Supporting Information to:

Phytomedicines Prepared from *Arnica* Flowers Inhibit the Transcription Factors AP-1 and NF-κB and Modulate the Activity of MMP1 and MMP13 in Human and Bovine Chondrocytes

Christoph Jäger[1], Andrea Hrenn[1], Jörn Zwingmann[2], Andreas Suter[3], and Irmgard Merfort[1]

Affiliation

[1] Institute for Pharmaceutical Sciences, Department of Pharmaceutical Biology and Biotechnology, University of Freiburg, Freiburg, Germany

[2] Department of Orthopaedics and Trauma Surgery, University Hospital Freiburg, Freiburg, Germany

[3] Bioforce AG, Roggwil, Switzerland

Correspondence

Prof. Dr. I. Merfort

Institute for Pharmaceutical Sciences

Department of Pharmaceutical Biology and Biotechnology

University of Freiburg

Stefan-Meier-Str. 19

79104 Freiburg

Germany

Tel.: +49/761/203 8373

Fax: +49/761/203 8383

irmgard.merfort@pharmazie.uni-freiburg.de

Cell Culture

Jurkat-T-cells

Cells were maintained in RPMI 1640 medium, supplemented with 10 % foetal calf serum, 2 mM L-glutamine (Biochrom), 100 IU/mL penicillin and 100 µg/mL streptomycin.

Preparation of nuclear protein extracts of Jurkat cells

Cell suspensions were transferred to Falcon tubes and centrifuged for 5 min at 4 °C and 300 g. The pelleted cells were washed with 1 mL of ice-cold PBS, resuspended in 400 µL ice-cold buffer A (10 mM Hepes, pH 7.6, 15 mM KCl, 2 mM $MgCl_2$ and 0.1 mM EDTA, pH 8.0) and incubated for 10 min on ice. The cells were then collected by centrifugation, resuspended in 200 µL of Igepal (buffer A + 0.2 % NP-40, 1 mM DTT, 0.0005 % PMSF and 1 % aprotinin-solution) and incubated for 10 min on ice. After a further centrifugation step (2000 g for 5 min at 4 °C), the pellets were resuspended by thorough vortexing in 48 µL buffer C (25 mM Hepes, 50 mM KCl, 0.1 mM EDTA, 10 % glycerol, 1 mM DTT, 0.0005 % PMSF and 1 % aprotinin-solution) and incubated 10 min on ice. Then, 4 µL of a 5 M NaCl solution were added and the samples were mixed for 30 min at 4 °C and 550 rpm. The nuclear suspensions were centrifuged (16000 g for 10 min at 4 °C), and the supernatants were collected and stored at -80 °C until EMSA analysis.

Electrophoretic mobility shift assay

Protein extracts were prepared as described above. Equal amounts of nuclear proteins (2-5 µg) were added to a reaction mixture containing 20 µg bovine serum albumin, 2 µg poly(dI-dC) (Roche Molecular Biochemicals), 2 µL buffer D+ (20 mM HEPES, pH 7.9, 20 % glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25 % NP-40, 2 mM DTT, 0.1 % PMSF), 4 µL buffer F (20 % Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, 0.1 % PMSF) and 100,000 cpm (Cerenkov) of a P^{33}-labeled oligonucleotide for NF-κB or AP-1 made up to a final volume of 20 µL with distilled water. For AP-1 EMSA experiments, the reaction mixture also contained $MgCl_2$ in a final concentration of 5 mM, and competition experiments contained a 100-fold excess of the respective non-radioactive labeled oligonucleotide. The NF-κB oligonucleotide

(5'-AGT TGA GGG GAC TTT CCC AGG C-3'; Promega), AP-1 oligonucleotide (5'-CGC TTG ATG AGT CAG CCG GAA-3'; Promega) as well as the Sp1 oligonucleotide (5'-ATT CGA TCG GGG CGG GGC GAG C-3'; Promega) were labeled using [γ^{33}P]ATP (3000 Ci/mmol; Amersham Biosciences) and a T4 polynucleotide kinase (New England Biolabs). The samples were resolved by non-denaturing 4 % polyacrylamide gel electrophoresis after 25 min of incubation at room temperature and the dried gel was then exposed to an Imaging Plate (BAS-MS 2340; Fujifilm) overnight; the plate was analyzed using a FLA-3000 (Fujifilm). Both the images as well as the quantified ^{33}P-stimulated luminescence (PSL) units of each specific shift are shown in the figures.

