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On the effects of the microsomal prostaglandin E synthase-1 inhibitors on the functional activity of inflammatory cells from RA patients

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Title (English)

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Title (Swedish)

This thesis work investigates the effects of microsomal Prostaglandin E Synthase-1 (mPGES-1) inhibition on the functional activity of synovial fibroblasts from patients with rheumatoid arthritis (RA). mPGES-1 is an inducible enzyme capable of converting prostaglandin H2 (PGH2) to prostaglandin E2 (PGE2) which in turn contributes to inflammation, pain and joint destruction in RA. Therefore, mPGES-1 inhibition is a potential novel target for the next-generation therapeutics for the treatment of inflammatory diseases. In this study, two mPGES-1 inhibitors (A and B) were tested for their ability to affect PG production and expression of pro-inflammatory molecules in synovial fibroblasts.

Keywords

PGE2, mPGES-1inhibitor, functional activity, rheumatoid arthritis

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Sammanfattning

Detta examensarbete undersöker hämning av microsomal Prostaglandin E Syntas-1 (mPGES-1) och dess effekt på funktionella aktiviteter av synovial fibroblast (SF) hos patienter med reumatoid artrit (RA). MPGES-1 är ett inducerbart enzym och kan katalysera prostaglandin H2 (PGH2) till prostaglandin E2 (PGE2) som är relaterad till en rad patologiska tillstånd såsom kronisk och akut inflammation, smärta, feber, ateroskleros, stroke, anorexi och cancer. PGE2 även bidrar till led skador i RA. Således är hämning av mPGES-1 en potential mål till nästa generation terapi speciellt för behandling av inflammatorisk sjukdom. Genom samarbete med Karolinska Institution, Actar AB har identifierade några mPGES-1 hämmare som dämpar mPGES-1 aktivitet i nanomolar koncentration. I den här studien, två mPGES-1 hämmare (A and B) var testade för deras förmåga att påverka prostaglandin (PG) produktion och uttryck av pro-inflammatory molekylär i SF.

SF från 4 RA patienter behandlades med mPGES-1 hämmare efter stimulation av IL-1β och TNFα. PGE2 produktion minskade signifikant efter behandlingen hos alla patienter. Produktionen av en av andra terminal produkt PGI2 höjdes dock av båda hämmare. Detta innebär att mPGES-1 hämning förmodligen höjde tillgängligheten till gemensam förfader PGH2 för andra terminala PG syntaser. Jämförd med hämmare A, hämmare B reducerade PGE2 produktion mer effektivt och hade mindre utökning av PGI2. Därför var hämmare B mer intressant för vidare forskning. Effekter av hammare B på mRNA uttryck av IL-6, IL-8, IL-23p19, MMP-1, MMP-3 and VEGF var analyserade i den här studien. Vi observerade att hämmare B nedreglerade mRNA utryck av IL-23p19 hos alla patienter och IL-6 mRNA uttryck hos tre av fyra patienter. Dessa resultat visar att mPGES-1 hämning skulle kunna ha välgörande effekt på inflammation och led förstörelse i RA patienter.

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Table of Contents

Abbreviations

AA:	Arachidonic Acid
COX:	Cyclooxygenase
cPGES:	Cytosolic Prostaglandin E Synthase
dNTP:	Deoxyribonucleotides
DTT:	Dithiothreitol
EIA:	Enzyme Immunoassay
FCS:	Fetal Calf Serum
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
LC-MS:	Liquid Chromatography- Mass Spectrometry
IL:	Interleukin
MMP:	Matrix Metalloproteinase
mPGES:	Microsomal Prostaglandin E Synthase
NCBI:	The National Center for Biotechnology Information
NSAID:	Nonsteroidal anti-inflammatory drug
PG:	Prostaglandin
PGD2:	Prostaglandin D2
PGE2:	Prostaglandin E2
PGF2α:	Prostaglandin F 2α
PGI2:	Prostacyclin
PLA2:	Phospholipase A2
RA:	Rheumatoid Arthritis
RA SF:	Rheumatoid Arthritis Synovial fibroblasts

RT-PCR:	Real Time Polymerase Chain Reaction
SD:	Standard Deviation
SF:	Synovial fibroblasts
TNFα:	Tumor Necrosis Factor Alpha
Trypsin-EDTA:	Trypsin Ethylenediamine Tetraacetic Acid
TX:	Thromboxane
TXA2:	Thromboxane A2
TXB2:	Thromboxane B2
VEGF:	Vascular Endothelial Growth Factor

Introduction

1. Eicosanoid biosynthesis from arachidonic acid

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease. It is characterized by systemic and local inflammation and results in joint destruction and severe disability. Prostaglandin E2 (PGE2) is considerably increased in RA and contributes to several pathological features of these diseases, such as pain, inflammation, angiogenesis and joint destruction. The regulation of the PGE2 synthesis or action has important implications as a possible means for treatment of RA.

PGE2 belongs to the family of eicosanoids, signaling molecules that are generated through oxidative pathways from arachidonic acid (AA) [2]. AA is a long chain omega 6 fatty acid and AA-derived eicosanoids regulate a wide variety of physiological functions and pathological processes. AA is generated from the membrane phospholipids of the cells by the enzyme phospholipase A2 (PLA2) and from diacylglycerol by diacylglycerol lipase. AA metabolites contribute to the main mechanisms of the pathogenesis of variety of diseases including cancer and arthritis [3]. AA can be converted to bioactive eicosanoids through the cyclooxygenase (COX), lipoxygenase (LOX) and P-450 epoxygenase pathways. The products from two main AA pathways include prostaglandin H2 (PGH2) that is generated by COX and subsequently converted to prostanoids (prostaglandins, prostacyclin (PGI2) and thromboxane (TXA2)), and leukotrienes and lipoxins, generated by LOX.

This study is within the COX pathway of AA. In the pathway, PGH2 itself does not act as an inflammatory mediator. However, it works like a substrate that is available for various particular enzymes. These enzymes catalyze the unstable PGH2 to more stable prostanoids. The prostanoids are involved in regulating different processes in our body. These processes include blood pressure, blood clotting, sleep, inflammation, etc. [2]

Prostanoid is a generalized term for products of the COX pathway including prostaglandins (PGE2, PGD2 and PGF2α), PGI2 and TXA2 (Figure 1). The list of prostanoids, their synthases together with tissue or cell specific expressions is shown in Table 1. In this study we focus on PGE2, the most important and abundant prostaglandin in the inflammation processes of rheumatoid arthritis.

Table 1. Major components of Prostanoids including their synthases, functions and specific tissue and cells where they are produced

Prostanoid	Synthases	Tissue/cell specific expression	functions
Prostaglandin E2 (PGE2)	Microsomal prostaglandin E synthase 1 (**MPGES1**) Microsomal prostaglandin E synthase 2 (**MPGES2**) Cytosolic prostaglandin E synthase (**CPGES**)	Synovial fibroblasts and macrophages	Inflammatory mediator Regulate inflammatory molecules such as IL-6, IL-8, MMP-1 and MMP-3, etc.
Prostaglandin F2α (PGF2α)	Prostaglandin F2α synthase (**PGFS**)	Uterus	Uterus constriction
Prostaglandin D2 (PGD2)	Prostaglandin D2 synthase (**PGDS**)	Brain and mast cells	Inhibitor of platelet aggregation
Prostacyclin (PGI2)	Prostacyclin synthase (**PGIS**)	Endothelial cells	vasodilator
Thromboxane A2 (TXA2)	Thromboxane A2 synthase (**TXAS**)	Platelets	vasoconstrictor

PGE2 is a terminal product of AA from PGH2 produced by several microsomal PGE synthases. PGE2 presents a great quantity in our body [1]. It has a lot of biological activities via its four receptors EP1, EP2, EP3 and EP4 [4] [5] [6]. PGE2 has been found to take part in different physiological actions such as inflammation, pain, fever, tumorigenesis, female reproduction, vascular regulation, neuronal functions and kidney function [7]. It is by far one of the major prostanoid synthesized in the joint and plays an important role in inflammation and pathogenesis of rheumatoid arthritis. The excessive production of PGE2 has been showed in serum and synovial fluids from rheumatoid arthritis patients and osteoarthritis patients. In addition to its own inflammatory actions PGE2 regulates inflammatory mediators such as IL-6, IL-8 [8], IL-23p19 [9], VEGF [10] and MMPs [11] [12].

