European Journal of Pharmacology 657 (2011) 41–50

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and Cellular Pharmacology

NF-κB-mediated anti-inflammatory activity of the sesquiterpene lactone 7-hydroxyfrullanolide

Lyle C. Fonseca [a,*], Shruta S. Dadarkar [a], Aurelio S. Lobo [a], Prabha B. Mishra [a], Arvind D. Thakkar [a], Shanthi Chandrababu [b], Muralidhara Padigaru [a,**]

[a] Department of Pharmacology, Piramal Life Sciences Limited, Mumbai-400063, Maharashtra, India
[b] School of Bio Sciences and Technology, Vellore Institute of Technology University, Vellore-632014, Tamil Nadu, India

ARTICLE INFO

Article history:
Received 3 November 2010
Received in revised form 6 January 2011
Accepted 20 January 2011
Available online 4 February 2011

Keywords:
Sesquiterpene lactone
Inflammation
Microarray
Adhesion molecules

ABSTRACT

Microarray technology can be used to study the molecular mechanisms of new chemical entities with the aim to develop effective therapeutics. 7-Hydroxyfrullanolide (7HF) is a sesquiterpene lactone that was found to be efficacious in multiple animal models of inflammation by suppression of pro-inflammatory cytokines; however, its molecular mechanism of action remains unclear. We investigated the effects of 7HF on lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells using microarray-based gene expression studies and explored the molecular targets affected. Gene expression profiles and pathway analysis revealed that 7HF potently suppressed multiple inflammatory pathways induced by LPS. More importantly, 7HF was found to inhibit NF-κB related transcripts. These transcripts were further validated using freshly isolated synovial cells from rheumatoid arthritis patients, thus clinically validating our findings. Cell-based imaging and subsequent Western blot analysis demonstrated that 7HF inhibited the translocation of NF-κB into the nucleus by directly inhibiting the phosphorylation of IKK-β. Since the transcription of adhesion molecules is regulated by NF-κB, further investigation showed that 7HF dose-dependently suppressed ICAM-1, VCAM-1 and E-selectin expression on LPS-stimulated endothelial cells as well as inhibited the adhesion of monocytes to LPS-stimulated endothelial cells. Taken together, our results reveal that 7HF possesses NF-κB inhibitory potential and suggest a likely molecular mechanism of its anti-inflammatory activity.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

7-Hydroxyfrullanolide (7HF; Fig. 1A) is a sesquiterpene lactone isolated from the methanolic extract of *Sphaeranthus indicus* (Atta et al., 1989; Sohoni et al., 1988). We have previously shown that 7HF inhibits pro-inflammatory cytokine production from lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells and freshly isolated synovial cells from rheumatoid arthritis patients and is orally efficacious in animal models of acute and chronic inflammation (Fonseca et al., 2010). In order to obtain new insights into the possible molecular basis of the anti-inflammatory action of 7HF, we conducted further studies using a genomic approach.

The genomic revolution has recently ushered the understanding of anti-inflammatory action of various compounds (Ben-Chetrit et al., 2006). Human peripheral blood mononuclear cells are the effector cells involved in inflammation, and gene expression profiling of peripheral blood using microarrays is already an established practice (Gu et al., 2002; Lindenmeyer et al., 2007). Microarray technology, which allows simultaneous monitoring of the expression levels of thousands of individual genes, provides comprehensive information to decipher how a compound affects cellular metabolism on a genomic scale (Gray et al., 1998).

Sesquiterpene lactones constitute a large and diverse group of biologically active plant chemicals that have been identified in several plant families (Neerman, 2003) and form one of the largest groups of bioactive compounds of plant origin. Sesquiterpene lactones have been reported in literature to be effective against inflammation, cancer, atherosclerosis, cardiac injury as well as bacterial, fungal and malarial diseases. (Lindenmeyer et al., 2007). It has been shown that sesquiterpene lactones exert most of their biological effects, by alkylating biological nucleophiles. The α,β-unsaturated carbonyl structures of sesquiterpene lactones, such as α-methylene-γ-lactone and α,β-en-one groups, react with thiols, like cysteine, of functional proteins and key enzymes via a Michael type addition, thus interfering with the functions of these biological targets (Picman et al., 1979). Prior microarray-based gene expression profiling studies have been conducted to determine the molecular effect of the sesquiterpene lactone parthenolide on epithelial cells (Lindenmeyer et al., 2007).

In the present study, we investigated the molecular basis of the action of 7HF by analyzing the effect of this molecule on global gene

* Corresponding author. Tel.: +91 22 30818417; fax: +91 22 30818036.
** Corresponding author. Tel.: +91 22 30818802; fax: +91 22 30818036.
E-mail addresses: lyle.fonseca@piramal.com (L.C. Fonseca), muralidhara.padigaru@piramal.com (M. Padigaru).

0014-2999/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2011.01.050

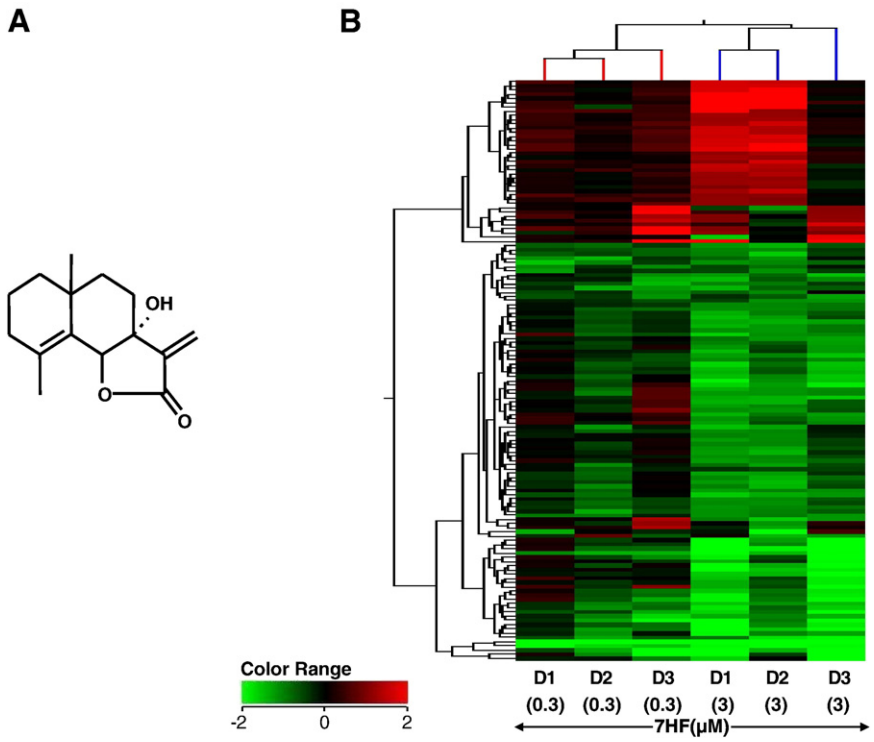


Fig. 1. Dendogram showing effect of 7HF treatment on LPS-stimulated human peripheral blood mononuclear cells. (A) Structure of 7HF. (B) Unsupervised hierarchical, uncentered Pearson distance correlation clustering was performed to classify the 280 genes into homogeneous clusters. Individual genes are represented in rows while the columns represent three individual donors at two concentrations of 7HF treatment. Black represents no change in expression relative to vehicle control. Augmented and diminished expression is represented by increasing intensity of red and green, respectively. The expression color bar demonstrates the limits of regulation on either direction.

expression. We exposed human peripheral blood mononuclear cells to different concentrations of 7HF followed by LPS stimulation and examined its effect on the induced inflammatory response using microarray-based transcript profiling. This approach enabled us to obtain a broad view of the anti-inflammatory activity of 7HF and allowed us to identify genes that are potentially related to its mechanism of action. In order to further probe the intracellular effect of 7HF, protein-based studies were performed to corroborate the microarray findings.

