Fibronectin Gene Up-regulation by *Arnica montana* in Human Macrophages: Validation by Real-Time Polymerase Chain Reaction Assay

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Abstract

Background and Aim *Arnica montana L.* (*Arnica m.*) is a popular traditional medicine, used for its therapeutic properties in healing traumas, but little is known about its biological action on tissue formation and repair. This new work tested the effects of *Arnica m.* homeopathic dilutions on human macrophages, key cells in tissue defence and repair.

Materials and Methods Macrophages derived from the THP-1 cell line were differentiated with interleukin-4 to induce a 'wound-healing'-like phenotype, and treated with various dilutions of *Arnica m*. centesimal (100 times) dilutions (2c, 3c, 5c, 9c, and 15c) or control solvent for 24 hours. RNA samples from cultured cells were analysed by real-time quantitative polymerase chain reaction in five separate experiments.

Results *Arnica montana* at the 2c dilution (final concentration of sesquiterpene lactones in cell culture = 10^{-8} mol/L) significantly stimulated the expression of three genes which code for regulatory proteins of the extracellular matrix, namely FN1 (fibronectin 1, % increase of 21.8 ± standard error of the mean 4.6), low-density lipoprotein-receptor-related protein 1 (% increase of 33.4 ± 6.1) and heparan sulphate proteoglycan 2 (% increase of 21.6 ± 9.1). Among these genes, the most quantitatively expressed was FN1. In addition, FN1, unlike other candidate genes, was upregulated in cells treated with higher dilutions/dynamisations (3c, 5c, and 15c) of *Arnica m.*

Conclusion The results support evidence that the extracellular matrix is a potential therapeutic target of *Arnica m.*, with positive effects on cell adhesion and migration during tissue development and healing.

Keywords
- *Arnica montana*
- macrophage
- homeopathic medicines
- wound healing
- gene expression
- inflammation
- extracellular matrix
- fibronectin

Introduction

Arnica montana L. (referred to here as *Arnica m.*) is widely used to treat various pathological conditions such as bruises, swelling associated with trauma, pain, inflammation, wounds and post-operative clinical conditions.[1–4] Investigations of its mechanisms of action have focused on inflammatory pathways,[5–11] but little is known about its possible action on the stages of tissue formation and renovation. In our laboratory,[11–14] we used the THP-1 human cell line, commonly employed to explore the function and regulation of monocytes and macrophages,[15] which are involved in inflammation and wound healing.[16] One of these articles[12] was recently retracted by the journal *PlosONE* due to criticisms raised about the concentration of *Arnica m.* used in the experiments and about the purported small gene expression changes. In a commentary,[17] we opposed these arguments on theoretical and methodological grounds and announced the publication of new data supporting our findings. These new data, based on reverse transcription quantitative polymerase chain reaction (RT-qPCR), are reported in the present article.

The previous RNA-seq analysis[12] highlighted some genes whose expression had increased when cells were incubated with *Arnica m.* 2c: low-density lipoprotein-receptor-related

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protein 1 (LRP1), fibronectin 1 (FN1), lysine (K)-specific methyltransferase (KMT2D), complement component receptor 1 (CR1), heparan sulphate proteoglycan (perlecan, HSPG2), microtubule-actin crosslinking factor 1 (MACF1) and fibrillin 2 (FBN2). Notably, the most enriched function was the 'extracellular matrix organization', which included four genes, namely FN1, LRP1, FBN2 and HSPG2. In this new work, the effects of *Arnica m.* dilutions on human macrophages were evaluated by RT-qPCR, which is considered the 'gold standard' tool for gene expression analysis associated with biological processes in cell cultures. Finally, we summarise the evidence outlining the complex network of biological processes influenced by *Arnica m.* during inflammation and wound healing.

