

Conference presentation

Study of the action mechanisms of *Arnica montana* effects on macrophages

Marta Marzotto, Clara Bonafini, Debora Olioso, Paolo Bellavite

Department of Medicine, University of Verona, Strada Le Grazie 8, 37134 Verona, Italy

Abstract

Objective: To test the effect of *Arnica montana* (*Arnica m.*) on human macrophages.

Method: The human monocytic leukaemia cell line THP-1 was cultured and differentiated in mature macrophages with PMA and other differentiating agents. Macrophages were exposed to *Arnica m.* homeopathic dilutions (2c, 3c, 5c, 9c and 15c) or Control solvent. Total RNA was isolated and sequenced to perform quantitative evaluation.

Results: Screening sorted out with a list of 20 genes that were significantly changed by *Arnica m.* 2c treatment: 7 up-regulated and 13 down-regulated. Most notably, a clearly up-regulated function concerned the proteinaceous extracellular matrix (ECM), including genes HSPG2, FBN2, FN1 (p<0.01). The down-regulated genes converge into the common pathway of oxidative phosphorylation and particularly into the cell component gene ontology of mitochondrial complex I (p<0.001). Higher *Arnica m.* dilutions were effective also by upregulating the same geneset in cells treated with LPS but not in resting cells.

Conclusion: In synthesis, we have identified a group of genes that are upregulated by *Arnica m.* in macrophages and many of them are involved in the processes of connective tissue remodelling and regeneration.

Background

Arnica montana L. (referred to here as *Arnica m.*) is widely employed to treat various pathological conditions, including pain, stiffness and swelling associated with trauma and postoperative clinical conditions. There is some experimental evidence of an anti-inflammatory action in laboratory animals and in *in vitro* models (macrophages and fibroblasts) but its action deserve further investigation. Given the central role of macrophages in tissue repair and regeneration, we decided to evaluate this plant's effects *in vitro* on the THP-1 human cell line differentiated to macrophages with PMA, a widely used model for immune modulation. In a first study, we used RT-PCR analysis to investigate changes in the expression of a panel of 28 genes focused on immune response (1). In a second series of experiments we decided to re-investigate the same cell extracts with the most high-throughput method, RNA-sequencing (RNA-seq), designed to evaluate the whole transcriptome.

Materials and Methods

The human monocytic leukaemia cell line THP-1 was purchased from DSMZ (Germany). *Arnica m.* was produced by Boiron Laboratoires (Lyon, France) according to the French Homeopathic pharmacopoeia and provided as a first centesimal dilution (*Arnica m.* 1c) of the hydroalcoholic extract (Mother Tincture, MT) in 30% ethanol/distilled water. Starting from a 1c solution, serial



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100x dilutions were prepared in 5ml of 30% ethanol/distilled water solvent followed by filtering and succussion. The last centesimal dilution step was always performed immediately before each experiment, in ultra-pure water. The THP-1 cell line was cultured in RPMI 1640 medium, supplemented with FBS 10% and 2mM final concentration of Ultraglutamine (Lonza), at 37°C in 5% CO₂ in a humidified incubator as described. On day 2 all the cell cultures were supplemented with 20 ng/mL of PMA and on day 3 the cultures were treated with IL-4 at a concentration of 50 ng/mL for 24h to differentiate them in “wound-healing” macrophages (M2-type). In separate experiments we also cultivated PMA-differentiate THP-1 cell in the absence of further stimulants (“Resting”) or in the presence of LPS at a concentration of 10 ng/mL (“Low-dose LPS”). Macrophages were exposed to *Arnica m.* dilutions or Control solvent (1ml cell culture + 110µl test solutions). Cell viability was checked by the Cell proliferation reagent WST-1 assay. Total RNA from cultured THP-1 cells was isolated using the RNeasy mini Kit (Qiagen). RNA aliquots were used to isolate poly(A) mRNA for the preparation of a directional Illumina RNA-Seq library using the TruSeq RNA Sample Prep Kit v2 (Illumina Inc., San Diego, CA, USA). Libraries were evaluated by RT-PCR and sequenced with a NextSeq500 sequencer. Genes with Log₂ Fold Change values that were significantly positive (up-regulated) or negative (down-regulated) were defined as differentially expressed genes (DEGs). RNA-seq was done on 5 separate experiments in IL4-cells treated with *Arnica m.* 2c dilution to identify DEGs with high statistical significance according to FDR scoring (2). We then tested pooled samples of the experiments carried out with 2c and higher dilutions, namely 3c, 5c, 9c and 15c. The statistical significance of the differences between expression profiles of gene groups (up-regulated) from cells treated with various *Arnica m.* dilutions (2c, 3c, 5c, 9c, 15c) was calculated by the Friedman multi-sample test followed by the Wilcoxon signed-rank test using the SPSS software, version 17 (SPSS Inc., Chicago, IL, USA).