2. Materials and methods

2.1. LPS stimulation of human peripheral blood mononuclear cells treated with 7HF

Peripheral blood was collected from healthy human donors after informed consent and Independent Ethics Committee approval. Human peripheral blood mononuclear cells were harvested using Ficoll–Hypaque density gradient centrifugation (1.077 g/ml; Sigma Aldrich, St. Louis, MO) (Dagia et al., 2009; Fonseca et al., 2010) and suspended in assay medium [RPMI 1640 culture medium (Sigma Aldrich) containing 10% heat inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KA), 100 U/ml penicillin (Sigma Aldrich) and 100 μg/ml streptomycin (Sigma Aldrich)]. A cell suspension containing 1×10^6 cells/ml was prepared, and 20 ml of the cell suspension was aliquoted into a cell culture flask. The cells were pre-treated with two different concentrations of 7HF or 0.05% dimethyl sulfoxide (DMSO) for 30 min at 37 °C, 5% CO_2 and stimulated with 1 μg/ml LPS (*Escherichia coli* Serotype 0127:B8; Sigma Aldrich). The cells were incubated for 2 h at 37 °C, 5% CO_2 following which both the adherent and suspension cells were harvested. The viability of cells after drug treatment was determined using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI).

2.2. Ex vivo treatment of synovial cells with 7HF

After informed consent and Independent Ethics Committee approval, synovial tissue was obtained from rheumatoid arthritis patients undergoing knee replacement surgery. Synovial cells were isolated as described earlier (Fonseca et al., 2010). For the experiment, a cell suspension containing 1×10^6 synovial cells/ml was prepared, and 20 ml of the cell suspension was aliquoted into a cell culture flask. The cells were pre-treated with 3 μM of 7HF or 0.05% DMSO (vehicle) for 16 h at 37 °C, 5% CO_2 following which both the adherent and suspension cells were harvested. The viability of cells after drug treatment was determined using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI).

2.3. RNA isolation

The procedure followed was similar to that described elsewhere (Dadarkar et al., 2011). Total cellular RNA was extracted from human peripheral blood mononuclear cells using TRIzol (Invitrogen Corporation, CA, USA) and purified using RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's recommendation. The concentration and purity of RNA samples was determined using NanoDrop ND-1000 spectrophotometer (NanoDrop products, DE, USA).

2.4. Gene expression profiling

The procedure followed was as described previously (Dadarkar et al., 2010; Thakkar et al., 2010). Microarray-based gene expression data for 7HF was generated by using 23 K human oligo chip based on 70-mer oligonucleotides (Operon, AL, USA). 20 μg of RNA from 7HF-treated human peripheral blood mononuclear cells and 20 μg of RNA from vehicle treated human peripheral blood mononuclear cells were reverse transcribed and labeled with Cy5 and Cy3 (GE Healthcare,

UK), respectively, according to the protocol established by Pronto Indirect Labeling kit (Promega Corporation, WI, USA). The labeled cDNA concentration and Cy5, Cy3 incorporation were assessed using the NanoDrop ND-1000 spectrophotometer. Pre-hybridization and post-hybridization of the spotted aminosilane slides was performed according to Universal Microarray Hybridization kit (Corning Inc., NY, USA). Hybridization was carried out for 6 h at 42 °C, 6 h at 35 °C, and 6 h at 30 °C. GeneTAC GT UC scanner (Genomic Solutions Inc., USA) was used to scan the hybridized slides. Image analysis was carried out using the GeneTAC Integrator software followed by global normalization. The gene array data was further analyzed using GeneSpring GX10 (Agilent Technologies, Santa Clara, CA).

2.5. RTQ-PCR analysis

For each sample, 2 μg of total RNA was reverse transcribed in a total volume of 20 μl using 200 units Superscript II reverse transcriptase (Invitrogen), oligo (dT)20, 50 pmol of random hexamer, and 10 mM of deoxynucleotide triphosphates (Invitrogen). PCR amplification was performed on an Eppendorf Mastercycler® realplex4 system (Eppendorf AG, Hamburg, Germany) using the Quanti-Fast SYBR Green PCR Kit (Qiagen). Briefly, each PCR reaction contained 1× master mix, 1 μl of the diluted cDNA, and 250 nM of forward and reverse primers. PCR was carried out through 40 cycles (95 °C for 10 s, 60 °C for 30 s) following initial 3 min enzyme activation at 95 °C. The primers used (Table 2) were designed using Primer 3 software and custom synthesized at Bioserve (Hyderabad, India). Melting curve analysis was performed at the end of PCR and changes in gene expression were analyzed using the 'Realplex' software (version 1.5). The relative expression of each gene was calculated using comparative Ct method with the formula: $2^{-\Delta\Delta Ct}$ (where ΔΔCt = ΔCt sample − ΔCt reference), where Ct is the number of cycles required to reach the threshold. Relative quantification of target mRNA expression was calculated and normalized to GAPDH expression. The results are presented as the $\log_2$ fold change of mRNA expression as compared to the amount present in vehicle control samples.

2.6. Cell-Based imaging

Human monocytic THP-1 cells (ATCC, Manassas, VA) were cultured in RPMI 1640 (Gibco BRL, Pasley, UK) supplemented with 10% heat inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (Dagia et al., 2010). Phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich) differentiation of THP-1 cells was carried out, the cells were examined for adherence and characteristic macrophage morphology before they were used for experiments. PMA-differentiated THP-1 cells were pre-treated with different concentrations of 7HF or 3 μM of 4(2'-Aminoethyl)amino-1,8-dimethylimidazo(1,2-a)quinoxaline [BMS-345541; a known IKK inhibitor; Sigma Aldrich] or 0.5% DMSO for 30 min at 37 °C, 5% CO_2 and stimulated with 1 μg/ml LPS for 30 min at 37 °C, 5% CO_2. Cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS; Sigma Aldrich), fixed with 3.7% formaldehyde (Merck Biosciences, Darmstadt, Germany) for 20 min and permeabilized with chilled permeabilization buffer (0.15% Triton X-100; Thermo Scientific, Waltham, MA) for 1.5 min. Wells were washed and subsequently blocked with DPBS containing 5% FBS and 5% goat serum (Vector Laboratories Inc., Burlingame, CA) for 1 h at room temperature. Appropriate dilution of rabbit anti-p65 (NF-κB) antibody (Primary antibody; Cell Signaling, Beverly, MA) was then added to the plates and incubated overnight at 4 °C. Following extensive washing, cells were incubated for 2 h at room temperature with staining solution containing 10 μM Hoechst 33342 (AnaSpec Inc., Fermont, CA) and 1 μg/ml DyLight™ 549-conjugated goat anti-rabbit antibody (Secondary antibody; Thermo Scientific). Finally, cells were washed with DPBS and scanned images of the cells were acquired on the Cellomics® ArrayScan® HCS reader (Thermo Scientific) as carried out by others (Tian et al., 2005). The Cellomics® BioApplication image processing software module was used for image analysis. Quantification of cytoplasmic and nuclear p65 (NF-κB) was carried out using the Cellomics® ArrayScan® VTI platform. All the data points were analyzed using the compartmental analysis and molecular translocation bio-algorithm of Cellomics and the quantitative data was expressed as percentage (%) activation in comparison to the DMSO control cells. One thousand cells were counted for each replicate well and the results were presented as an average ± S.E.M. The 50% inhibitory concentration (IC_{50}) values and 95% confidence intervals (95% CI) were calculated by a nonlinear regression method using GraphPad software (Prism 3.03). In all experiments, a parallel plate was run to ascertain the toxicity of 7HF. The toxicity was determined using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI). In every experiment, each condition was run in triplicate wells.

2.7. Western blot analysis

PMA-differentiated THP-1 cells were pre-treated with different concentrations of 7HF or 0.5% DMSO for 30 min, subsequent to which the cells were stimulated with 1 μg/ml LPS. Following incubation at 37 °C for 30 min, the cells were washed extensively and nuclear and cytoplasmic extracts were obtained using the NE-PER® Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology, Rockford, IL). Lysates were used immediately or stored at −80 °C for later use. The protein levels in lysates were quantified using Bradford Reagent (Bio-Rad Laboratories, Hercules, CA). SDS-PAGE and Western blotting was performed in a manner similar to that described previously (Dagia et al., 2010). The antibodies utilized included: rabbit anti-p65 (NF-κB; Cell Signaling), rabbit anti-IκBα (Cell Signaling), rabbit anti-phospho IκBα (Cell Signaling) rabbit anti-phospho IKK-β (Cell Signaling), rabbit anti-β-actin (Sigma Aldrich), rabbit anti-histone deacetylase (Sigma Aldrich) and horseradish peroxidase-conjugated polyclonal (secondary) antibody to rabbit IgG (Calbiochem, Darmstadt, Germany). BMS-345541 was used as a positive control.