Materials and Methods

Materials

The human monocytic leukaemia cell line THP-1 was purchased from DSMZ (Leibniz Institute, Braunschweig, Germany). Growth media RPMI 1640 and Ultraglutamine 20 mM solution were purchased from Lonza (Belgium). Foetal bovine serum (FBS), phorbol 12-myristate 13-acetate (PMA), pure ethanol and ultra-pure water (W3500) were purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA). Human interleukin-4 (IL-4) was purchased from Miltenyi Biotec (Germany). RNeasy Mini kit was from Qiagen (California, USA). RNA was reverse-transcribed into cDNA using PrimeScript RT reagent Kit (Takara Bio; Kusatsu, Japan), while RT-qPCR was performed using TB Green Premix Ex Taq (Tli RNase H Plus; Takara Bio, Jesi, Ancona, Italy). Sequences of the gene-specific primers were from ThermoFisher Scientific, Waltham, Massachusetts, USA.

Test Solutions

Arnica montana 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 hydro-alcoholic extract (mother tincture, MT) in 30% ethanol/distilled water. The conformity of the whole plant extract to pharmacopoeia standards was checked by thin-layer chromatography, and the assay of sesquiterpene lactones in the MT was determined by liquid chromatography with a Lichros 100 RP18 column in a HPLC1100 instrument (Agilent) using the method described in the French Pharmacopoeia.[18] The content of total sesquiterpene lactones, expressed as dihydrohelenalin tiglate, was 0.0355%, calculated from the areas of the indicated peaks. Since the mean molecular weight of *Arnica m.* sesquiterpene lactones is 340 g/mol,[19] this amount is equivalent to 1.05×10^{-3} mol/L in MT and to 1.05×10^{-5} mol/L. in *Arnica m.* 1c.

Arnica montana 1c was used to prepare the second centesimal dilution (*Arnica m.* 2c) by adding 50 µL of 1c solution to 4.95 mL of distilled ultra-pure water. This solution was filtered with a 0.22 µm Millipore filter and subjected to vigorous shaking with a Dyna-A mechanical shaker delivering 20 strokes per second over an 11 mm travel distance. Therefore, the 2c dilution corresponds to 10^{-4} of the MT and the calculated concentration of *Arnica m.* sesquiterpene lactones is 1.05×10^{-7} mol/L. The control test solution was prepared using 30% ethanol/distilled water diluted 100 times in distilled ultra-pure water and succussed as described for the *Arnica m.* 2c sample. Higher dilutions of *Arnica m.* were prepared as described previously.[11] Starting from a 1c solution, further serial 100 times dilutions were prepared in 5 mL of 30% ethanol/distilled water solvent followed by filtering and succussion. Stock *Arnica m.* dilutions in 30% ethanol/distilled water were wrapped in aluminium foil, stored at room temperature in the dark, and used within 12 months of preparation. The last centesimal dilution step (e.g. the 15c from the stock 14c) was always performed immediately before each experiment, in ultra-pure water. Among the *Arnica m.* dilutions prepared in this way, those tested with the cells were: 2c, 3c, 5c, 9c and 15c. All procedures for drug preparation and cell treatments were performed in sterile conditions.

Cell Cultures and Treatments

The THP-1 cell line was cultured in RPMI 1640 medium, supplemented with FBS 10% and 2 mM final concentration of Ultraglutamine (Lonza), at 37°C in 5% CO_2 in a humidified incubator as previously described.[11] Briefly, on day 1 the cells were seeded at a density of 2.5×10^5 cells/mL in 24-well plates in 1 mL medium with 2 mM Ultraglutamine and 2% FBS. 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 24 hours. On day 4, the plates were washed twice with culture medium and the cultures were again supplemented with 50 ng/mL IL-4 and incubated for 24 hours. Macrophages were exposed for 24 hours to *Arnica m.* dilutions or control solvent (1 mL cell culture + 110 µL test solutions). We performed a total of five complete separate experiments; in each experiment, every treatment was performed in triplicate wells.