Prostacyclin (PGI2) is a member of prostanoid family and is derived from arachidonic acid. It is produced in the endothelial cells from PGH2 by prostacyclin synthase. Prostacyclin is not stable and it may degrade to 6-keto-PGF1α that is a stable end product of PGI2 [13]. PGI2 chiefly prevents the formation of platelet plugs in blood clotting, and it also functions as an effective vasodilator. Non-steroid anti-inflammatory drugs (NSAIDs) or COX inhibitors inhibit not only PGE2 but also PGI2. Blocking of PGI2 production plays an important role in the cardiovascular side effects. Therefore, selective inhibition of downstream enzyme mPGES-1 has been considered a potential novel treatment for the inflammatory diseases and pain without the side effects associated with COX inhibition [1]. Recent studies suggested that PGI2 is also a significant contributor to the inflammation process in RA. So it is very interesting to study the effect of PGE2 reduction on the PGI2 production [14].

Thromboxane is another member of prostanoids. It is produced in platelets by thromboxane synthase from PGH2. There are two major thromboxanes, thromboxane A2 and thromboxane B2. Thromboxane A2 is a major component in blood clotting and very unstable in aqueous mixture. It is produced by thromboxane synthase catalyzing PGH2 to TXA2. TXB2 is a stable metabolite of TXA2. Thromboxane is involved in increasing platelet aggregation as a powerful vasoconstrictor. Thromboxane and prostacyclin function as antagonists. They keep homeostatic balance in the circulatory system.

Prostaglandin D2 (PGD2) is involved in the central nervous system as a neuroregulator and platelet aggregation as a strong inhibitor. Prostaglandin F 2α (PGF2α) has two main functions: uterus constriction and bronchoconstriction. PGF2α is widely used in the medicine to stimulate labor and as an abortifacient. The receptors of both PGD2 and PGF2α are members of G- protein coupled receptor family.

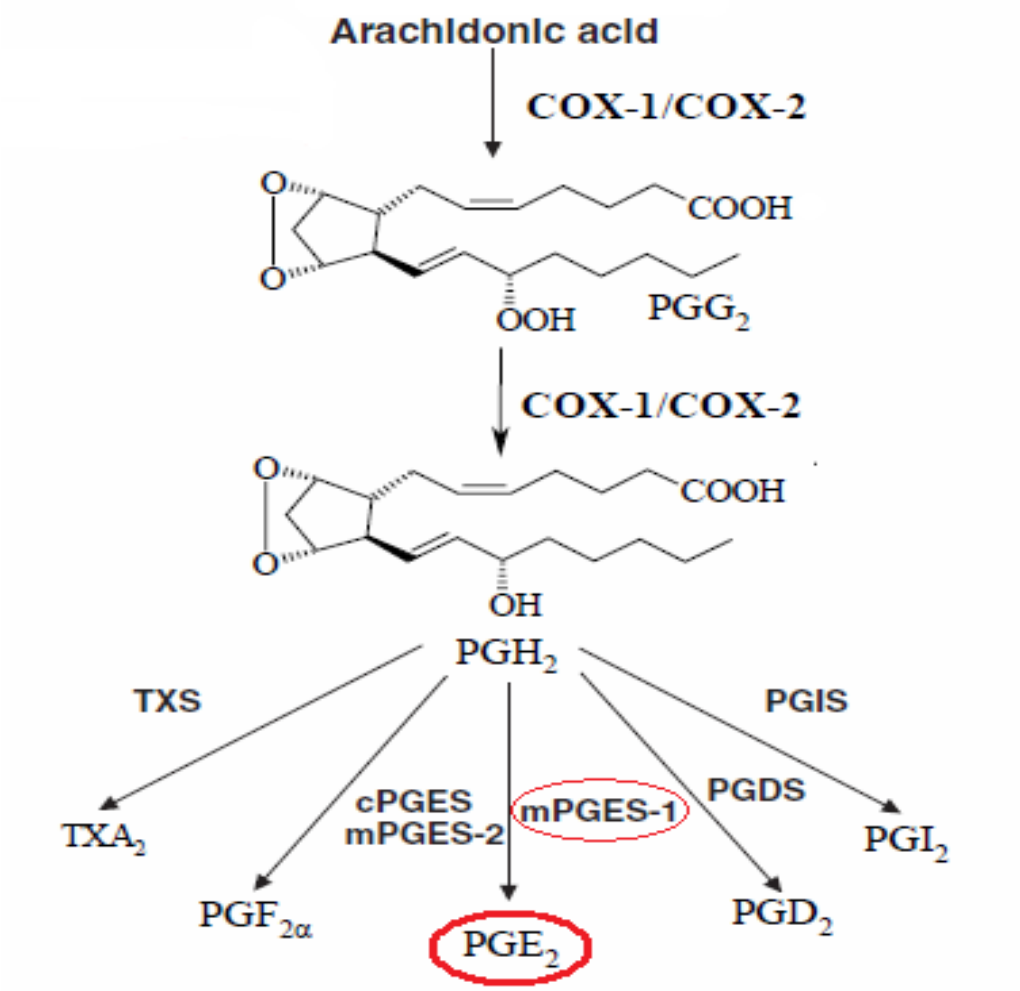


Figure 1. Prostaglandin synthesis pathway. COX and PGES are coordinated to produce PGE2. There are two isoforms of COX (COX-1 and COX-2) and three of PGES (mPGES-1, mPGES-2 and cPGES). COX-2 and mPGES-1 are able to be induced by inflammatory stimuli. Modified from [15]

2. Role of synovial fibroblasts and important inflammatory molecules in the pathogenesis of RA

RA is a complex multisystem disease characterized by an abnormal proliferation of synovial tissue called pannus where the immune response and joint damage take place. The pannus consists of immune part and erosive part (Figure 2). The components of the immune part are macrophages, T cells, B cells and dendritic cells. They are involved in various biological activities, such as antigen presentation, immunoglobulin production and cytokine generation. The T cell is the key component to coordinate these activities. The erosive part is comprised of cells such as osteoclasts and synovial fibroblasts. These particular cells are very close to bone and cartilage. So they are able to be involved in erosion and destruction of these particular tissues directly [13].

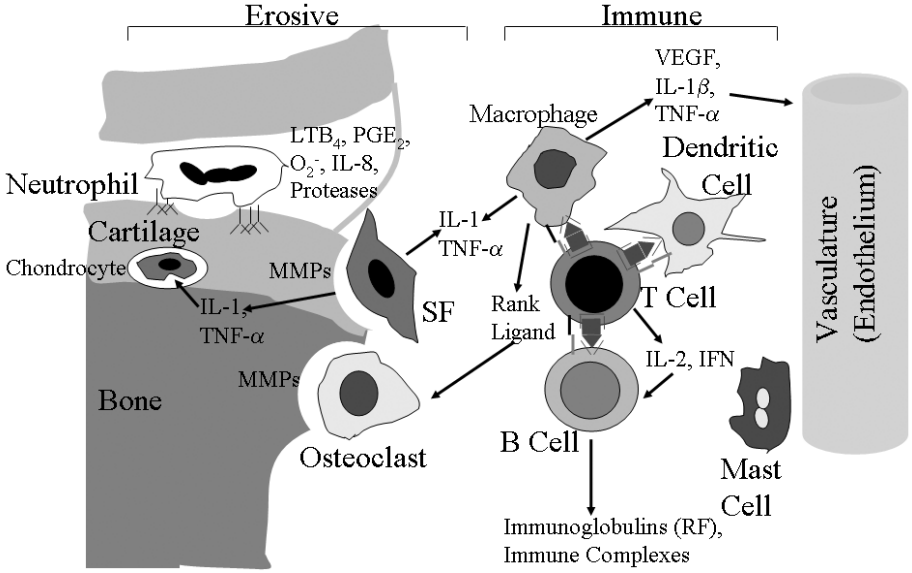


Figure 2. Pathophysiology of rheumatoid arthritis [13] **synovial tissue consists of erosive part and immune part. The components of immune part are macrophages, T cells, B cells and dendritic cells. T cells plays key role in the pathological and physiological processes. Synovial fibroblasts are one component in the erosive part. It is involved in the direct and indirect in the inflammation, bone and cartilage destruction.**

Synovial fibroblasts (SF) function as both directly and indirectly roles in inflammation and joint destruction in RA. RASF is largely responsible for the production and the secretion of pro-inflammatory mediators such as matrix metalloproteinases (MMPs). That is another reason that cartilage and bone could be eroded by RASF. Thus synovial fibroblasts are a good model to analyze molecular mechanisms of the pathogenesis of rheumatoid arthritis.