2.8. IKK-β kinase assay

A commercially available kit (HTScan® IKK-β Kinase Assay Kit; Cell Signaling) was utilized to perform this assay. Briefly, 25–50 ng of recombinant human IKK-β kinase was treated with various concentrations of 7HF or 0.02% DMSO or BMS-345541 for 5 min at room temperature. Subsequently, a cocktail of ATP/IκBα (Ser32) biotinylated peptide ([ATP] = 200 μM, [IκBα] = 1.5 μM) was added and the reaction mixture (total volume 50 μl) incubated at room temperature for 30 min. The reaction was stopped by addition of 50 μl of Stop Buffer (50 mM EDTA, pH 8). The phosphorylation status of IκBα (a downstream target of activated IKK-β) was ascertained using a streptavidin-coated plate (Perkin Elmer, Waltham, MA) and mouse anti-human phospho-IκBα (Ser32/36) (5A5; Cell Signaling) antibody in a colorimetric Enzyme-Linked Immunosorbent Assay (ELISA). In all experiments, a non-enzyme blank (i.e., reaction mixture containing ATP/IκBα(Ser32) biotinylated peptide but no IKK-β) was used as a negative control. The absorbance values of this negative control were considered as background. The absorbance values of DMSO-treated IKK-β sample were utilized to calculate% IKK-β inhibition.

2.9. Endothelial cell adhesion molecule assay

Human umbilical vein endothelial cells (HUVEC; Cascade Biologics, Portland, OR) were cultured as described by others (Dagia et al., 2006). For the experiment, confluent HUVEC in fibronectin-coated 96-well plates were pre-treated with 7HF or 0.5% DMSO. After a 30-min pre-treatment at 37 °C, 5% CO_2, HUVEC were stimulated with 1 μg/ml LPS for 6 h at 37 °C, 5% CO_2. Subsequently, cell surface expression of

E-selectin, ICAM-1, and VCAM-1 was determined using ELISA, as described by others (Dagia et al., 2004). The reagents utilized in ELISA were from R&D Systems and included mouse monoclonal antibodies to ICAM-1 (clone BBIG-I1), VCAM-1 (clone BBIG-V1), E-selectin (clone BBIG-E4), isotype control mouse IgG1, and horseradish peroxidase-conjugated polyclonal (secondary) antibody to mouse IgG. In every experiment, 1 μM (E)-3-(4-methylphenylsulfonyl)-2-propenenitrile [BAY 11-7082; Calbiochem] was used as a positive control for inhibiting induced endothelial cell adhesion molecule (ECAM) expression. Furthermore, in every experiment, each condition was run in triplicate wells.

2.10. Monocyte–endothelial cell adhesion assay

The procedure followed was as reported by others (Yamaguchi et al., 1997) with slight modifications. Briefly, THP-1 cells were harvested, washed and resuspended to 6×10^5 cells/ml in HUVEC culture medium. Subsequently, THP-1 cells were labeled with 10 μg/ml 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Sigma Aldrich) for 30 min at room temperature in dark. Following the incubation, the reaction was quenched with 0.1% bovine serum albumin (Sigma Aldrich) and the THP-1 cells resuspended at 6×10^5 cells/ml in HUVEC culture medium. For the experiment, confluent HUVEC in fibronectin-coated six-well plates were pre-treated with 7HF (3 μM) or 0.5% DMSO. After a 30-min pre-treatment at 37 °C, 5% CO_2, HUVEC were stimulated with 1 μg/ml LPS for 6 h at 37 °C, 5% CO_2. The HUVEC were washed, and 1.8×10^6 labeled THP-1 cells added per well. After a 10 min incubation, HUVECs were washed extensively and THP-1 cell adhesion to HUVEC visualized using a fluorescence microscope (Nikon 80i with DXM1200 camera). In every experiment, BAY 11-7082 (1 μM) was used as a positive control for inhibiting induced monocyte–endothelial cell adhesion.

2.11. Statistical analysis

One-way ANOVA was performed for gene expression analysis using GeneSpring GX10. For analyzing differences between two groups, Student's *t*-test was used. For analyzing differences among multiple (more than two) groups, a single factor ANOVA followed by Dunnett's multiple comparison tests was used. P values <0.05 were considered statistically significant. Unless stated otherwise, all error bars represent standard error of mean.

3. Results

3.1. 7HF suppresses expression of inflammatory related genes in LPS-stimulated human peripheral blood mononuclear cells

As a first step towards ascertaining the mechanism for anti-inflammatory potential of 7HF, we investigated its effect on induced changes in the genome of human peripheral blood mononuclear cells. LPS-stimulated human peripheral blood mononuclear cells were pre-treated with two different concentrations of 7HF in order to study its transcriptional effect on the inflammatory response. Data analysis was carried out using GeneSpring GX10 to identify genes dysregulated by 7HF treatment, and genes which exhibited ≥2-fold differential expression were considered differentially expressed. These analyses led to identification of a set of 280 genes which showed a significantly differential expression by 7HF pre-treatment. Unsupervised clustering was carried out using a hierarchical algorithm and Pearson-based distance approach. These results are concised in a dendogram (Fig. 1B) created using GeneSpring GX10.

Subsequently, biological enrichment analysis of the microarray data was performed to identify the primary transcriptional target down-regulated by 7HF. We checked for over-representation of transcription factor binding sites in up- or down-regulated genes using the over-representation analysis tool 'oPOSSUM' (Ho Sui et al., 2005) and performed Gene Set Enrichment Analysis (GSEA) to interpret the gene expression data (Subramanian et al., 2005). Our analyses revealed that a number of key genes down-regulated by 7HF correlated with the transcription factor NF-κB. The 280 differentially expressed genes were first classified into biological processes using PANTHER analysis and further sub-classified on the basis of biology of immune processes (Table 1). We observed that the down-regulated genes obtained belonged to different groups including cytokines (e.g., TNF, IL6, IL1A), chemokines (e.g., CCL2, CCL26, CCL7), stress response (e.g., GADD45B, NFKBIA, TNFAIP8), blood clotting (e.g., F3, PLSCR1, THBS1), immune and defense (e.g., ANTXR2, IFNGR2, FOS), macrophage-mediated immunity (e.g., ALOX5AP, GBP1, GBP4) and T-cell-mediated immunity (e.g., CD69, SLAMF7).

From the set of 280 genes obtained after gene expression analysis, a panel of 14 genes related to the NF-κB pathway was selected for validation by RTQ-PCR analysis. The candidate genes related to NF-κB and down-regulated by 7HF included BIRC3, CCL7, CXCL1, EHD1, EIF4A2, FOS, IL1A, IL1RN, IL6, NFKB1, NFKBIA, NFKBIZ, TANK and TNF. Details of the selected genes are given in Table 2. RNA isolated from human peripheral blood mononuclear cells from each of the three donors was subjected to RTQ-PCR analyses using appropriate primers, which resulted in the amplification of the selected genes (Fig. 2A). In accordance with the microarray data, 7HF showed ≥2 fold down-regulation of all 14 genes (Fig. 2A). This data was consistent across cells from all three donors thereby confirming the microarray data.

3.2. 7HF down-regulates NF-κB-related transcripts in synovial cells obtained from active rheumatoid arthritis patients

Having established that 7HF down-regulates LPS-induced inflammatory response in human peripheral blood mononuclear cells, we wanted to evaluate their effect in a physiologically relevant cell system such as freshly isolated synovial cells. It is well-known that synovial cells from rheumatoid arthritis patients secrete soluble pro-inflammatory cytokines spontaneously in the absence of an extrinsic stimulus for a prolonged period of time, and hence it is likely that most of the inflammatory genes especially those of the NF-κB cascade are continually up-regulated (Marok et al., 1996). Thus, we used RTQ-PCR to validate the effect of 7HF on the 14 selected genes (identified by microarray-based studies; Table 2) in synovial cells (Fig. 2B). Similar to our observation with human peripheral blood mononuclear cells, synovial cells also displayed ≥2-fold down-regulation of transcripts in presence of 7HF (Fig. 2B). This data was consistent across synovial cells obtained from all three patients. These results suggest that 7HF causes down-regulation of NF-κB-related transcripts in pathologically relevant synovial tissue cells and confirms that the down-regulation of genes related to NF-κB plays an important role in pro-inflammatory cytokine inhibition in diseased patients.