Reverse Transcription Quantitative Polymerase Chain Reaction

Total RNA from cultured THP-1 cells was extracted using the RNeasy mini Kit (Qiagen), according to the manufacturer instructions. An on-column DNase digestion with the RNase-free DNase set (Qiagen) was also performed during total RNA isolation to completely remove any possible contaminating DNA. The integrity of total RNA samples was checked by a Fragment Analyzer (Agilent, Wokingham, UK) before use. RNA was then reverse-transcribed into cDNA and analysed by RT-qPCR. Sequences of the gene-specific primers (ThermoFisher Scientific, Waltham, Massachusetts, USA) used in this study are the following: GAPDH, forward AACAGCCTCAAGATCATCAGC and reverse GGATGATGTTCTGGAGAGCC; FN1, forward CGTTGGTTTGTACTTGTTATGG and reverse TCTCTTCAGCTTCAGGTTTACTC KMT2D forward CTGCTTACCAAGAATAACCTGAG and reverse ACTCTTCACCTCTGAAGTATCC; HSPG2 forward GGACACATTCGTACCTTTCTG and reverse GGCTCGGAAATAAACCATCTG; MACF1 forward CAGCAGCACATCCATACGAG and reverse GACATCTTTCCCTCATACAACCT; LRP1 forward CTGTGTCTCATCAACTACAACC and reverse GGAACTTCTTAAACTCATAGCAGG; FBN forward GGCTCCAGATCCATACAACAC and reverse CACACCTTCCTCCATTGAGAC.

Statistics

PCR amplification efficiency was calculated by LinRegPCR[20] and data were then calculated by Q-Gene software (http://www.gene-quantification.de/download.html) and expressed as mean normalised expression (MNE) units after glyceraldehyde 3-phosphate dehydrogenase (GAPDH) normalisation. Q-gene calculates the MNE by averaging the three concomitant CT values (triplicate of the same sample) of the target gene and of the reference gene, respectively, taking into account the difference of the PCR amplification efficiency between the target gene and the reference gene.[21] Statistical evaluation was performed by one-way analysis of variance followed by Fisher's least significant difference test or by Student's paired *t*-test where indicated. Values of $p < 0.05$ were considered as statistically significant.

To investigate the function of the expressed genes from this work and a previous one,[11] Gene Ontology (GO) consortium database was accessed using the clusterProfiler (Version 3.10.1) R/Bioconductor package.[22] Gene Ontology by biological process was queried using the *enrichGO* function from the clusterProfiler for the upregulated genes, with pAdjustMethod = 'BH'.

Results

As reported in ►**Table 1**, RT-qPCR confirmed the significant upregulation of 3 relevant genes previously found with the RNA-seq. *Arnica montana* 2c treatment significantly induced the expression of these genes as follows: FN1 (% increase of 21.8 ± standard error of the mean 4.6), HSPG2 (% increase of 21.6 ± 9.1) and LRP1 (% increase of 33.4 ± 6.1).

The changes due to treatment were relatively small, but the effect was highly reproducible in the five separate experiments performed. In the presence of *Arnica m.*, the FN1 expression values (MNE= 0.1646) were much higher than those of HSPG2 (MNE= 0.0180) and LRP1 (MNE= 0.079). Fibrillin 2 and MACF1 genes were slightly stimulated, but the absolute values of MNE were very low and the changes were not statistically significant. CR1 gene was omitted from the RT-qPCR analysis because in RNA-seq results[12] its expression level was extremely low (between 1 and 2.5 reads per kilobase million).

The expression level of the three genes (FN1, HSPG2 and LRP1), whose upregulation by *Arnica m.* 2c treatment was validated with RT-qPCR, was investigated in a five further separate series of experiments on macrophages treated for 24 hours continuously with serial 100-times (centesimal, 'c') dilutions/dynamisations: namely *Arnica m.* 2c, 3c, 5c, 9c, 15c or with the control solvent used for the dilutions (Ctrl, 30% ethanol in pure water). *Arnica montana* 2c increased the FN1 gene expression by ~30%, confirming the data of the experiments of ►**Table 1**. All the subsequent dilutions/dynamisations significantly upregulated the expression of FN1 compared with Ctrl, with the exception of 9c whose statistical significance was borderline ($p = 0.052$) (►**Fig. 1**).