There are a number of pro-inflammatory cytokines and mediators that are involved in the pathological and physiological process. The cytokines and mediators were included in this work due to their essential roles in the RA, and also that RASF are able to produce these mediators.

Tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β) are the two most important mediators in the pathogenesis of chronic inflammatory joint diseases such as rheumatoid arthritis (Probert, et al. 1995). There are markedly up-regulated levels of these mediators in the synovial fluid of RA patients. In animal models of arthritis it was shown that TNFα is able to increase inflammation while overexpressed IL-1β leads to cartilage destruction. The studies have provided convincing evidence that these cytokines play important roles in RA pathogenesis, and that they could promote production of PGE2 and other pro-inflammtory mediators resulting in inflammation, damage of bone and cartilage.

RASF produces and secretes IL-6 and IL-8. Production is up-regulated by IL-1β and TNFα. IL-6 is an inflammatory cytokine that has a wide range of biological activities, such as remodeling bone, generation of inflammatory responses and immune responses. IL-8 is a chemokine within a family of small cytokines. In RASF, IL-8 is able to promote chemotaxis which is movement by cells or organisms in reaction to chemical stimuli. It has been reported that PGE2 induces IL-6 expression in mouse osteoclasts and IL-8 expression in the synovial fibroblasts [8].

VEGF promotes angiogenesis that is a process of formation of new blood vessels under pathological and physiological conditions. Remarkably high levels of VEGF have been found in the RA synovial tissues, which suggested that VEGF might be involved in the inflammatory angiogenesis in RA. PGE2, the most abundant prostaglandin, is able to induce production of VEGF. In addition, it has been reported that NSAIDs inhibit VEGF in RA synovial fibroblasts [16].

IL-23 is a heterodimeric cytokine, consisting of two subunits. One subunit is p40 shared with IL-12, while the other subunit p19 is specific for IL-23. IL-23p19 is a human gene also known as IL-23A [17]. IL-23 is produced by antigen-presenting cells such as dendritic cells and macrophages, Th-1 lymphocytes and RASF. IL-23 is

a key molecule in the expansion and survival of Th17 cells, which are essential in the pathogenesis of autoimmune diseases. IL-23 has been indicated in the development of cancerous tumor. Interestingly, PGE2 induces the production of IL-23 in bone marrow dendritic cells [18].

Matrix metalloproteinases is a family of enzymes that are able to degrade the components of extracellular matrix not only in normal physiological processes like reproduction but also in disease processes like arthritis [19]. Most of MMPs are inactive proteins secreted in the extracellular spaces so that the level of MMP activity in the healthy condition is low. The expression of MMPs is increased generally by pro-inflammatory cytokines and growth factor in arthritis, which results in bone and cartilage destruction [12].

MMP-1 is a collagenase, one of the primary enzymes responsible for the degradation of type II collagen. MMP-1 is increased in response to IL-1β and TNFα in the synovial tissues from RA patients. MMP-3 is a ubiquitous MMP, which activates MMP-1 and cleaves many matrix proteins [20]. Recent studies have shown that PGE2, the inflammatory modulator, inhibits the production of MMP-1 in RASF [21]. A number of reports suggest that PGE2 might modulate the production of MMP-3. However, the effects depend on cell type and conditions.

3. Role of mPGES-1 in RA

In the PGE2 synthesis pathway, cyclooxygenase (COX) is a key enzyme that metabolizes AA to PGG2 and to PGH2. There are two isoforms of COX, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-1 is constitutively expressed in various cells and tissues. COX-2 is able to be induced by various stimuli including cytokines in inflammatory cells and tissues [22].

PGE synthases act downstream of cyclooxygenases and catalyze the conversion of PGH2 to PGE2. There are three distinct PGES isoforms that have been identified. They include cytosolic PGES (cPGES), microsomal Prostaglandin E2 Synthase 1 (mPGES-1) and microsomal Prostaglandin E2 Synthase -2 (mPGES-2). cPGES is constitutively expressed in various tissues. It is preferentially coupled with COX-1, and involved in native production of PGE2. mPGES-2 is also constitutively expressed in diverse tissue and is coupled with both COX-1 and COX-2 functionally. mPGES-1 is induced by pro-inflammatory cytokines such as interleukin-1β or tumor necrosis factor (TNF-α) and is coordinated with COX-2 to produce PGE2 [1]. It was shown that PGE2 production is increased in correspondence with over-expression of COX-2

and mPGES-1 [23]. Therefore, both COX-2 and mPGES-1 have key roles in the inflammation in RA [24] [1].

Non-steroid anti-inflammatory drugs function by inhibition of COX-1 and COX-2 activity resulting in suppression of all downstream products of AA. Most of NSAIDs may be able to result in serious side effects such as formation of ulcers [25] and an increased risk of thrombosis and cardiovascular complications [26]. This is because normal physical condition in our body could not be kept in balance. Cardiovascular side effects are due to changed balance between pro-thrombotic tromboxane A2 and anti-thrombotic prostacyclin [27].

mPGES-1 deficient mice showed a significant reduction of inflammation in experiment arthritis [28]. mPGES-1 is also believed to play a key role in the chronic inflammatory diseases like rheumatoid arthritis [29]. The antirheumatic treatment with TNF blockers suppressed PGE2 production did not suppress expression of mPGES-1 in vivo [30]. These studies suggest that mPGES-1 constitutes an attractive novel therapeutic target for the treatment of inflammatory diseases possibly without any side effects associated with COX-inhibitors [7]. Inhibitors of mPGES1 are presently under development.

In collaboration with Karolinska Institutet, Actar AB has identified several mPGES-1 inhibitors which suppress mPGES-1 activity in nanomolar concentrations. In this study we have studied two inhibitors from Actar AB, which we refer to as inhibitor A and inhibitor B.

4. Aim of the project

The aim of this study was to investigate the effects of two mPGES-1 inhibitors on the functional activity of synovial fibroblasts from RA patients. The study includes the effects on the biosynthesis of different prostanoids and examination of mRNA expression of pro-inflammatory mediators. With a small scale experiment consisting of four RA patients, we aim to conclude the potential of the two inhibitors for further investigation.

Materials and methods

1. Synovial fibroblast culture

Primary synovial fibroblasts were isolated from synovial membranes of knee joints from 4 patients with RA (bought from Dominion Pharmakine, S.L., Bizkaia, Spain). Cells were cultured in Dulbecco´s Modified Eagle´s Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100U/ml penicillin and 100ug/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37ºC in plastic culture flasks (175 cm^2). Synovial fibroblasts from passages 3-6 were used for experiments. When the cells reached the confluence, they were trypsinised. Before trypsinisation the medium was removed and the cells were washed with PBS. The trypsin-EDTA was used to detach the cells from the flasks. The cells were collected and centrifuged. Cell number in suspensions was counted in counting chamber (haemocytometer).

2. Cell stimulation and treatment

The cells (1 million cells/2ml/well) were put in the 6-wells cell culture plates. The cells in complete medium were allowed to adhere overnight in 5% CO_2 incubator at 37ºC. Thereafter, the cells were divided into three groups and processed as following:

i) Maintained as un-stimulated and untreated controls in presence of vehicle (1% DMSO).
ii) Stimulated by IL-1β (10ng/ml) and TNFα (10ng/ml) for 24h
iii) Stimulated by IL-1β (10ng/ml) and TNFα (10ng/ml) and treated with inhibitors (A and B, 10μM dissolved in DMSO) for 24h.

The optimal concentrations of IL-1β, TNFα and inhibitors and the optimal time for induction were determined by previous experiments (performed by Marina Korotkova). After treatment the cell supernatants were collected and store at -20ºC for prostanoids analysis.

3. RNA isolation and cDNA synthesis

After culturing the synovial fibroblasts were lysed and total RNA was extracted using RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer´s protocol. Samples were incubated with DNase (Qiagen RNase free DNase set) for 20 min in order to avoid contamination with genetic DNA. Total RNA concentrations were quantified using spectrophotometer (Thermo Scientific NanoDrop™ Spectrophotometer). Approximately 1ug RNA was converted to cDNA using SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA, USA). For each reaction,

4 µl 5× First strand buffer, 2 µl dNTPs (10 mM) and 1 µl random hexamer primers (0.1 mg/ml) were mixed with 10ul RNA. The reaction was then incubated in 70ºC heating blocks for 5 minutes and transferred on ice immediately. 1 µl dithiothreitol (DTT, 0.1M), 1 µl RNAguard (Pharmacia) and 1 µl SuperScript (200U/ml) were added to the samples. The samples were first incubated at room temperature for 10 minutes, then at 42ºC for 60 minutes and finally at 70ºC for 10 minutes. 50 µl of sterile water was added afterwards for final mixing. The final cDNA products were stored at -20ºC until needed. The remaining RNA was stored at -80ºC.