ATP assay with bovine articular chondrocytes

Bovine articular chondrocytes from the first passage were seeded out into 96-well plates (10000 cells/well) in Ham's F12 with 2.5 % FCS, 100 IU/mL penicillin and 100 µg/mL streptomycin and allowed to recover overnight. Then the cells were treated as described for the AP-1 and NF-κB EMSA as well as for the real time quantitative PCR analyses. ATP content of the cells was determined using the ViaLight® Plus Kit accordingly to the instructions of the manufacturer (Lonza). This assay is based upon the bioluminiscent measurement of ATP that is present in metabolically active cells. The bioluminiscent method utilizes luciferase which catalyzes the formation of light from ATP and luciferin. The emitted light intensity is linearly related to the ATP concentration and measured using a luminometer (Sirius HT-TRF microplate reader; MWG). Data are expressed as mean ± SD of duplicate measurements.

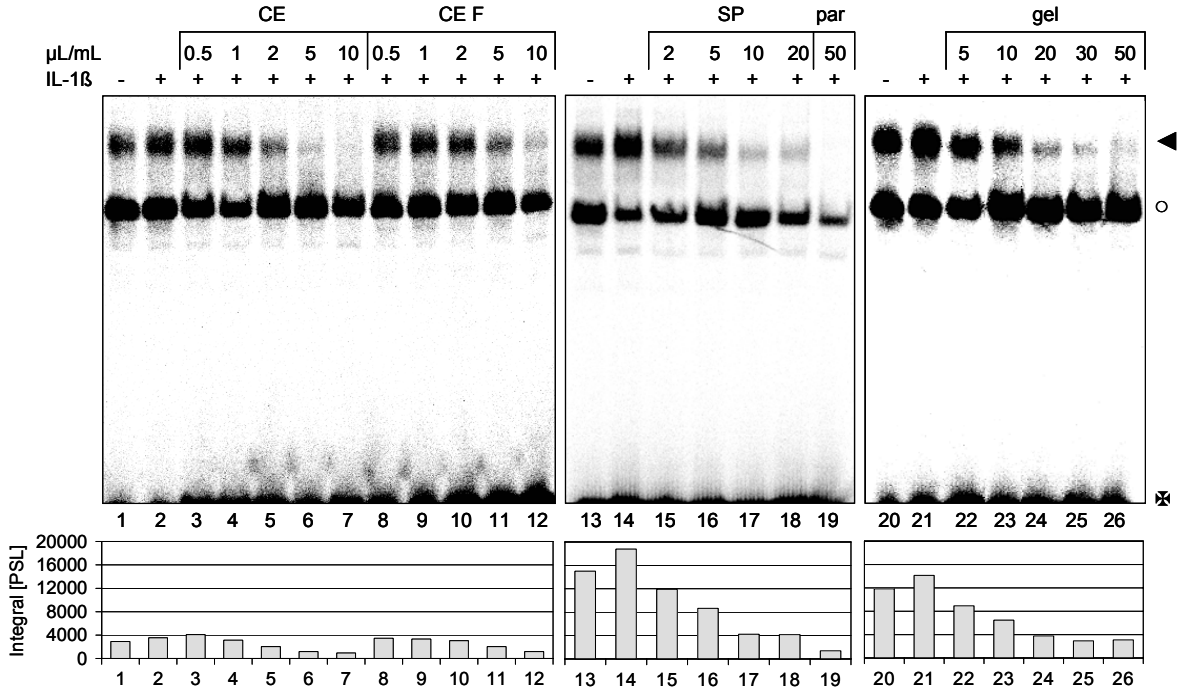

Fig. 1S Effect of *Arnica* tinctures and *Arnica* gel on AP-1 DNA binding activity in Jurkat T-cells.

