induction of macrophage-inflammatory protein-3alpha gene expression by TNF-dependent NF-kappaB activation. Journal of Immunology 168, 5621–5628.

Uchi, H., Arrighi, J.F., Aubry, J.P., Furue, M., Hauser, C., 2002. The sesquiterpene lactone parthenolide inhibits LPS-but not TNF-alpha-induced maturation of human monocyte-derived dendritic cells by inhibition of the p38 mitogen-activated protein kinase pathway. Journal of Allergy and Clinical Immunology 110, 269–276.

Wagner, S., Hofmann, A., Siedle, B., Terfloth, L., Merfort, I., Gasteiger, J., 2006. Development of a structural model for NF-kappaB inhibition of sesquiterpene lactones using self-organizing neural networks. Journal of Medicinal Chemistry 49, 2241–2252.

Wuyts, A., Proost, P., Van Damme, J., 1998. In: Thomson, A.W. (Ed.), The Cytokine Handbook. Academic Press, San Diego, Calif, pp. 271–311.

Zhou, A., Scoggin, S., Gaynor, R.B., Williams, N.S., 2003. Identification of NF-kappa B-regulated genes induced by TNFalpha utilizing expression profiling and RNA interference. Oncogene 22, 2054–2064.

Zipfel, P.F., Skerka, C., Hellwage, J., Jokiranta, S.T., Meri, S., Brade, V., Kraiczy, P., Noris, M., Remuzzi, G., 2002. Factor H family proteins: on complement, microbes and human diseases. Biochemical Society Transactions 30, 971–978.