Supporting Information to:

Screening of five essential oils for identification of potential inhibitors of IL-1-induced NF-κB activation and NO production in human chondrocytes: characterization of the inhibitory activity of α-pinene

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Methods

Isolation and cell culture

Articular chondrocytes were isolated from the cartilage samples by enzymatic digestion as described previously [7], and used to evaluate the ability of the essential oils and their fractions to inhibit IL-1-induced NO production. Non-proliferating monolayer cultures were established from each cartilage sample, allowed to recover in medium containing 5% fetal bovine serum for 24h, serum-starved overnight and maintained thereafter in serum-free culture medium. The cells were subsequently treated with 30 ng.ml\(^{-1}\) recombinant human IL-1 (Peprotech), for the periods indicated in the figure legends, in the presence or absence of the essential oils and fractions or of control compounds, namely the specific NF-κB inhibitor, Bay 11-7082 (≥95% purity; Calbiochem) and DMSO (Merck) used as solvent. Culture supernatants and the adherent cells were used, respectively, to measure NO production and to evaluate cell viability.

The human chondrocytic cell line C-28/I2 (a kind gift from Prof. M. Goldring, Beth Israel Deaconess Medical Center and New England Baptist Bone and Joint Institute, Boston, MA, USA) was used to evaluate NF-κB activation. This cell line fully responds to IL-1 in terms of NF-κB activation, but fails to express iNOS. C28/I2 cells were cultured in DMEM/Ham’s F-12 (1:1, v/v) containing 10% FBS and antibiotic solution (100 U/mL penicillin and 100 μg/mL streptomycin) and passaged every four days. For experiments, 25x10^4 cells.mL\(^{-1}\) were plated in 6-well plates (2.5 mL/well) and allowed to recover for 24 hours. Then, cells were serum-starved overnight and thereafter kept in serum-free medium with 1% antibiotic solution. Treatment conditions for evaluation of
IκB-α phosphorylation, total IκB-α and NF-κB-DNA binding activity are indicated in the figure legends.

Assessment of cell viability
The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma Chemical Co.) reduction assay [24] was used to evaluate cell viability in order to assess the cytotoxicity of the essential oils and their fractions in the concentrations used. The adherent cells remaining after collection of the culture supernatants used to measure NO production, were incubated, for 1 h, at 37°C, with a 0.5 mg.mL^{-1} MTT solution in Ham F-12 medium. The dark blue crystals of formazan produced were dissolved in acidified isopropanol, and formazan quantification was performed by measuring the absorbance of the corresponding solution, using an automatic plate reader (SLT) set at a test wavelength of 570 nm and a reference wavelength of 620 nm.

Western blot analysis
Cytoplasmic extracts were diluted in sodium dodecylsulfate (SDS) sample buffer (2.5% SDS, 0.0625 M Tris-HCl, 10% glycerol, 5% 2-mercaptoethanol, 0.05% bromophenol blue, pH 6.8) and boiled for 5 minutes. Samples (25 μg protein) and molecular weight markers (All blue, Precision Plus molecular weight markers; Bio-Rad Laboratories Inc.) were subjected to SDS/PAGE and electroblotted onto PVDF membranes, which were probed with anti-IκB-α or anti-phospho-IκB-α antibodies (Cell Signaling Technology, Inc.) and then with an anti-rabbit alkaline phosphatase-conjugated secondary antibody (1:20,000 dilution; Amersham Biosciences). Immune complexes were detected with the Enhanced ChemiFluorescence reagent (Amersham Biosciences) in a Storm 840 scanner (Amersham Biosciences). A mouse anti-actin monoclonal antibody (1:10,000 dilution;
Chemicon International, Inc.) was used to measure the expression of this house-keeping gene product as an internal control. The intensity of the bands was analyzed using ImageQuant™ TL (Amersham Biosciences).
Effect of the essential oils on the viability of human chondrocytes. C: control, untreated cells; IL-1, cells treated with IL-1, 30 ng.mL^{-1} for 18h; the remaining bars represent the results obtained in cells treated for 2h with the indicated concentrations of each essential oil followed by addition of IL-1, 30 ng.mL^{-1} and incubation for 18 h. Each bar represents the mean ± SD of 3 independent experiments each performed in duplicate.