3.3. 7HF inhibits NF-κB translocation by inhibiting phosphorylation of IKK-β

In order to validate the microarray data and subsequent RTQ-PCR profiles, we carried out protein studies to determine the effect of 7HF on the NF-κB signaling cascade. Given that stimulation of THP-1 macrophages with LPS leads to the activation of NF-κB, as a first step towards further characterization of 7HF, we examined its effects on the translocation of NF-κB into the nucleus. Consistent with the results of earlier studies (Zhou et al., 2004), high content cell-based imaging analysis of LPS-stimulated human monocytic THP-1 cells showed significant nuclear translocation of NF-κB (p65 subunit) compared with naïve cells (Fig. 3). More importantly, pre-treatment of THP-1 cells with 7HF inhibited the LPS-induced translocation of

Table 1
Classification of genes into immune processes based on PANTHER analysis. Gene expression profiling of 7HF-treated human peripheral blood mononuclear cells was carried out and the 280 differentially expressed genes obtained were first classified into biological processes using PANTHER analysis and further sub-classified on the basis of immune processes. Expression values depicted are $\log_2$ fold change in gene expression by 7HF treatment and are averages of three donors. Many of the classified genes are NF-κB-related.

Immune process	Accession ID	Gene symbol	7HF (0.3 μM)	7HF (3 μM)	NF-κB regulated
Cytokine	NM_000594	TNF	−1.374	−3.108	+
	NM_000577	IL1RN	−1.856	−2.993	+
	NM_000575	IL1A	−1.236	−3.447	+
	NM_000600	IL6	−0.444	−2.626	+
Chemokine	NM_002982	CCL2	−1.116	−3.975	+
	NM_006072	CCL26	−0.463	−2.523	−
	NM_006273	CCL7	0.118	−2.029	−
	NM_001511	CXCL1	−0.048	−1.802	+
Stress response	NM_015675	GADD45B	−0.708	−1.056	+
	NM_020529	NFKBIA	−0.333	−1.614	+
	NM_002135	NR4A1	−0.978	−1.265	+
	NM_006186	NR4A2	−1.015	−1.469	+
	NM_006981	NR4A3	−0.957	−0.974	+
	NM_002852	PTX3	0.044	−0.989	+
	NM_014445	SERP1	−0.540	−0.961	−
	NM_014350	TNFAIP8	−0.200	−1.715	+
Blood clotting	NM_001993	F3	−0.636	−2.262	+
	NM_021105	PLSCR1	−0.885	−1.541	−
	NM_003246	THBS1	−0.799	−3.594	+
Immune and defense	NM_058172	ANTXR2	−0.184	−1.004	+
	NM_002110	HCK	0.286	−1.055	−
	NM_005534	IFNGR2	−0.103	−1.865	−
	NM_000963	PTGS2	−0.288	−1.974	+
	NM_002922	RGS1	−1.077	−1.491	+
	NM_005252	FOS	−0.281	−1.276	+
	NM_181657	LTB4R	0.116	−1.078	+
Macrophage-mediated immunity	NM_001629	ALOX5AP	0.237	−1.014	+
	NM_002053	GBP1	−1.190	−1.718	+
	NM_052941	GBP4	−0.752	−1.391	−
	NM_052942	GBP5	−0.580	−1.252	−
	NM_002964	S100A8	0.416	−0.979	−
T-cell-mediated immunity	NM_001781	CD69	−1.779	−2.286	+
	NM_021181	SLAMF7	−0.218	−1.275	−
Granulocyte-mediated immunity	NM_000759	CSF3	−0.539	−1.337	+
MHCII-mediated immunity	NM_004079	CTSS	0.171	−1.062	−
Natural killer cell-mediated immunity	NM_002287	LAIR1	0.163	−1.137	−
Interferon-mediated immunity	NM_001547	IFIT2	−0.497	−1.828	−
B-cell and antibody-mediated immunity	NM_003998	NFKB1	−0.293	−1.210	+

NF-κB with an IC_{50} of 0.3 μM (95% CI, 0.05–1.1 μM) (Fig. 3). Western blot analysis of nuclear and cytoplasmic extracts from LPS-stimulated THP-1 cells showed distinct presence of NF-κB in the nuclear extracts compared to naïve cells. In cells pre-treated with 7HF a concentration-dependent inhibition of NF-κB translocation was observed (Fig. 4A). It is well established that activation of NF-κB is brought about by the phosphorylation and subsequent degradation of IκBα which sequesters NF-κB in the cytoplasm and prevents its translocation into the nucleus. Accordingly, to further unravel the upstream effects of 7HF-mediated NF-κB inhibition, we probed its effects on phosphorylation of IκBα. PMA-differentiated THP-1 cells were pre-treated with or without 7HF and then stimulated with LPS. Western blot analysis of lysates from LPS-stimulated THP-1 cells showed distinct up-regulation in the phosphorylation status of IκBα compared with lysates from naïve cells (Fig. 4B). Importantly, pre-treatment with 7HF led to abrogation of LPS-induced IκBα phosphorylation (Fig. 4B).

Table 2
Candidate gene set for RTQ-PCR analysis. List of genes and their primer sequences used for the RTQ-PCR analysis along with their accession numbers. The primer sequences were designed using Primer-3.

Gene name	Gene symbol	Accession number	Forward primer	Reverse primer
Baculoviral IAP repeat-containing 3	BIRC3	NM_001165	TGCAAGAAGCTGAAGCTGTG	GTCTCCGCAATTGTTCTTCC
Chemokine (C-C motif) ligand 7	CCL7	NM_006273	ACCACCAGTAGCCACTGTCC	TTTCTTGTCCAGGTGCTTCA
Chemokine (C-X-C motif) ligand 1	CXCL1	NM_001511	CCCCAAGAACATCCAAAGTG	ACTATGGGGGATGCAGGATT
EH-domain containing 1	EHD1	NM_006795	GGTGAACAACCTGGGAGAGA	TGCTGAAGTCCTGGGTCTG
Eukaryotic translation initiation factor 4A2	EIF4A2	NM_001967	TGTGCAACAAGTGTCTTTGGT	ACCTTTCCTCCCAAATCGAC
FBJ murine osteosarcoma viral oncogene homolog	FOS	NM_005252	AAGGAGAATCCGAAGGGAAA	TAGTTGGTCTGTCTCCGCTTG
Interleukin 1, alpha	IL1A	NM_000575	TGTGACTGCCCAAGATGAAG	CCGTGAGTTTCCCAGAAGAA
Interleukin 1 receptor antagonist	IL1RN	NM_000577	GGAATCCATGGAGGGAAGAT	TGTTCTCGCTCAGGTCAGTG
Interleukin 6	IL6	NM_000600	AGTGAGGAACAAGCCAGAGC	GTTGGGTCAGGGGTGGTTAT
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NFKB1	NM_003998	GGTCTCTGGGGGTACAGTCA	TCGGAGCTCGTCTATTTGCT
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NFKBIA	NM_020529	CTCCATCCTGAAGGCTACCA	TCCTGAGCATTGACATCAGC
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	NFKBIZ	NM_031419	GCCTAATTCAAATGGGAGCA	AGAGGCGAATGAGTTCCAGA
TRAF family member-associated NFKB activator	TANK	NM_004180	GACCCATCTGATGCACCTTT	CCACCGAGTCACTGTCTTGA
Tumor necrosis factor	TNF	NM_000594	CCCCAGGGACCTCTCTCTAA	CAGCTTGAGGGTTTGCTACA

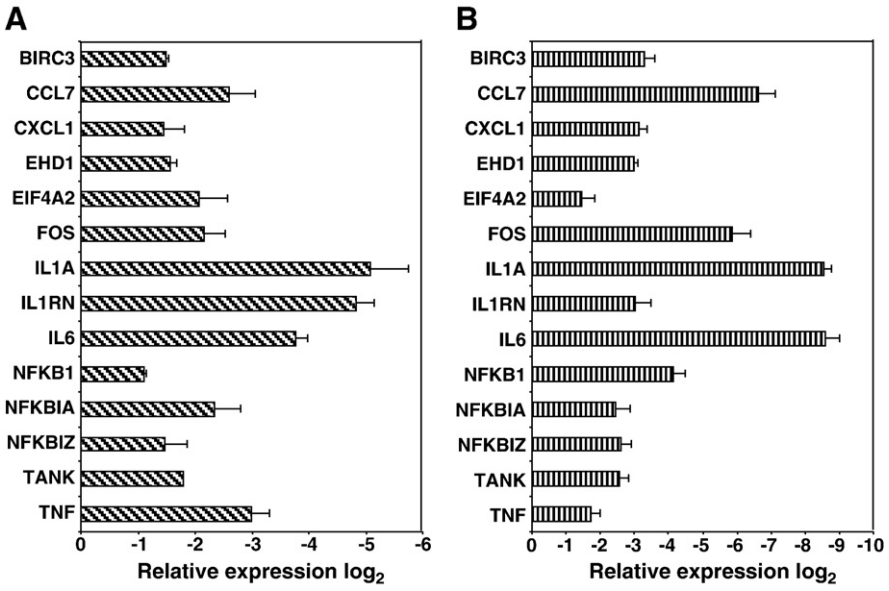


Fig. 2. RTQ-PCR analysis of 14 down-regulated NF-κB related genes. (A) NF-κB related genes down-regulated by 7HF in LPS-stimulated human peripheral blood mononuclear cells. (B) NF-κB related genes down-regulated by 7HF in synovial cells obtained from rheumatoid arthritis patients. Results are expressed as average ± S.E.M. of three separate donors based on $\log_2$ transformation of normalized RTQ-PCR values of the assayed genes. GAPDH gene was used as normalization control.