4. Primer design and Real time PCR Assay

The primers were designed using Beacon Designer 6.0 software (PRIMER Biosoft International, Palo Alto, CA, USA) or was obtained from Wang et al (2006). The mRNA sequences were retrieved from the National Center for Biotechnology Information (NCBI) website. The amplicons which span over exon-exon junctions or two exons were selected when possible to be able to distinguish between amplification of mature RNA and genomic DNA. The NCBI blast server [31] was used to confirm the specificity of all the primers. A list of the primer sequences and product sizes for mRNA analysis by real time PCR is shown in Table 2.

Real-time PCR was performed using The ABI PRISM 7900HT Fast Real-Time PCR System with a three-step protocol (95ºC for 15 min, followed by 40 cycles of 95ºC for 15s, 65ºC for 30s, and 72ºC for 30s) and with SYBR green fluorophore. The reactions were performed in optical 384-well plates (Applied Biosystems) in a total volume 12ul including 6ul 2x SYBR Green Master Mix (Applied Biosystems), 0.72ul of each primer (5mM), 2.56ul of water and 2ul of cDNA template. The SYBR green method is the most popular recently because of its low cost, ease of use and reliability.

All PCR-products were separated on 2% agarose gel to analyse the specificity of amplification process. Agarose gels were stained with ethidium bromide.

The plates were read on The ABI PRISM 7900HT and the data analysed in SDS .2.1 software. We have used Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. It is constitutively expressed in many cell types and is commonly used to normalize the signal value of samples and correct for possible differences in RNA quantity and quality. The differences between the normalized values reflect the real biological difference. Gene expression levels were evaluated with help of the cycle threshold (Ct). The cycle threshold is the cycle number when the exponential phase of amplification rises above the background signal.

Table 2. **Primer sequences and product sizes for mRNA analysis by using Real time PCR**

* - primers from publication of [32]

Target Gene (biomarker)	Forward primer (5´-3´)	Reverse Primer(3´-5´)	cDNA product size(bp)	GenBank accession number
IL-6*	GACAGCCACTCACCTCTTCA	TTCACCAGGCAAGTCTCCTC	211	NM_000600
IL-8*	CTGCGCCAACACAGAAATTATTGTA	TTCACTGGCATCTTCACTGATTCTT	170	NM_000584
IL-23p19	CAAGTGGAAGTGGGCAGAG	CAGCAACAGCAGCATTACAG	114	NM_016584
MMP-1*	CATGCCATTGAGAAAGCCTTCC	AGAGTTGTCCCGATGATCTCC	123	NM_002421
MMP-3*	GACAAAGGATACAACAGGGACCAAT	TGAGTGAGTGATAGAGTGGGTACAT	122	NM_002422
GAPDH	AGGGCTGCTTTTAACTCTGGTAAA	CATATTGGAACATGTAAACCATGTAGTTG	91	NM_002046
mPGES-1	GAAGAAGGCCTTTGCCAAC	CCAGGAAAAGGAAGGGGTAG	137	NM_004878
COX-2	TGCATTCTTTGCCCAGCACT	AAAGGCGCAGTTTACGCTGT	146	NM_000963
VEGF	AGAAGGAGGAGGGCAGAATC	GCACACAGGATGGCTTGAA	146	NM_001025366

There were two alternative ways to determine expression levels. The first approach was to include efficiency of PCR reaction, then fold increase defined as $(2\times\text{efficiency})^{\Delta\Delta Ct}$, where $\Delta\Delta Ct = [(Ct_{sampel\_ctrl} - Ct_{sampel\_stim.}) - Ct_{HK\_ctrl} - Ct_{HK\_stim.}$) and HK is

housekeeping gene. Efficiency can be determined by slope of a standard curve, defined as Efficiency $=10^{-(1/slope)}-1$. A standard curve represents the relationship of Ct value and the log value of starting copy numbers of cDNA templates [33].

The second approach is to approximate fold increase measurement without efficiency coefficient. Fold increase is then defined as $2^{\Delta\Delta Ct}$.

Data was expressed as fold changes relative to unstimulated conditions (control). In this study we have used $2^{\Delta\Delta Ct}$ to calculate fold increase. Fold increase of the unstimulated conditions is one.

5. Enzyme Immunoassay

Enzyme immunoassay (EIA) was used to analyze production of PGE2 and PGI2 in supernatants from RA SF (Prostaglandin E2 EIA kit and 6-keto Prostaglandin F1α EIA Kit, Cayman Chemical, USA). We developed plate according to the kit's protocol. Each plate contains two blank wells, two non-specific binding (NSB) wells, one Total Activity, three maximum binding (B_0) and an eight point standard curve in duplicates. Samples were applied on the plate in duplicates as well. The plate was covered with plastic film and incubated 18 hours at 4ºC. After incubation the plate was emptied and washed by wash buffer five times. The unbound reagents were removed. The plate was developed by adding 200µl of Ellman´s reagent to each well and 5µl of tracer to the Total Activity well. Samples were developed further on a shaker in the dark in 60-90 minutes finally.

The assay results were calculated using software Profox. The NSB average value was subtracted from the B_0 average value to get the correct maximum binding. The $\%B/B_0$ (% Samples or Standard Bound / Maximum Bound) values were calculated for each well. The standard curve was determined by plotting the $\%B/B_{0\ values}$ for standards versus PGE2 concentrations (in pg/ml) with the correlation coefficient 0.99. The concentration of each sample was obtained by identifying the $\%B/B_0$ on the standard curve and reading the corresponding values on the x-axis.

Results and discussion

1. Induction of prostanoid in RA synovial fibroblasts by IL-1β and TNFα.

Synovial fibroblasts without stimulation produced relatively low PGE2 levels. Treatment of RA synovial fibroblasts with TNFα and IL-1β for 24h resulted in substantial increase of PGE2 production in cells from all patients (Figure 3). The increase in PGE2 production was associated with significant up-regulation of mPGES-1 and COX-2 mRNA expression in synovial fibroblasts (Figure 4A and B). COX-2 and mPGES-1 act in concert to produce inducible PGE2 and both of them have very important roles in the inflammation in RA.

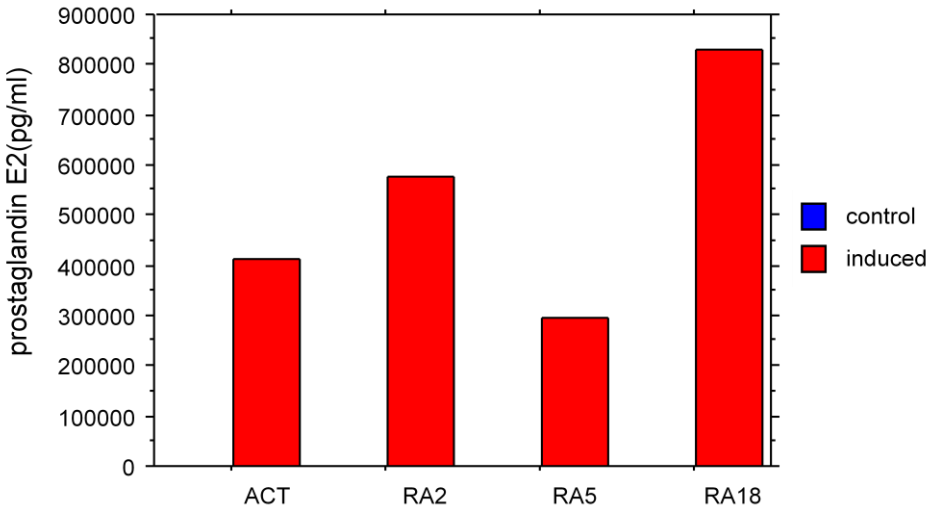
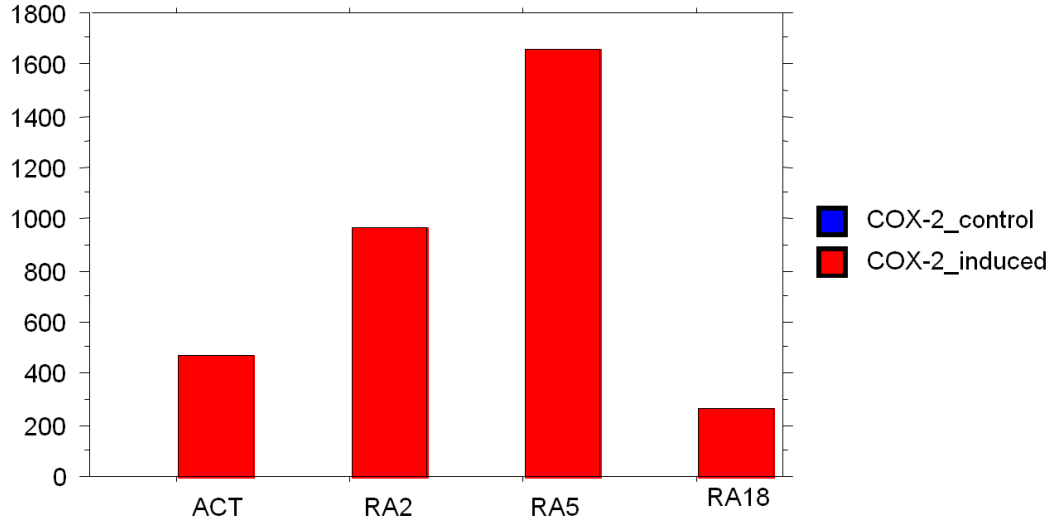