Results

The WST assay of cell viability showed that the metabolic activity of macrophages, both in the resting state and after IL-4 differentiation, was slightly increased after 24h incubation with *Arnica m.* dilutions 2c and 3c, but the difference with the Control vehicle was not statistically significant.

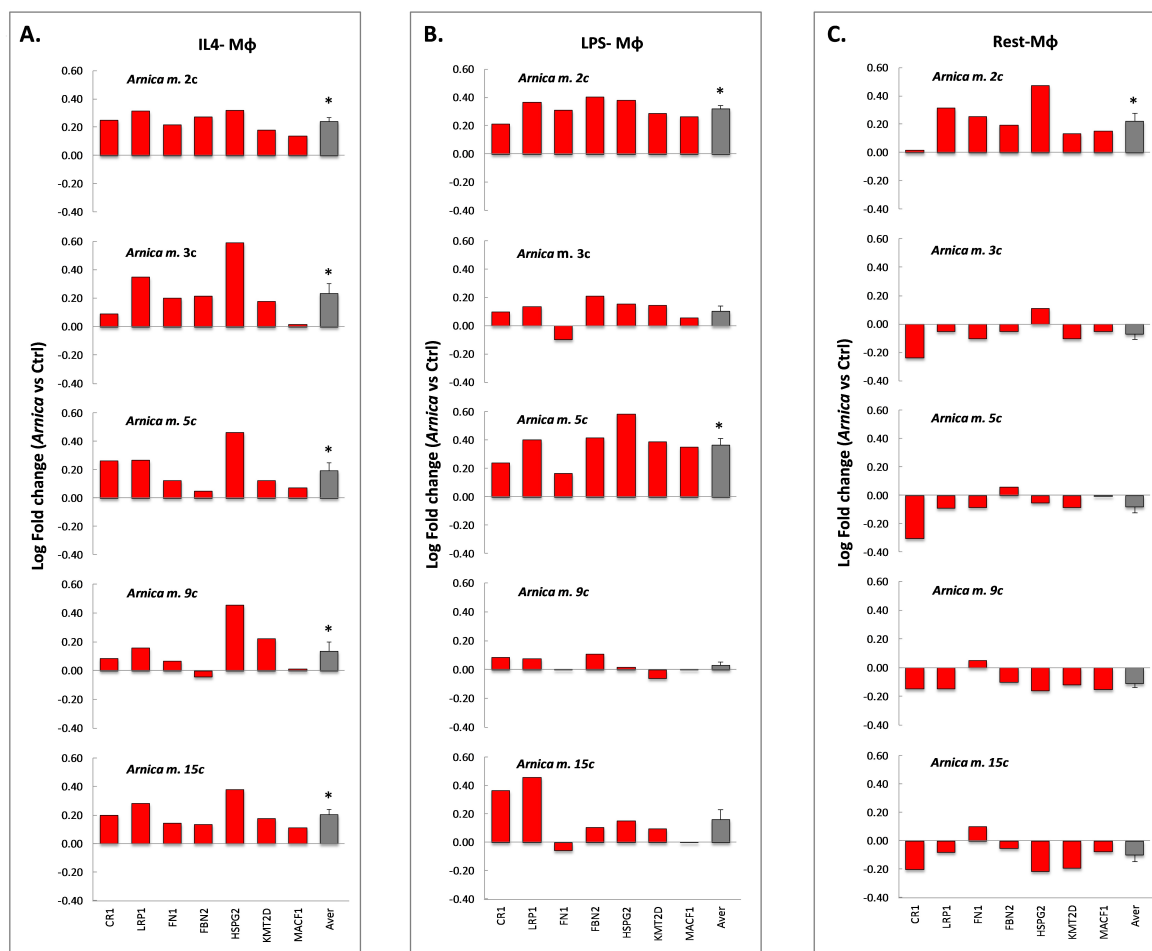
The RNA-seq analysis performed on IL-4 differentiated cells treated with *Arnica m.* 2c (final concentration of 10⁻⁸ sesquiterpene lactones) sorted out a list of 20 genes which expression was significantly changed (2): 7 up-regulated and 13 down-regulated. The 7 up-regulated genes included low-density lipoprotein-receptor-related protein 1 (LRP1), fibronectin 1 (FN1), lysine (K)-specific methyltransferase (KMT2D), complement component receptor 1 (CR1), heparan sulfate proteoglycan (perlecan, HSPG2), microtubule-actin crosslinking factor 1(MACF1), and fibrillin 2 (FBN2). The set of down-regulated DEGs (13 genes) were mitochondrial genes coding for proteins of the mitochondrial respiratory chain complex.