The phosporylation of IκBα is brought about by IKK-β which is one of the key kinases known to modulate the NF-κB signal transduction pathway (May and Ghosh, 1998). This fact, combined with our observations that 7HF inhibits induced IκBα phosphorylation, led us to posit that 7HF might inhibit the phosphorylation of IKK-β. To investigate this hypothesis, we utilized two different approaches. In the first approach, we studied the effect of 7HF on phosphorylation of IKK-β. Western blot analysis showed that 7HF pre-treatment of THP-1 cells blocked the LPS-induced phosphorylation of IKK-β (Fig. 4C). In contrast, DMSO pre-treatment of THP-1 cells had little, if any, effect (Fig. 4C). In the second approach, we studied the effect of 7HF on activity of IKK-β in a cell-free system. Recombinant human IKK-β kinase was treated with various concentrations of 7HF or DMSO and subsequently, IκBα biotinylated

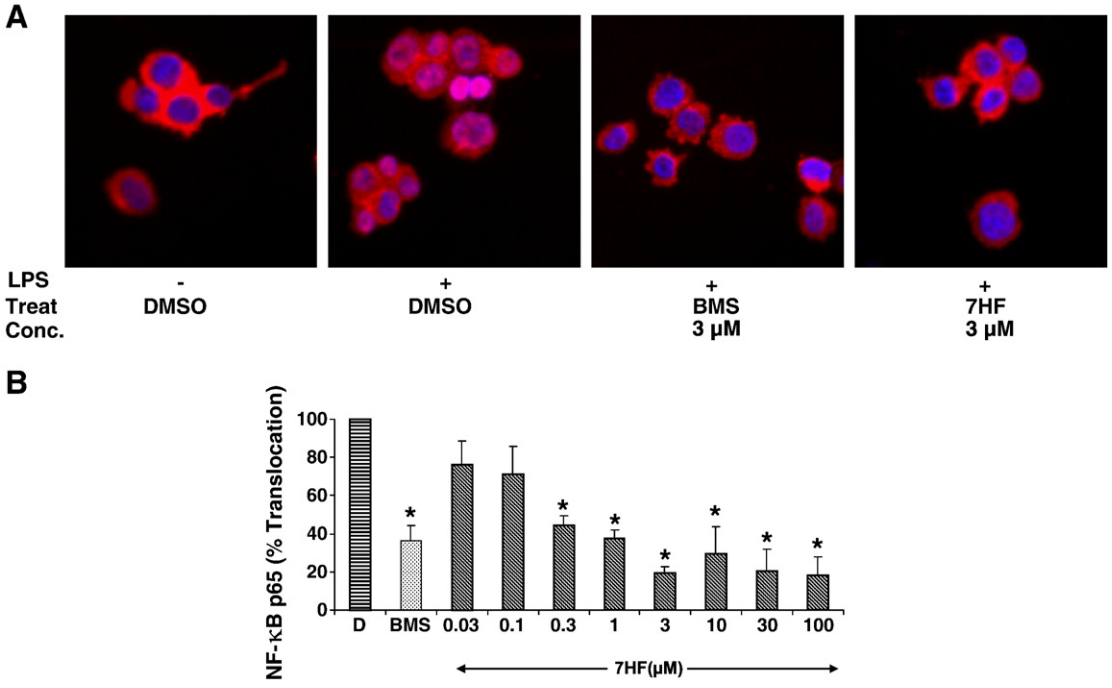


Fig. 3. 7HF inhibits the LPS-induced translocation of NF-κB into the nucleus. PMA-differentiated THP-1 cells were pre-treated with 7HF or BMS-345541 (BMS) or DMSO and subsequently challenged with LPS. Following a 30-min incubation, the extent of activation and subsequent translocation of NF-κB (p65 subunit) was determined using the Cellomics ArrayScan HCS reader. (A) Representative images of NF-κB localization in the cytoplasm and nucleus are presented. Nucleus is stained blue, cytoplasmic NF-κB is indicated by red, translocation of NF-κB into the nucleus is visualized by pink coloration of the nucleus and a concomitant decrease in cytoplasmic NF-κB. (B) NF-κB nuclear translocation in LPS-stimulated THP-1 cells. The pink fluorescence intensity in the nucleus of DMSO pre-treated, LPS-stimulated THP-1 cells was set as 100% translocation in every experiment and used to normalize the other data. All values are average ± S.E.M. from a single experiment. Results presented are representative of three separate experiments. * indicates $P<0.05$ compared to DMSO pre-treated control cells.