Figure 3. PGE2 levels in supernatants from RA synovial fibroblasts before and after stimulation with IL-1 and TNF. RASF are obtained from 4 patients (Act, RA2, RA5 and RA18). Control concentration of PGE2 was too low to see in the graph. The concentrations of PGE2 were determined by EIA

A



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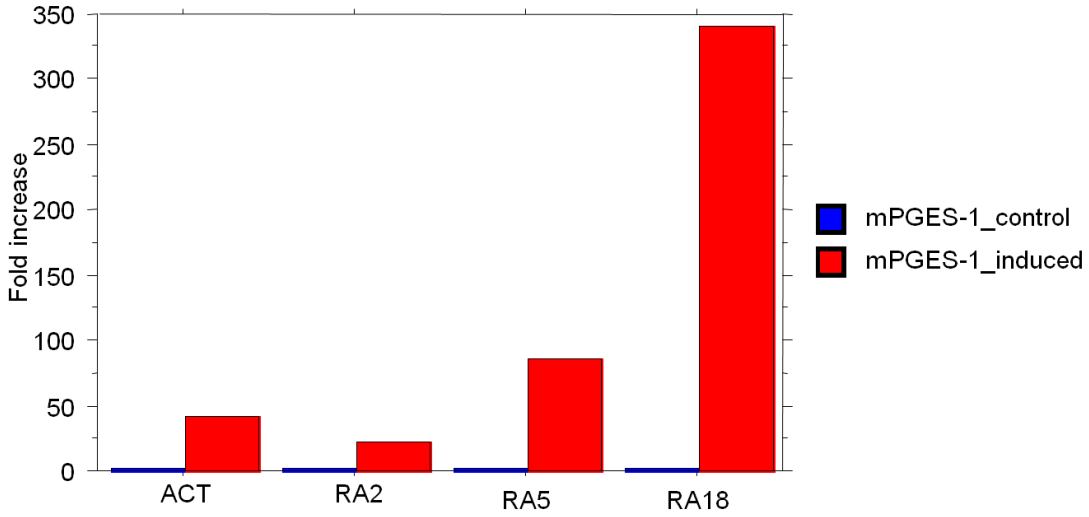
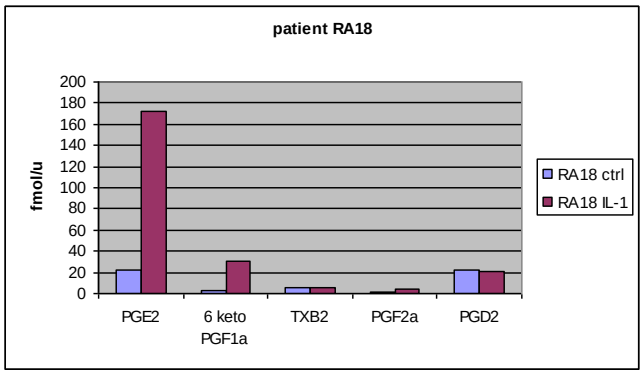


Figure 4. The COX-2 (A) and mPGES-1 (B) mRNA expression before and after stimulation with IL-1β and TNFα. Real- time PCR was used to validate gene expression.

The production of prostanoid was measured in supernatants of synovial fibroblasts from 3 patients before and after stimulation with IL-1β and TNFα and treatment with inhibitors. Analysis of prostanoid profile in cell supernatants using liquid chromatography – mass spectrometry (LC-MS) method was performed by another researcher in our group. In this experiment only three patients were analyzed (Figure 5). Under controlled conditions synovial fibroblasts produced low levels of PGE2, 6 keto-PGF1α (stable metabolite of PGI2), PGD2, PGF2α and TXB2 (stable metabolite of TXA2). Induction with IL-β and TNFα resulted in significant increase of PGE2 and PGI2 production in the cells from all patients, while the levels of other prostanoid were not significantly changed (Figure 5A, B and C). Thus in the following experiments we focused on analysis of these two prostaglandins, PGE2 and PGI2.

In the separate experiment we have analyzed prostanoid profile in supernatants from synovial fibroblasts using two different methods, separation lipid extracts with LC-MS and analysis with EIA. We compared production of PGE2 and PGI2 measured by LC-MS and EIA (Figure 6 and Figure 7). Since only three patients were included in LC-MS, in the Figure 7 there are three patients. The patterns of PGE2 and PGI2 production under different conditions measured by two methods were similar. PGE2 levels in cell supernatants were significantly increased after stimulation with pro-inflammatory cytokines and were suppressed by inhibitors (Figure 6A and Figure 6B). The inhibitor B showed higher efficiency of PGE2 suppression. In contrast, both inhibitors A and B increased PGI2 production. Therefore, inhibition of mPGES-1 increases availability of the common precursor PGH2 to the other PG synthases and redirect prostanoid production towards PGI2 (Figure 7A and Figure 7B). A shift from PGE2 production to PGI2 production may change the functional activity of cells, and should be avoided or reduced if possible. Particularly, recently studies based on PGI2 receptor deficient mice showed that PGI2 is also a significant contributor to the inflammation process [14]. Results from both LC-MS and EIA analysis have shown that the inhibitor B increased PGI2 production to a less extent than the inhibitor A, while still providing efficient suppression of PGE2. Therefore, we focused on the inhibitor B in our further investigation.

A



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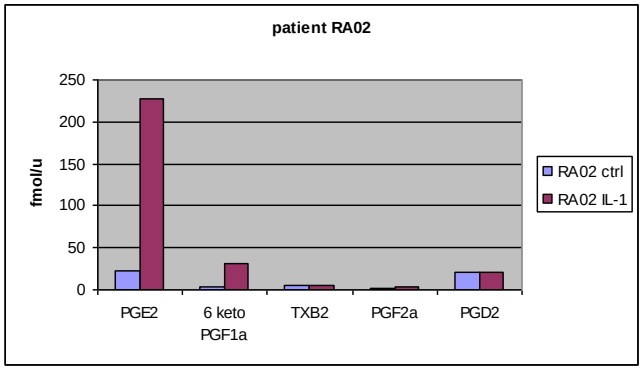

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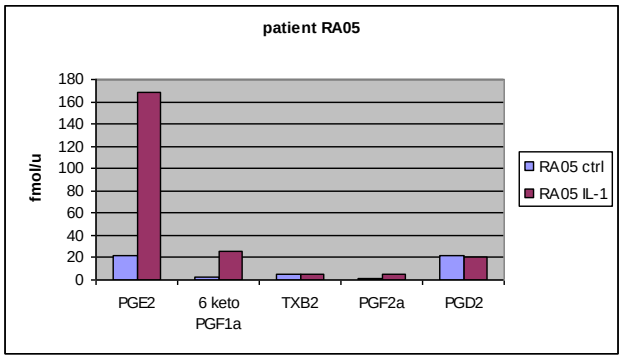
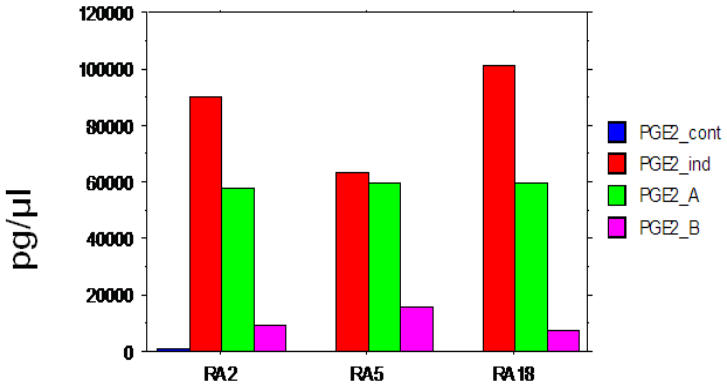


Figure 5 Production of prostanoid by synovial fibroblasts measured by LC-MS. Synovial fibroblasts from three RA patients (A, B and C) were stimulated by using IL-1β and TNFα.