Functional gene enrichment analysis showed that among the genes stimulated by *Arnica m.*, a statistically significant enrichment of genes with EGF-like (LRP1, HSPG2, FBN2, FN1) and Calcium ion binding motifs (LRP1, MACF1, FBN2) emerged (p<0.001 and <0.05 respectively). Most notably, a clearly up-regulated function concerned the proteinaceous extracellular matrix (ECM), including genes HSPG2, FBN2, FN1 (p<0.01). Fibronectin was identified in considerable amounts in cell culture supernatants and was increased by *Arnica m.* 2c treatment (2). The down-regulated genes converge into the common pathway of oxidative phosphorylation and particularly into the cell component gene ontology of mitochondrial complex I (p<0.001). However, the decrease of mitochondrial genes did not impair cell viability.



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We investigated the up-regulating changes induced by increasingly higher *Arnica m.* dilutions (Figure 1).



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Figure 1. Effects induced by increasing dilutions of *Arnica m.* on gene expression in THP-1 macrophages (Mφ). Red bars report log fold changes of single genes, grey bars report the mean fold changes ± SE of the whole group considered as geneset at each dilution tested. Panel A: IL4-differentiated cells. Panel B: LPS-activated cells. Panel C: Resting cells. * Wilcoxon p values < 0.05.

These studies were carried out in pooled samples of RNA extracted from 5 separate experiments. IL-4 treated macrophages (Figure 1, panel A) showed an increase of the indicated genes at all dilutions, suggesting that these cells are extremely sensitive to the effect of the medicinal plant. *Arnica m.* 3c similarly showed a prevailing stimulating effect in the previously up-regulated geneset: 6 genes out of a total of 7 were here also be found up-regulated. The average changes of each geneset (grey bars) were approximately of the same magnitude as those induced by *Arnica m.* 2c and were statistically significant. *Arnica m.* 5c, 9c and 15c stimulated all genes of the up-regulated geneset in a statistically significant way, although with a slightly lower effect size. In summary, up-regulating effects were maintained across the increasing dilutions. Interestingly, LPS-treated cells (Figure 1, panel B) were sensitive to this remedy at 2c as IL4-differentiated macrophages, but also at the higher dilution 5c. Paradoxically, the effect decreased from 2c to 3c as expected according to a conventional pharmacological



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wisdom, but, from 3c to 5c, the effect of *Arnica m.* increased again, even if the dilution between the two drug samples is 4 orders of magnitude (10,000 times). Resting cells (**Figure 1**, panel C) showed a up-regulating effect of the same gene group as observed in the other conditions, thus confirming the sensitivity of those genes to the stimulus by *Arnica m.*. However, in resting cells *Arnica m.* higher dilutions did not have significant effects and, on the contrary, a trend to

inhibition was evident, even if it did not reach the statistical threshold. Inspection of dose responses of individual genes and of the averages in all described conditions (**Figure 1** panels A, B, C) reveals a marked non-linearity: in several instances (but not in all the cases) alternating peaks of different activities with pseudo-sinusoidal trends are observed, typical of homeopathic paradoxical phenomena (3). Down-regulation did not give consistent results in different conditions: in resting cells and in cells treated with LPS, no or very little and variable down-regulation of mitochondrial genes appeared (data not shown).

In synthesis, we have identified a group of genes that are upregulated by *Arnica m.* in macrophages and many of them are involved in the processes of connective tissue remodelling and regeneration. The fact that the same group of genes undergo to the same trend of regulation in different conditions of cell cultivation reinforces the hypothesis that these are true “target” genes of this remedy. Furthermore, our data suggest that the state of cell differentiation is a fundamental factor in determining the sensitivity to ultra-low doses and high dilutions.

Conclusions

The results of this work indicate that homeopathic *Arnica m.* acts on macrophages by modulating a number of genes. RNA-seq analysis allowed the identification of several genes which are particularly sensitive to ultra-low doses and high dilutions of this plant extract. The main and novel finding is the increased expression of several genes of tissue matrix proteins and particularly of fibronectin. This evidence indicates some relevant targets of *Arnica m.* in wound-healing processes.

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