Cells were pretreated for 1 h with the indicated concentrations of different *Arnica* preparations and subsequently stimulated with 20 ng/mL human IL-1β for 3 h (lanes 3 – 12, 15 – 18, 22 – 26). Lanes 1, 13, 20 showed unstimulated control cells, and cells in lanes 2, 14 and 21 were treated with IL-1β alone. In lane 19, cells were pretreated with parthenolide as positive control. Nuclear protein extracts were prepared and equal amounts were analysed for AP-1 binding activity by EMSA.

A PhosphoImager was used for detection and quantification. Shown are 33P-stimulated luminescence (PSL) units. A filled arrowhead indicates the position of AP-1-DNA complexes, and the open circle denotes a non-specific binding to the probe. The open arrowhead indicates unbound oligonucleotide.

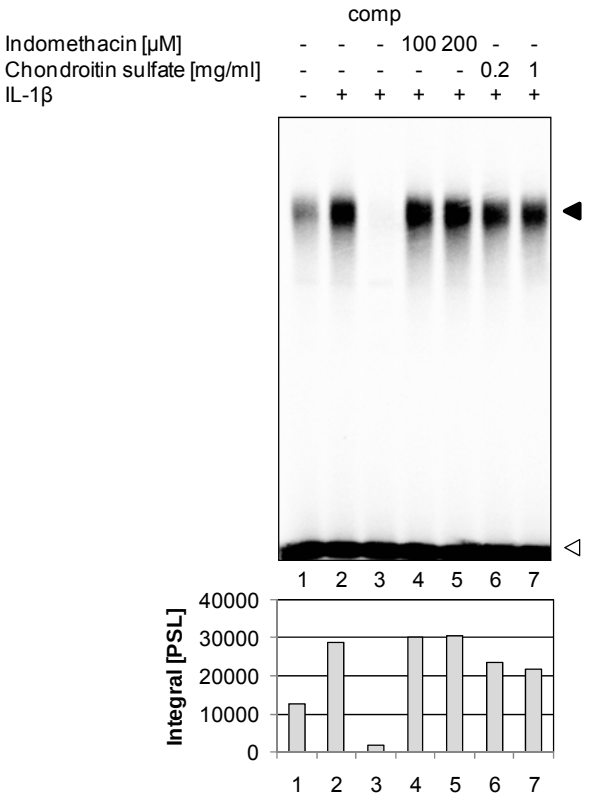

Fig. 2S Effect of indomethacin and chondroitin sulphate on AP-1 DNA binding activity in bovine chondrocytes.

Confluent bovine articular chondrocytes were pretreated for 1 h with the indicated concentrations of indomethacin or chondroitin sulphate and subsequently stimulated with 20 ng/mL bovine IL-1β for 3 h (lanes 4-7). Lane 1 shows unstimulated control cells, in lane 2 cells were treated with IL-1β for 3 h alone. Lane 3 shows the competition assay. Nuclear protein extracts were prepared and equal amounts were analysed for AP-1 binding activity by EMSA. A PhosphoImager was used for detection and quantification. Shown are 33P-stimulated luminescence (PSL) units. A filled arrowhead indicates the position of the AP-1-DNA complex. The open arrowhead denotes unbound oligonucleotide.

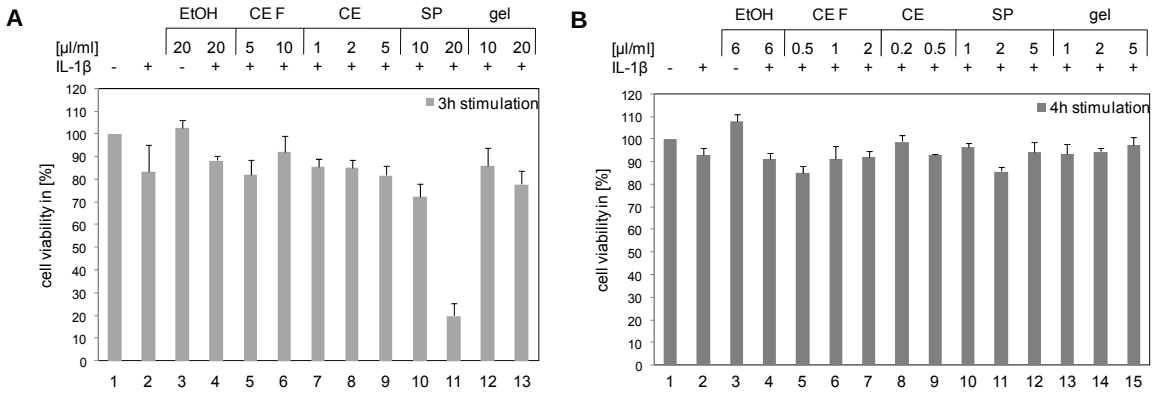

Fig. 3S ATP assay with bovine articular chondrocytes.