A



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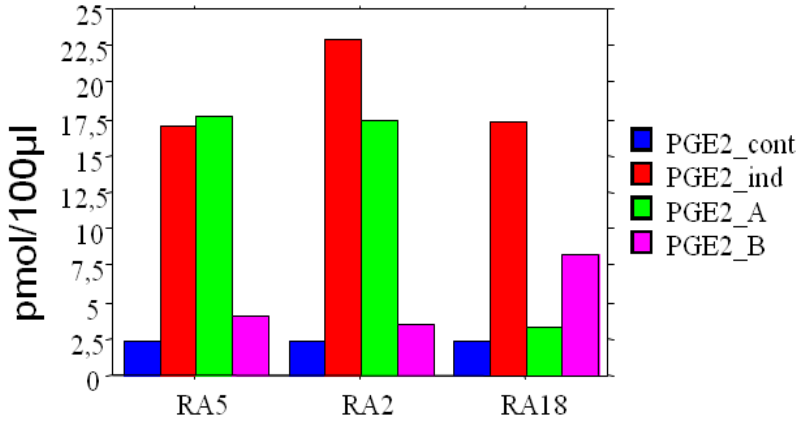
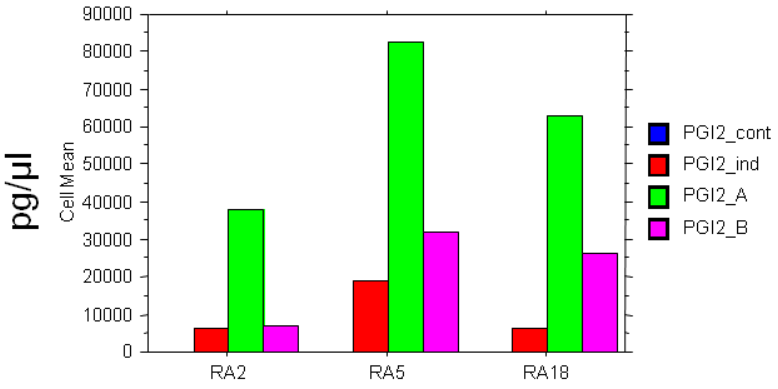


Figure 6. Production of PGE2 by synovial fibroblasts measured by EIA (A) and LC-MS (B). Synovial fibroblasts were obtained from 3 RA patients (RA2, RA5, RA18) and stimulated without (control) or with IL-1b and TNF-a and treated with inhibitor A or inhibitor B. Because only three patients were analyzed using LC-MS (B), in A there are also only three patients. It showed that inhibitor B had more effect on suppressing PGE2.

A



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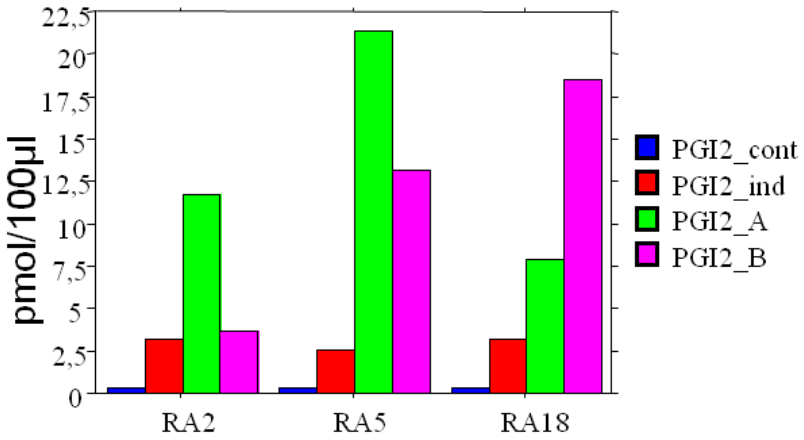


Figure 7. Production of PGI2 by synovial fibroblasts measured by EIA (A) and LC-MS (B). Synovial fibroblasts were obtained from 3 RA patients (RA2, RA5, RA18) and stimulated without (control) or with IL-1b and TNFa and treated with inhibitor A or B. Inhibitor B increased PGI2 less than inhibitor A. So inhibitor B is more promising.

2. Induction of mRNA expression of pro-inflammatory molecules in RA synovial fibroblasts by IL-1β and TNFα.

In initial experiments we analyzed primer specificity, real-time PCR efficiency and linearity for IL-6, IL-8, IL-23p19, MMP-1, MMP3, VEGF-A, mPGES-1 and COX-2. The specificity of amplification reaction was confirmed using 2% agarose gel electrophoresis. All amplicons had a single band of expected size confirming that we got the correct products of real time PCR (IL-6 211bp, TNF 114 bp, IL-8 170bp, IL-23p19 114 bp, MMP-1 123bp, VEGF-A 146 bp, MMP-3 122bp, mPGES-1 137bp, GAPDH 91bp and COX-2 146 bp) (Figure 8).

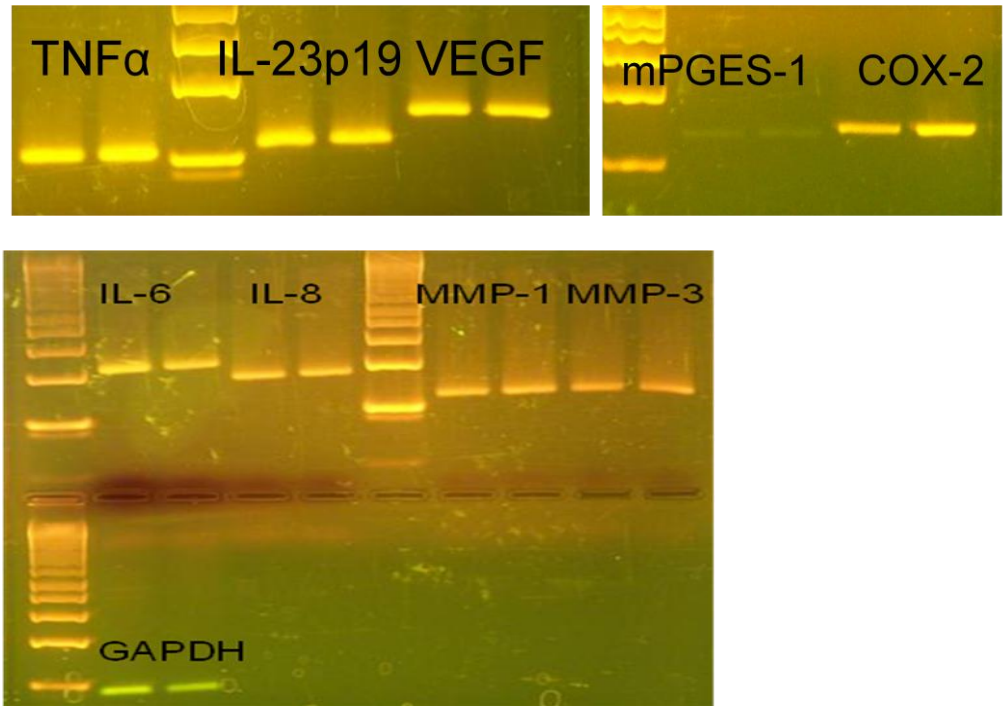


Figure 8. Agarose gel electrophoresis shows a single band of expected size for each PCR product. Expected size of different molecules: TNF is 114bp, IL-23p19 is 114bp, VEGF is 146bp, mPGES-1 is 137bp, COX-2 is 146bp, IL-6 is 211bp, IL-8 is 170bp, MMP-1 is 123bp, MMP-3 is 122, GAPDH is 91bp.