The extent of the change induced by *Arnica m.* 2c is comparable to that of the higher *Arnica m.* potencies, indicating an absence of linearity between the effect size of

Table 1 Effects of *Arnica m.* on gene expression of human macrophages

mRNA expression (MNE)															
	FN1			FBN2			HSPG2			MACF1			LRP1		
Experiment	Ctrl	*Arnica m.* 2C	% Effect	Ctrl	*Arnica m.* 2C	% Effect	Ctrl	*Arnica m.* 2C	% Effect	Ctrl	*Arnica m.* 2C	% Effect	Ctrl	*Arnica m.* 2C	% Effect
1	0.1343	0.1614	20.2	0.0007	0.0009	28.6	0.0095	0.0150	57.9	0.0034	0.0041	20.6	0.0060	0.0084	40.0
2	0.1361	0.1607	18.1	0.0005	0.0007	40.0	0.0109	0.0121	11.0	0.0041	0.0046	12.2	0.0043	0.0049	14.0
3	0.1379	0.1657	20.2	0.0008	0.0008	0.0	0.0197	0.0223	13.2	0.0050	0.0060	20.0	0.0065	0.0081	24.6
4	0.1284	0.1785	39.0	0.0003	0.0005	66.7	0.0172	0.0196	14.0	0.0037	0.0045	21.6	0.0068	0.0094	38.2
5	0.1405	0.1568	11.6	0.0009	0.0006	−33.3	0.0188	0.0210	11.7	0.0052	0.0038	−26.9	0.0060	0.0089	48.3
Mean	0.1354	0.1646	21.8	0.0006	0.0007	20.4	0.0152	0.0180	21.6	0.0043	0.0046	9.5	0.0059	0.0079	33.0
±SEM	0.0020	0.0038	4.6	0.0001	0.0001	17.2	0.0021	0.0019	9.1	0.0004	0.0004	9.3	0.0004	0.0008	6.1
Student's *t*-test	*p* = 0.006			*p* = 0.641			*p* = 0.018			*p* = 0.522			*p* = 0.006		

Abbreviations: Ctrl, control; FBN2, fibrillin 2; FN1, fibronectin 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-2, interleukin-2; LRP-1, lipoprotein receptor-related protein 1; MACF1, microtubule-actin crosslinking factor 1; MNE, mean normalised expression; mRNA, messenger ribonucleic acid; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SEM, standard error of the mean.
Note: IL-4 differentiated THP-1 macrophages were cultured with or without *Arnica m.* 2c for 24 hours and their FN1, FBN2, HSPG2, MACF1 and LRP1 mRNA expression was evaluated by RT-qPCR. Gene expression is reported as MNE units after GAPDH mRNA normalisation. Student's paired *t*-test was used as statistical test.

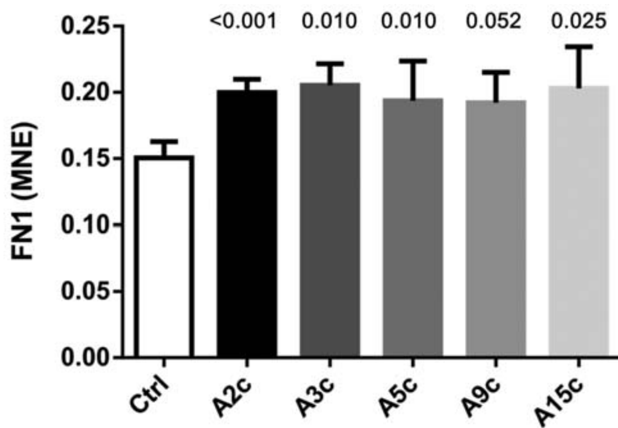

Fig. 1 Effects of increasing dilutions of *Arnica montana* on the expression of FN1 by human macrophages. IL-4-differentiated THP-1 macrophages were cultured with *Arnica montana* 2c, 3c, 5c, 9c, 15c or control solvent (30% EtOH, Ctrl) for 24 hours continuously to evaluate their FN1 mRNA expression by RT-PCR. Gene expression is depicted as mean normalised expression (MNE) units after GAPDH mRNA normalization (mean ± SEM, $n = 5$). Data above each bar report *p*-value by one-way ANOVA, followed by the least significant difference post-hoc test comparing each dilution to the control. ANOVA, analysis of variance; A, Arnica; Ctrl, control; EtOH, ethanol; FN1, fibronectin 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; RT-PCR, real-time polymerase chain reaction; SEM, standard error of the mean.