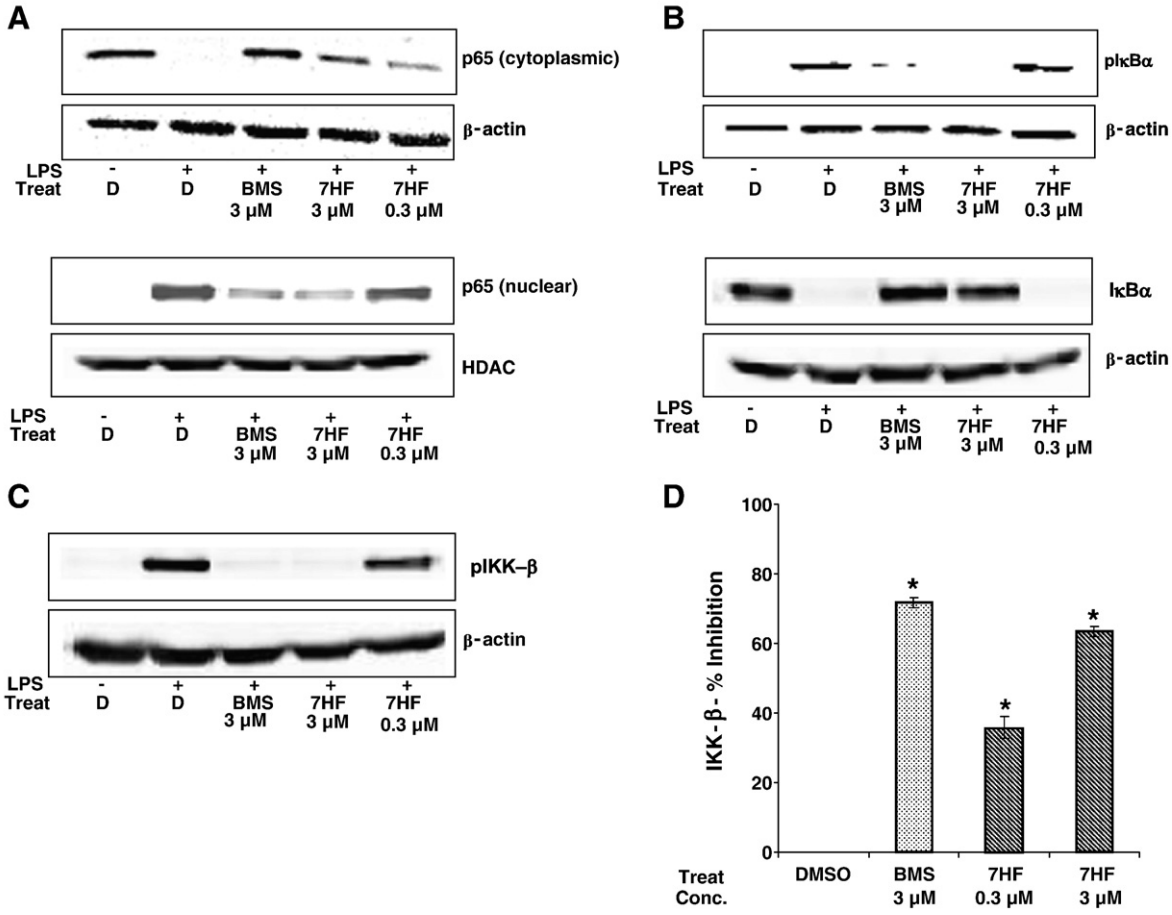


Fig. 4. 7HF blocks NF-κB activation pathway. (A–C) PMA-differentiated THP-1 cells were pre-treated with 7HF or BMS-345541 (BMS) or 0.5% DMSO (D) followed by stimulation (+) or no stimulation (−) with LPS. Equal amounts of lysates from various treatment groups were separated by SDS-PAGE, transferred onto PVDF membrane and blotted with antibody to (A) Cytoplasmic p65 or β-actin and nuclear p65 or histone deacetylase (HDAC) or (B) IκBα or pIκBα or β-actin or (C) pIKK-β or β-actin. Blots presented are representative of $n=3$ experiments. (D) IKK-β was treated with various concentrations of 7HF or 0.02% DMSO or BMS-345541 (BMS) (positive control) for 5 min. Subsequently, a cocktail of ATP/IκBα(Ser32) biotinylated peptide was added. Following a 30-min incubation, the reaction was stopped and the phosphorylation status of IκBα (a downstream target of activated IKK-β) ascertained using a colorimetric ELISA assay. The absorbance values of DMSO-treated IKK-β sample was used to calculate% IKK-β inhibition. Values are average ± S.E.M. of three separate experiments. * indicates $P<0.05$ compared to DMSO-treated IKK-β.

peptide was added to the reaction mixture. The phosphorylation status of IκBα was measured by ELISA and was used to indicate the extent of IKK-β inhibition. 7HF (but not DMSO) pre-treatment robustly inhibited the activity of IKK-β in a dose-dependent manner (Fig. 4D).

3.4. 7HF potently inhibits expression of cell adhesion molecules and prevents monocyte–endothelial cell adhesion

Having established that 7HF-mediated IKK-β inhibition is associated with dramatic suppression of downstream signaling events, including NF-κB activation, we next investigated the effects of 7HF on induced production of endothelial cell adhesion molecules (ECAM). LPS stimulation induces/up-regulates the expression of adhesion molecules on the damaged microvascular endothelium that leads to recruitment and infiltration of inflammatory leukocytes (Dagia et al., 2004). The aforementioned fact, combined with the observation that 7HF inhibits the activation of NF-κB, led us to examine the effects of 7HF on LPS-induced ECAM expression and resultant leukocyte adhesion. Unactivated HUVEC did not appear to express E-selectin or VCAM-1 but did express ICAM-1. Six-hour treatment of HUVEC with LPS induced E-selectin and VCAM-1 expression and increased ICAM-1 expression. Pre-treatment of HUVEC with 7HF significantly reduced LPS-induced expression of E-selectin, ICAM-1, and VCAM-1 relative to pre-treatment with 0.5% DMSO [IC_{50}: 0.6 μM (95% CI, 0.1–3.1 μM), 0.6 μM (95% CI, 0.1–1.2 μM) and 0.3 μM (95% CI, 0.06–0.4 μM) for inhibition of ICAM-1, VCAM-1 and E-selectin production, respectively; IC_{50}: 15.0 μM (95% CI, 5.2–44.7 μM) for cytotoxicity; Fig. 5A–C and data not shown]. To determine the functional consequence of these findings, we studied the adhesion of THP-1 cells to HUVEC. As shown in Fig. 5D, a significant number of THP-1 cells adhered to LPS-activated HUVEC. Given that human monocyte–endothelial cell adhesion is mediated by both E-selectin and VCAM-1 (Dagia et al., 2004), based on our ECAM ELISA results, we hypothesized that 7HF might attenuate THP-1 cell adhesion to HUVEC. Indeed, pre-treatment of HUVEC with 7HF significantly reduced THP-1 cell adhesion (Fig. 5D), whereas pre-treatment with 0.5% DMSO had little, if any, effect on THP-1 cell adhesion (Fig. 5D).

4. Discussion

In this study, we report that 7HF, an anti-inflammatory sesquiterpene lactone (Fonseca et al., 2010), suppresses the inflammatory response induced by LPS. Gene expression studies reveal that 7HF potently down-regulates NF-κB-related transcripts in LPS-induced human peripheral blood mononuclear cells. Furthermore, 7HF down-regulates spontaneous induction of NF-κB-related genes from pathologically relevant synovial tissue cells isolated from patients with active rheumatoid arthritis. Most importantly, 7HF inhibits the

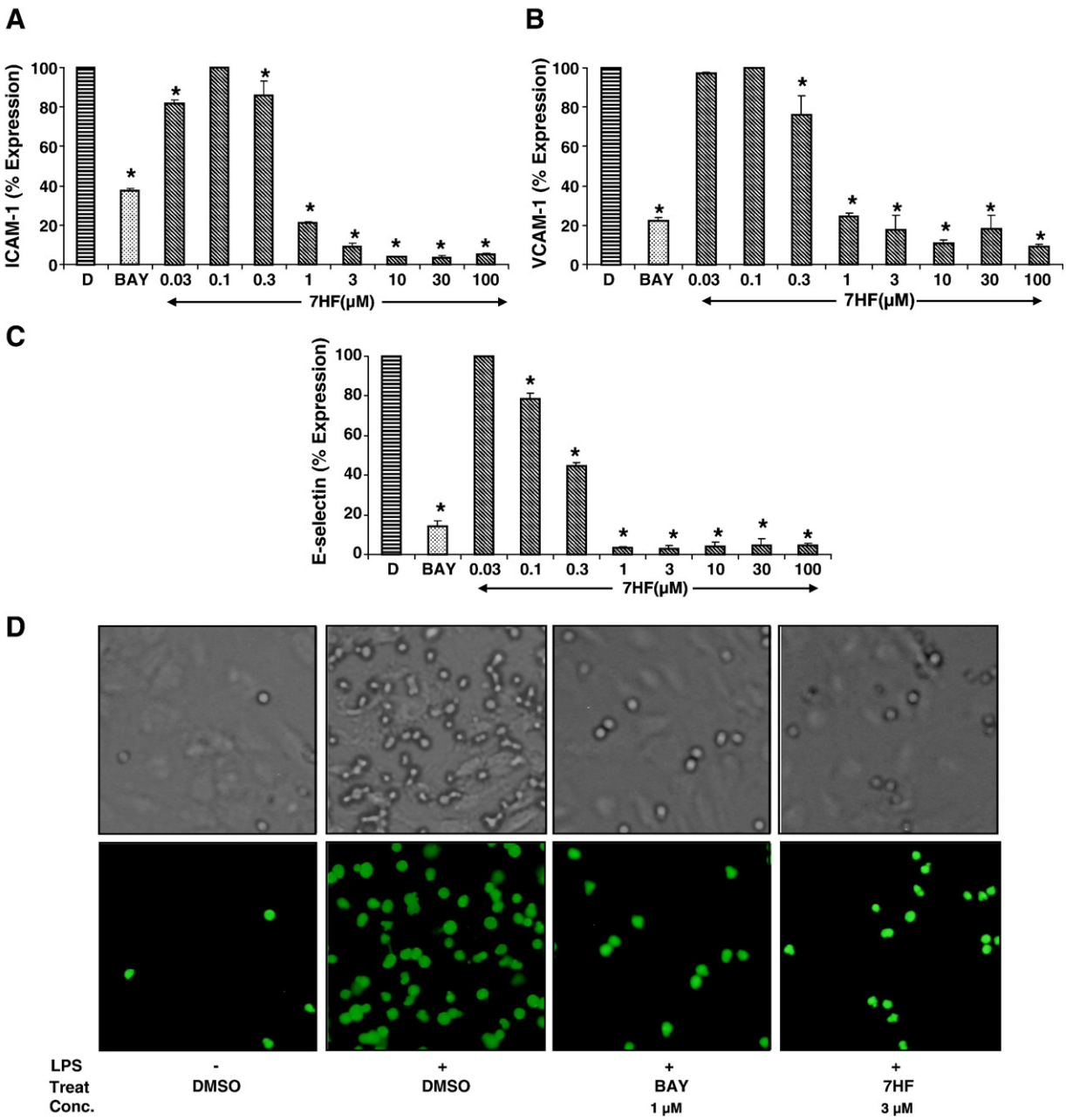


Fig. 5. 7HF diminishes LPS-induced NF-κB-dependent expression of endothelial cell adhesion molecules (ECAM) and resultant monocyte–endothelial cell adhesion. HUVEC were pre-treated with 7HF or BAY 11-7082 (BAY) or DMSO and subsequently challenged with LPS. Following a 6-h incubation, the surface expression of (A) ICAM-1, (B) VCAM-1 and (C) E-selectin was determined using ELISA. The ECAM expression on DMSO-treated, LPS-stimulated HUVEC was set at 100% in every experiment and used to normalize the other data. Values presented are average ± S.E.M. of triplicate wells from a single experiment. Results presented are representative of three separate experiments. (D) BCECF-AM labeled THP-1 monocyte adhesion to LPS-stimulated HUVEC treated with or without 7HF (3 μM) or BAY (1 μM) or 0.5% DMSO was studied using fluorescence microscopy. (Top panel) Phase contrast images. (Bottom panel) Fluorescence photomicrographs.

induced expression of endothelial cell adhesion molecules as well as subsequent adhesion of monocytes to activated endothelial cells.