In addition, melting curve analysis performed after final amplification period showed a single peak for all PCR assays, confirming the specificity of amplification (Figure 9).

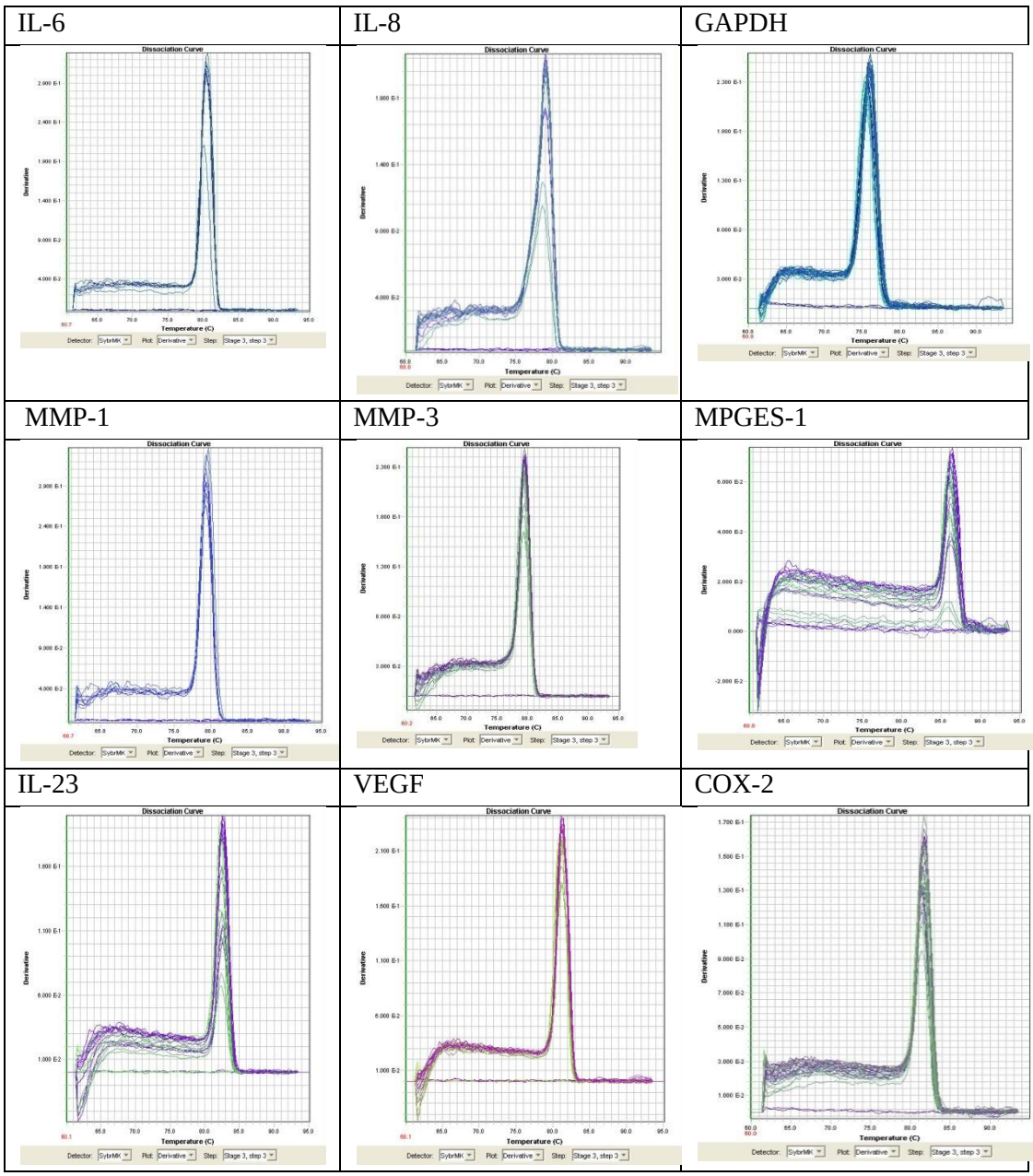


Figure 9 Dissociation curves of all PCR products, each graph showed only one significant peak. The results confirmed the specificity of amplification.

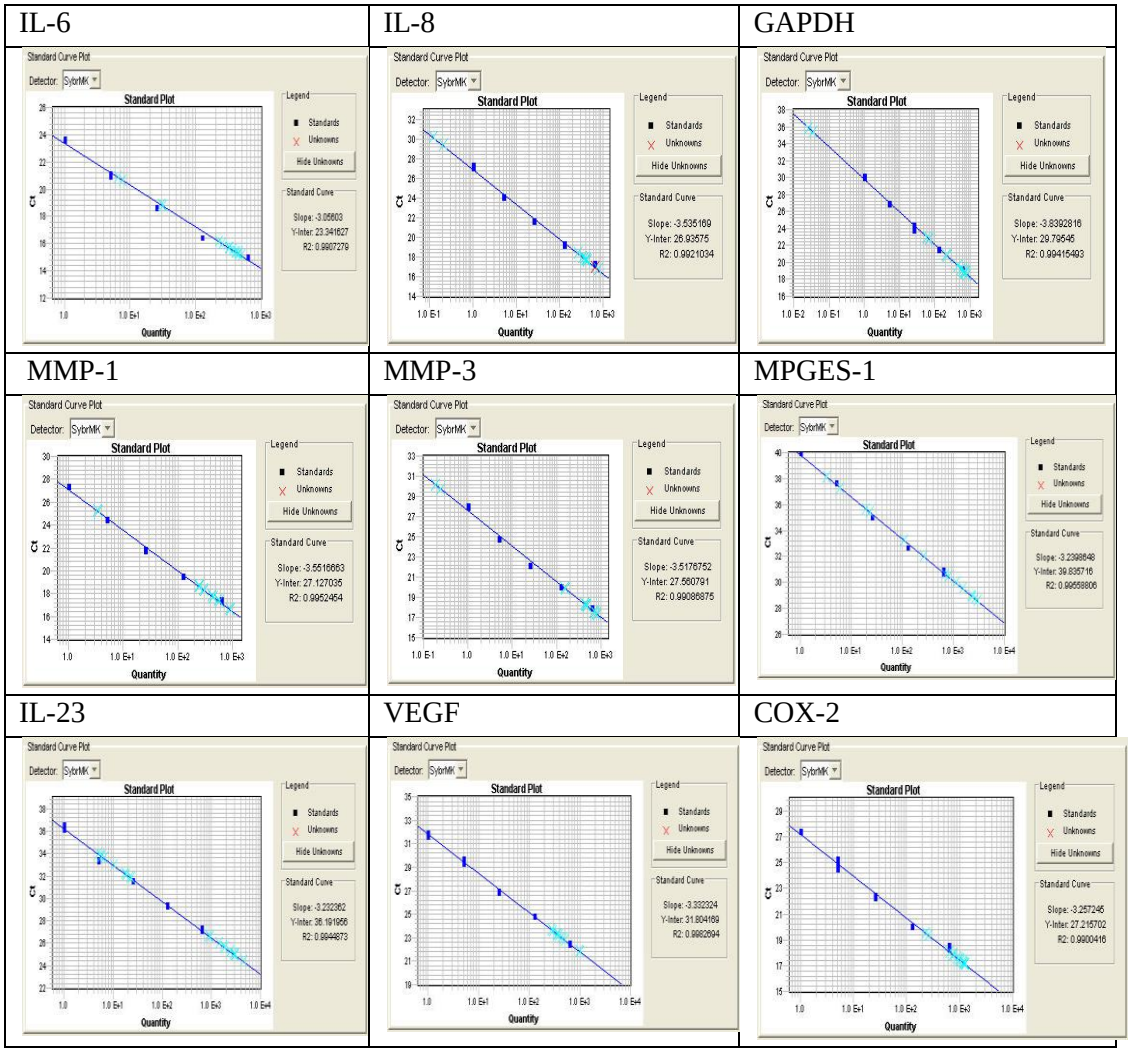


Figure 10 Calibration curves for each PCR assay, the correlation coefficient of each curve is 0.99.

To test for linearity of PCR reaction we generated calibration curves using cDNA pulled from different experiments and serial 5-fold dilutions (Figure 10). The correlation coefficients of the calibration curves were 0.99. From the calibration curves, we computed the efficiency of each PCR reaction, defined as $10^{-(1/slope)}$-1, that was close to 0.9 (Table 3). If the efficiency is more than one we assume the PCR reaction efficiency is one. There are two ways to calculate fold increase, by using calibration curve or by approximation method delta- delta Ct. We compared these two ways and got very similar patterns in the 11 PCR experiments. The delta-delta Ct

method was used in the remaining experiments since it is less costly and gives almost the same accuracy.