Arnica m. and the increasing dilution of the theoretical concentrations of plant molecular components in the solution. LRP1 and HSPG2 gene expression were also investigated in the same RNA samples from cells treated with highly diluted *Arnica m.* In both genes, the maximum up-regulation was observed with *Arnica m.* 2c but the higher dilutions exerted very little stimulatory effects, which were not statistically significant (data not shown).

Discussion

The main finding of this investigation is the increased expression of several genes of tissue matrix proteins and particularly that of FN, whose increase was confirmed also by protein assay in culture supernatants.[12] Fibronectin is a multi-domain protein with an essential role in the extracellular matrix (ECM) since it binds to both cell receptors and connective tissue fibres. Fibronectin 1 is overexpressed in macrophages during the healing phase of inflammation, suggesting it has important roles in ECM deposition and tissue re-modelling in acute inflammatory reactions and in chronic inflammatory diseases.[23]

Our previous study with RNA-seq[12] reported the upregulation of FN1, LRP1, HSPG2, KMT2D, CR1, MACF1 and FBN2 in cells treated with *Arnica m.* 2c. In the present work, FN1 showed the highest value of expression, followed by LRP1 and HSPG2, supporting the previous evidence, while other candidate genes showed low expression and their changes were not statistically significant. Since the difference of outcomes between RNA-seq and RT-qPCR was observed only in genes that were expressed to a minor extent, it is conceivable that the apparent discrepancy is due to the different sensitivity level and precision of the two methods. In fact, RNA-seq is followed by bioinformatics analysis, which can be done by using different mapping, different reads quantification tools and various analytical approaches. In this context, the output of differentially expressed genes can be slightly different depending, for example, on whether or not the mapping is done on the genome or on the transcriptome.[24,25] For this reason, validation of RNA-seq output of differentially expressed genes by RT-qPCR is highly recommended.

The changes of gene expression induced by *Arnica m.* are notably low, ranging from 20 to 30% of the basal expression in the absence of drugs. It is conceivable that this small effect is due to the very low doses (or high dilutions) used, since the 2c dilution corresponds to a 10^{-8} mol/L final concentration of active principles. However, even a small increase in macrophage activity in production of key proteins such as FN may have a decisive positive outcome for tissue healing and repair. It should be noted that conventional anti-inflammatory drugs are designed to suppress the underlying enzymatic mechanism of inflammation (e.g. prostaglandins, cytokines) and act at considerably high doses, whereas homeopathic treatment is designed to regulate only the pathological aspects and malfunctioning tissues, because the inflammatory process per se is seen as an expression of natural healing dynamics. Moreover, given the variety of *Arnica m.* effects and the multiplicity of its alkaloids, flavonoids and sesquiterpene lactones,[26] it is conceivable that the full picture of its action is much more complex and could involve modulation of different cells and further pathways. Complex dynamics of the ECM in inflammatory and repair events after *Arnica m.* treatment have been observed using immunohistochemistry in a rat model, concurring with the results of the present work.[9]

In an RT-PCR array of a limited set of genes,[11] we observed an increase in gene expression for CXC chemokines and bone morphogenetic protein after treatment with *Arnica m.*, and a decrease of metalloproteinase-1. Taken together with the evidence provided in the present analysis, a complex and interesting picture emerges of the action of this plant on the regulation of cell migration, on mechanisms of connective tissue defence (inflammation) and on repair and healing (regulation of ECM) (►**Fig. 2**). Fibronectin 1 is central in this regulation, being connected with all three biological processes.