Bovine articular chondrocytes were pre-treated for 1 h with the indicated concentrations of different *Arnica* preparations and subsequently stimulated with 20 ng/ml bovine IL-1β for 3 h (**A**) and 4 h (**B**) respectively. Bars 1 to 4 in both diagrams show the respective controls including ethanol 70 % in the highest concentration used as solvent control. Relative ATP contents of the cells were determined (ViaLight® Plus Kit; Lonza) and the amount in untreated cells was set to 100 % viability. Values shown are means +/- SD of two independent experiments.

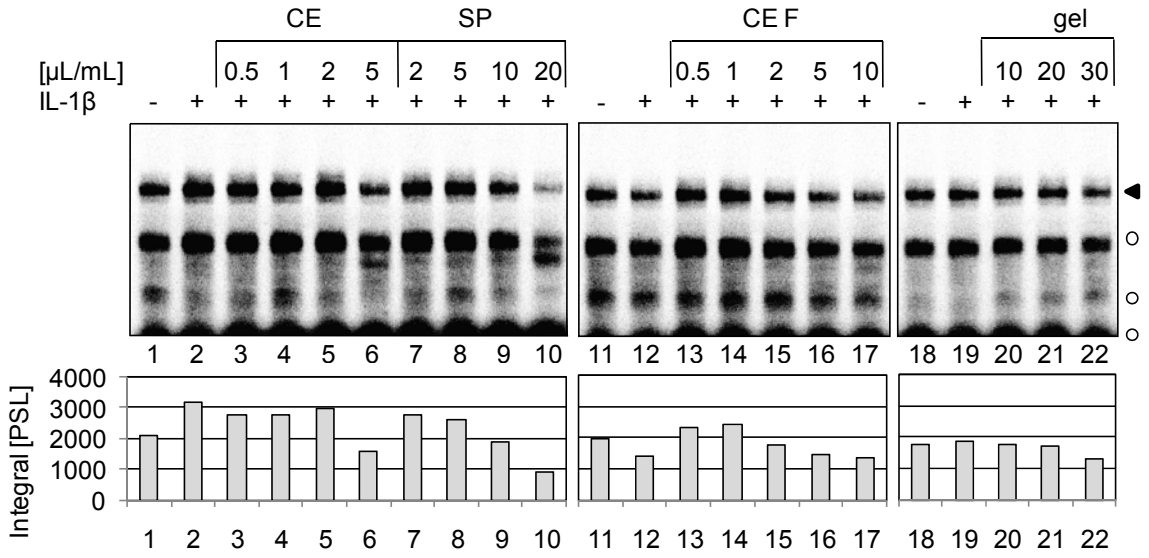

Fig. 4S. Effect of *Arnica* tinctures and *Arnica* gel on Sp1 DNA binding activity in bovine chondrocytes.

Confluent bovine articular chondrocytes were pretreated for 1 h with the indicated concentrations of different *Arnica* preparations (CE: tincture prepared from dried *Arnica* flowerheads of Central Europe; CE F: tincture made from fresh flowerheads from Central European *Arnica*; SP: tincture prepared from dried Spanish *Arnica* flowerheads; gel: *Arnica* gel, containing CE F tincture) and subsequently stimulated with 20 ng/mL bovine IL-1β for 3 h (lanes 3 – 10, 13 – 17, 20 – 22). Lanes 1, 11 and 18 show unstimulated control cells, and cells in lanes 2, 12 and 19 were treated with bovine IL-1β alone. Nuclear protein extracts were prepared and equal amounts were analysed for Sp1 binding activity by EMSA. A PhosphoImager was used for detection and quantification. Shown are 33P-stimulated luminescence (PSL) units. A filled arrowhead indicates the position of Sp1 DNA complexes, and an open circle denotes unspecific binding to the probe.