We have previously shown that 7HF was found to be efficacious in multiple animal models of inflammation and suppresses TNF-α and IL-6 secretion (Fonseca et al., 2010). It is also well established that the induced production of TNF-α and IL-6 is controlled at the gene level by the activity of transcription factors (Vallabhapurapu and Karin, 2009). Our observations that 7HF inhibits (both) TNF-α and IL-6 suggest that it is targeting transcription factor(s) which are common in the promoter regions for these pro-inflammatory cytokines. Therefore, to decipher the mechanism by which inhibition of TNF-α and IL-6 production occurs and identify the transcription factor targeted by 7HF, we examined the effects of 7HF on the mRNA of LPS-stimulated human peripheral blood mononuclear cells using gene array technology. Microarray data analysis using bioinformatics tools like 'oPOSSUM' and 'Gene set enrichment analysis' revealed that 7HF down-regulated NF-κB-related transcripts.

NF-κB, a ubiquitous transcription factor, is one of the key regulators of genes involved in the inflammatory process and is one of the major intracellular mediators of LPS-induced responses (Baldwin, 1996). In the majority of cell types this dimeric protein is composed of a p50 and a p65 subunit. In the present study, 7HF markedly inhibited the translocation of NF-κB (p65 subunit) from the cytoplasm into the nucleus (Fig. 3). Under physiological

conditions, NF-κB is sequestered in an inactive cytoplasmic complex by non-covalent interactions with IκBα, its inhibitory subunit (Ghosh et al., 1998). In response to various pro-inflammatory stimuli like LPS or TNF-α, IκBα is phosphorylated at serine residues by the IKK complex (Brown et al., 1995), leading to its rapid ubiquitination and subsequent degradation by the 26 S proteasome (DiDonato et al., 1996). The IKK complex consists of two catalytic subunits, IKK-α and IKK-β, and a regulatory subunit, IKK-γ (Maniatis, 1997). The release of NF-κB from its inhibitory subunit IκBα, results in the translocation of NF-κB into the nucleus, where it binds to specific sequences in the promoter regions of target genes. The target genes whose transcription is primarily regulated by NF-κB include many cytokines (e.g., TNF-α, IL-6, IL-1), chemokines (e.g., IL-8, MCP-1), cell adhesion molecules (e.g., ICAM-1, VCAM-1, E-selectin), as well as acute-phase proteins, inducible nitric oxide synthetase, hematopoetic growth factors, growth factor receptors and immunoreceptors (Baeuerle and Baltimore, 1996; Baeuerle and Henkel, 1994).

To further determine the mechanistic basis of 7HF blocking of NF-κB activation, the effect of 7HF on phosphorylation of IκBα was assessed. Phosporylation of the IκBα protein by LPS was almost completely abolished in THP-1 cells treated with 7HF at 3 μM concentration. Since the IKK complex is responsible for the phosporylation of IκBα, we determined the effect of 7HF on phosphorylation of the catalytic subunit IKK-β. Western blot analysis of the cytoplasmic extracts shows that 7HF effectively inhibits the phosporylation of IKK-β, thus demonstrating that 7HF acts at the level of IKK-β. This finding was further validated by the fact that 7HF inhibited the kinase activity of IKK-β in a cell-free assay, indicating that 7HF targets IKK-β directly. These result combined with the fact that the promoter regions for TNF-α and IL-6 have consensus binding sites for NF-κB (Sanceau et al., 1995; Trede et al., 1995) are evidence that the reported inhibition of TNF-α and IL-6 production by 7HF (Fonseca et al., 2010) is mediated, at least in part, due to its ability to suppress IKK-β phosphorylation. Our observations are not entirely surprising particularly since prior *in vitro* studies have shown that other sesquiterpene lactones having anti-inflammatory activity are known to directly target IKK-β (Kwok et al., 2001).

Cell-based assays showed that 7HF significantly inhibits LPS-induced endothelial cell adhesion molecule expression and resultant monocyte–endothelial cell adhesion. These data complement and extend the findings of an earlier study wherein marked decrease in neutrophil infiltration was observed in inflamed lungs of NF-κB knockout mice (Alcamo et al., 2001). Of note, NF-κB is known to be involved in chemokine-induced migratory responses of leukocytes (Mizgerd et al., 2004). Prior studies in our laboratory have shown that 7HF markedly inhibited induced production of chemokines, MCP-1 and IL-8, from LPS-stimulated human peripheral blood mononuclear cells [IC_{50}: 0.2 μM (95% CI, 0.1–1.1 μM) and 3.2 μM (95% CI, 1.9–8.1 μM) for inhibition of MCP-1 and IL-8 production, respectively]. The findings from this study have important implications for oncology too. It is well established that NF-κB is a critical integrator of various signals emanating from growth factors, cytokines, and chemokines (Baeuerle and Baltimore, 1996; Dolcet et al., 2005). Of note, sesquiterpene lactone derived drugs from thapsigargin, artemisinin, and parthenolide are now in cancer clinical trials (Ghantous et al., 2010).

In summary, the findings from our study provide mechanistic insights into the anti-inflammatory efficacy of 7HF and its effect on the NF-κB signaling cascade.

Given the importance of NF-κB as one of the key mediators of pro-inflammatory responses, its pharmacological inhibition is an attractive target for therapeutic intervention against inflammatory processes and diseases. Furthermore, sesquiterpene lactones, like 7HF, may serve as lead structures for the development and generation of cytokine suppressing agents and therapeutics valuable for the treatment of various inflammatory disorders.

References

Alcamo, E., Mizgerd, J.P., Horwitz, B.H., Bronson, R., Beg, A.A., Scott, M., Doerschuk, C.M., Hynes, R.O., Baltimore, D., 2001. Targeted mutation of TNF receptor I rescues the RelA-deficient mouse and reveals a critical role for NF-kappa B in leukocyte recruitment. J. Immunol. 167, 1592–1600.

Atta, U.R., Shekhani, M.S., Perveen, S., Habib, U.R., Yasmin, A., Haque, A.Z., Shaikh, D., 1989. 7-Hydroxyfrullanolide, an antimicrobial sesquiterpene lactone from *Sphaeranthus indicus* Linn. J. Chem. Res. Synop. 68.

Baeuerle, P.A., Baltimore, D., 1996. NF-kappa B: ten years after. Cell 87, 13–20.

Baeuerle, P.A., Henkel, T., 1994. Function and activation of NF-kappa B in the immune system. Annu. Rev. Immunol. 12, 141–179.

Baldwin Jr., A.S., 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu. Rev. Immunol. 14, 649–683.

Ben-Chetrit, E., Bergmann, S., Sood, R., 2006. Mechanism of the anti-inflammatory effect of colchicine in rheumatic diseases: a possible new outlook through microarray analysis. Rheumatol. Oxford. 45, 274–282.

Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., Siebenlist, U., 1995. Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. Science 267, 1485–1488.

Dadarkar, S.S., Fonseca, L.C., Mishra, P.B., Lobo, A.S., Doshi, L.S., Dagia, N.M., Rangasamy, A.K., Padigaru, M., 2011. Phenotypic and genotypic assessment of concomitant drug-induced toxic effects in liver, kidney and blood. J Appl Toxicol. 31, 117–130.

Dadarkar, S.S., Fonseca, L.C., Thakkar, A.D., Mishra, P.B., Rangasamy, A.K., Padigaru, M., 2010. Effect of nephrotoxicants and hepatotoxicants on gene expression profile in human peripheral blood mononuclear cells. Biochem. Biophys. Res. Commun. 401, 245–250.