In recent years, progress has been made in understanding the mechanisms of biological action of homeopathic medicines at the molecular level. A modulating action of gene expression has been detected in cell cultures of macrophages,[27] neurocytes,[28] epithelial cells,[29] embryonic kidney,[30] and even plant cells.[31] In microbiological models, *Arnica m.* 30c modified the expression of specific genes that are the targets of ultraviolet irradiation injury.[32] Anti-tumour homeopathic medicines induce expression changes in the whole genome of cancer cells.[33] These and other findings, reviewed elsewhere,[34–39] support the hypothesis that highly diluted homeopathic medicines are able to turn some important genes on or off, initiating a cascade of gene actions to correct the gene expression changes that produced the disorder or disease.

In conclusion, the modulation of expression of a series of ECM genes in IL-4-differentiated macrophages suggests that

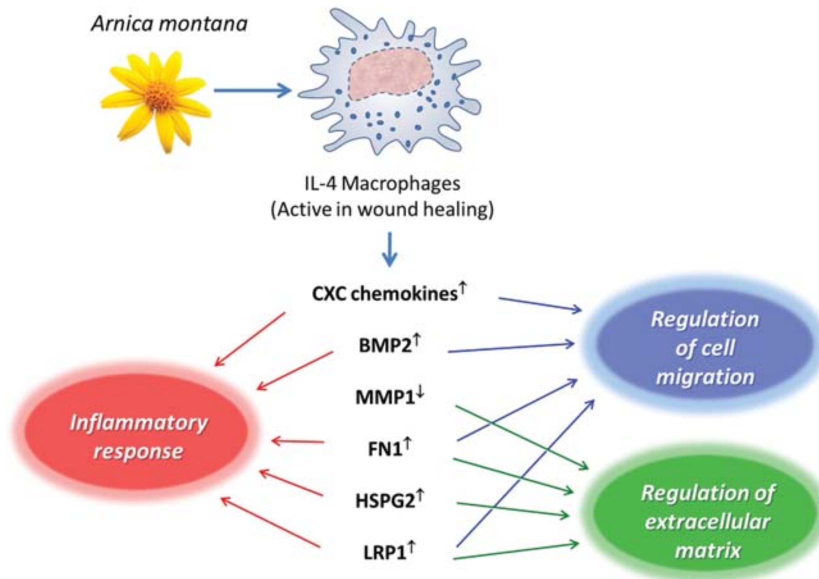

Fig. 2 Hypothetical effects of *Arnica montana* in human macrophages based on gene expression studies (this paper and ref. 13) and their functions in wound healing. The gene functions were identified by accessing the Gene Ontology (GO) consortium database using the clusterProfiler R/Bioconductor package[22] as described in Methods section. The graph was constructed using the significantly upregulated genes described in this work (FN1, HPSG2, LRP1) and those from a RT-PCR array used in previous work on the same cell model (CXC chemokines, BMP2, MMP1).[13] BMP2, bone morphogenetic protein 2; FN1, fibronectin 1; HPSG2, heparan sulphate proteoglycan 2; IL-4, interleukin-4; LRP1, lipoprotein receptor-related protein 1; MMP1, matrix metalloproteinase 1; RT-PCR, real-time polymerase chain reaction.

the known beneficial properties of *Arnica m.* are mediated by modulation of relevant biological functions that are involved in inflammation and connective tissue healing after injury.

Highlights

- Macrophages derived from the THP-1 cell line were differentiated with IL-4 to induce a 'wound-healing'-like phenotype.
- Measurements using RT-qPCR assay indicated that macrophages are a pharmacological target of *Arnica m.*
- A slight but statistically significant up-regulation of three ECM genes was observed.
- FN1 (fibronectin 1) is the major gene whose expression was stimulated by *Arnica m.*
- We conclude that *Arnica montana* impacts a network of biological functions including inflammation and extracellular matrix regulation.

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Conflict of Interest

None declared.

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THIEME