Dagia, N.M., Harii, N., Meli, A.E., Sun, X., Lewis, C.J., Kohn, L.D., Goetz, D.J., 2004. Phenyl methimazole inhibits TNF-alpha-induced VCAM-1 expression in an IFN regulatory factor-1-dependent manner and reduces monocytic cell adhesion to endothelial cells. J. Immunol. 173, 2041–2049.

Dagia, N.M., Gadhoum, S.Z., Knoblauch, C.A., Spencer, J.A., Zamiri, P., Lin, C.P., Sackstein, R., 2006. G-CSF induces E-selectin ligand expression on human myeloid cells. Nat. Med. 12, 1185–1190.

Dagia, N.M., Kamath, D.V., Bhatt, P., Gupte, R.D., Dadarkar, S.S., Fonseca, L., Agarwal, G., Chetrapal-Kunwar, A., Balachandran, S., Srinivasan, S., Bose, J., Pari, K., C, B.R., Parkale, S.S., Gadekar, P.K., Rodge, A.H., Mandrekar, N., Vishwakarma, R.A., Sharma, S., 2009. A fluorinated analog of ISO-1 blocks the recognition and biological function of MIF and is orally efficacious in a murine model of colitis. Eur. J. Pharmacol. 607, 201–212.

Dagia, N.M., Agarwal, G., Kamath, D.V., Chetrapal-Kunwar, A., Gupte, R.D., Jadhav, M.G., Dadarkar, S.S., Trivedi, J., Kulkarni-Almeida, A.A., Kharas, F., Fonseca, L.C., Kumar, S., Bhonde, M.R., 2010. A preferential p110alpha/gamma PI3K inhibitor attenuates experimental inflammation by suppressing the production of proinflammatory mediators in a NF-kappaB-dependent manner. Am. J. Physiol. Cell Physiol. 298, C929–C941.

DiDonato, J., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S., Karin, M., 1996. Mapping of the inducible IkappaB phosphorylation sites that signal its ubiquitination and degradation. Mol. Cell. Biol. 16, 1295–1304.

Dolcet, X., Llobet, D., Pallares, J., Matias-Guiu, X., 2005. NF-κB in development and progression of human cancer. Virchows Arch. 446, 475–482.

Fonseca, L.C., Dadarkar, S.S., Lobo, A.S., Suthar, A.C., Chauhan, V.S., Chandrababu, S., Sharma, S.D., Dagia, N.M., Padigaru, M., 2010. 7-hydroxyfrullanolide, a sesquiterpene lactone, inhibits pro-inflammatory cytokine production from immune cells and is orally efficacious in animal models of inflammation. Eur. J. Pharmacol. 644, 220–229.

Ghantous, A., Gali-Muhtasib, H., Vuorela, H., Saliba, N.A., Darwiche, N., 2010. What made sesquiterpene lactones reach cancer clinical trials? Drug Discov. Today 15, 668–678.

Ghosh, S., May, M.J., Kopp, E.B., 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 16, 225–260.

Gray, N.S., Wodicka, L., Thunnissen, A.M., Norman, T.C., Kwon, S., Espinoza, F.H., Morgan, D.O., Barnes, G., LeClerc, S., Meijer, L., Kim, S.H., Lockhart, D.J., Schultz, P.G., 1998. Exploiting chemical libraries, structure, and genomics in the search for kinase inhibitors. Science 281, 533–538.

Gu, J., Marker-Hermann, E., Baeten, D., Tsai, W.C., Gladman, D., Xiong, M., Deister, H., Kuipers, J.G., Huang, F., Song, Y.W., Maksymowych, W., Kalsi, J., Bannai, M., Seta, N., Rihl, M., Crofford, L.J., Veys, E., De Keyser, F., Yu, D.T., 2002. A 588-gene microarray analysis of the peripheral blood mononuclear cells of spondyloarthropathy patients. Rheumatol. Oxford. 41, 759–766.

Ho Sui, S.J., Mortimer, J.R., Arenillas, D.J., Brumm, J., Walsh, C.J., Kennedy, B.P., Wasserman, W.W., 2005. oPOSSUM: identification of over-represented transcription factor binding sites in co-expressed genes. Nucleic Acids Res. 33, 3154–3164.

Kwok, B.H., Koh, B., Ndubuisi, M.I., Elofsson, M., Crews, C.M., 2001. The anti-inflammatory natural product parthenolide from the medicinal herb Feverfew directly binds to and inhibits IkappaB kinase. Chem. Biol. 8, 759–766.

Lindenmeyer, M.T., Kern, C., Sparna, T., Donauer, J., Wilpert, J., Schwager, J., Porath, D., Kreutz, C., Timmer, J., Merfort, I., 2007. Microarray analysis reveals influence of the sesquiterpene lactone parthenolide on gene transcription profiles in human epithelial cells. Life Sci. 80, 1608–1618.

Maniatis, T., 1997. Catalysis by a multiprotein IkappaB kinase complex. Science 278, 818–819.

Marok, R., Winyard, P.G., Coumbe, A., Kus, M.L., Gaffney, K., Blades, S., Mapp, P.I., Morris, C.J., Blake, D.R., Kaltschmidt, C., Baeuerle, P.A., 1996. Activation of the transcription

factor nuclear factor-kappaB in human inflamed synovial tissue. Arthritis Rheum. 39, 583–591.
May, M.J., Ghosh, S., 1998. Signal transduction through NF-kappa B. Immunol. Today 19, 80–88.
Mizgerd, J.P., Lupa, M.M., Spieker, M.S., 2004. NF-kappaB p50 facilitates neutrophil accumulation during LPS-induced pulmonary inflammation. BMC Immunol. 5, 10.
Neerman, M.F., 2003. Sesquiterpene lactones: a diverse class of compounds found in essential oils possessing antibacterial and antifungal properties. Int. J. Aromath. 13, 114–120.
Picman, A.K., Rodriguez, E., Towers, G.H., 1979. Formation of adducts of parthenin and related sesquiterpene lactones with cysteine and glutathione. Chem. Biol. Interact. 28, 83–89.
Sanceau, J., Kaisho, T., Hirano, T., Wietzerbin, J., 1995. Triggering of the human interleukin-6 gene by interferon-gamma and tumor necrosis factor-alpha in monocytic cells involves cooperation between interferon regulatory factor-1, NF kappa B, and Sp1 transcription factors. J. Biol. Chem. 270, 27920–27931.
Sohoni, J.S., Rojatkar, S.R., Kulkarni, M.M., Dhaneshwar, N.N., Tavale, S.S., Gururow, T.N., Nagasampagi, B.A., 1988. A new Eudesmanolide and 2-hydroxycostic acid from *Sphaeranthus indicus* linn:X-ray molecular structure of 4-alpha 5-alpha epoxy-7-alpha-hydroxyeudesmanolide. J Chem Soc Perkin Trans. 1 (2), 157–160.
Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., Mesirov, J.P., 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl Acad. Sci. USA 102, 15545–15550.
Thakkar, A.D., Raj, H., Chakrabarti, D., Ravishankar, P., Saravanan, N., Muthuvelan, B., Balakrishnan, A., Padigaru, M., 2010. Identification of gene expression signature in estrogen receptor positive breast carcinoma. Biomark. Cancer. 2, 1–15.
Tian, Q., Li, J., Xie, X., Sun, M., Sang, H., Zhou, C., An, T., Hu, L., Ye, R.D., Wang, M.W., 2005. Stereospecific induction of nuclear factor-kappaB activation by isochamaejasmin. Mol. Pharmacol. 68, 1534–1542.
Trede, N.S., Tsytsykova, A.V., Chatila, T., Goldfeld, A.E., Geha, R.S., 1995. Transcriptional activation of the human TNF-alpha promoter by superantigen in human monocytic cells: role of NF-kappa B. J. Immunol. 155, 902–908.
Vallabhapurapu, S., Karin, M., 2009. Regulation and function of NF-kappaB transcription factors in the immune system. Annu. Rev. Immunol. 27, 693–733.
Yamaguchi, M., Suwa, H., Miyasaka, M., Kumada, K., 1997. Selective inhibition of vascular cell adhesion molecule-1 expression by verapamil in human vascular endothelial cells. Transplantation 63, 759–764.
Zhou, J.Y., Tang, F.D., Mao, G.G., Bian, R.L., 2004. Effect of alpha-pinene on nuclear translocation of NF-kappa B in THP-1 cells. Acta Pharmacol. Sin. 25, 480–484.