Table 3 PCR reaction efficiency determined by slop of calibration curve (Figure 9)

	IL-6	IL-8	MMPS-1	MMPS-3	mPGES-1	IL-23p19	VEGF	GAPDH	COX-2
Slope	-3.05	-3.53	-3.55	-3.51	-3.23	-3.23	-3.33	-3.38	-3.26
Reaction efficiency	1,12	0.92	0.91	0.92	1.03	1.03	1.00	0.98	1.03

In the next experiments we quantified mRNA expression of inflammatory molecules in RA synovial fibroblasts from four RA patients. Induction of synovial fibroblasts with IL-1β and TNFα was performed for 24 hours. mRNA expression of IL-6, IL-8, IL-23p19, MMP-3, MMP-1 and VEGF was strongly up-regulated (Figure 11). The variations are rather high since the patient group consists of only four patients. mRNA expression of IL-8 and MMP-3 has the strongest response, while mRNA expression of MMP-1 and VEGF was increased much less.

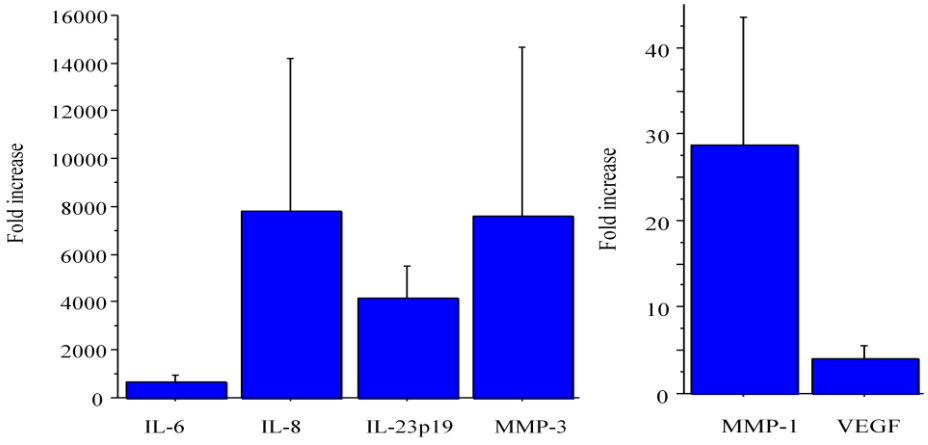


Figure 11. Up-regulation of gene expression of the pro-inflammatory mediators after induction by IL-1β and TNFα. The graphs show the average values and variations (standard deviation) of fold increase.

3. The effects of mPGES-1 inhibitor B on prostaglandin production in RA synovial fibroblasts.

Synovial fibroblasts from the four RA patients were induced with cytokines IL-1β and TNFα and simultaneously treated with inhibitor B and specific COX-2 inhibitor NS-398. We measured the production of PGE2 in supernatant from cells by using Enzyme Immunoassay. Production of PGE2 was significantly up-regulated by cells from all patients with some inter-individual variations (Figure 3). The highest production was observed in patients RA18, which had almost 3 fold higher PGE2 levels compared to patient RA5 and twice more than patient ACT (Table 4). The inhibitor B strongly suppressed induced PGE2 production by cells though to different extent in different patients. However specific COX-2 inhibitor reduced PGE2 production even more effectively than inhibitor B.

Table 4. Production of PGE2 by RA synovial fibroblasts from 4 RA patients induced with IL-1β and TNFα and treated with mPGES-1 inhibitor B and COX-2 inhibitor NS-398

PGE2, pg/ml	RA 2	RA 5	RA 18	RA Act
Control	175	84	67	127
Induced	574280	295259	829159	414441
Induced + inhibitor B	64871	12139	3468	11634
Induced +NS-398	1107	1568	317	NA

To normalize for inter-individual variations in PGE2 production we expressed the PGE2 levels in supernatants from synovial fibroblasts induced with IL-1β and TNFα as 100 % (Figure 12). We can see that although mPGES-1 inhibitor B suppressed PGE2 production remarkably (by 89%-99.6% in different patients), specific COX-2 inhibitor NS-398 down-regulated PGE2 production even more efficiently (by 99.5-99.9%)

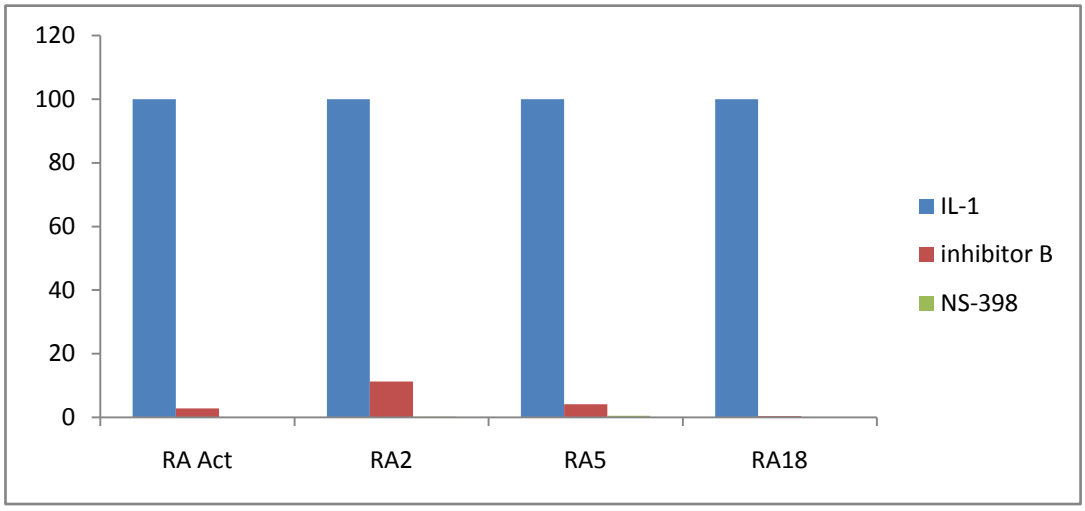


Figure 12 The normalized PGE2 production by synovial fibroblasts from 4 patients induced by IL-1β and TNFα and treated with inhibitor B and NS-398. Inhibitor NS-398 could almost completely suppressed PGE2 production (by 99.5%-99.9%), so PGE2 production by using NS-398 inhibitor is not visible on this scale.

4. The effect of mPGES-1 inhibitor on gene expression of inflammatory cytokines and mediators by synovial fibroblasts

The effects of inhibitors on mRNA expression of inflammatory molecules are presented in two graphs (Figure 13). The top graph presents the fold increase in mRNA expression of the respective inflammatory molecules for each patient. In order to normalize for inter-individual difference in mRNA levels the mRNA fold increase in the cells induced with IL-1β and TNFα was expressed as 1.

The botten graph presents the normalized levels of mRNA in stimulated cells and relative increase and decrease of mRNA expression by the inhibitors. Results are expressed as box plots that indicate median and lower and upper quartiles (25th and 75th). Variations are rather high because data include the values from only four patients.

In synovial fibroblasts from patient RA ACT induction with pro-inflammatory cytokines caused only a weak increase in IL-6 mRNA expression, which was not changed after the treatment with both inhibitors. For the other three patients, inhibitor A reduced the expression of IL-6 mRNA, although to a different extent. The most pronounced suppression of IL-6mRNA was observed in the cells from patient RA18 (blue one). The inhibitor B reduced mRNA expression of IL-6 in synovial fibroblasts

from two patients (RA ACR, RA 2 and RA18) while in the cells from patient RA 5 the expression was increased. In 3 out of 4 patients (RA2, RA5 and RA18) mRNA expression was down-regulated by Inhibitor A. (Figure 13A).

Gene expression of IL-8 was up-regulated for all patients remarkably induced by IL-1β and TNFα in RA SF. RA 18 (blue) had most pronounced gene expression of IL-8 compared to other three patients. However, all of them had higher level compared to other mediators (Table 5). Both inhibitor A and B suppressed expression of IL-8 in RA2 (red) and RA18, but they haven´t effect on gene expression of IL-8 in RA ACT (pink) and increased gene expression of RA5 (green) significantly. The bottom graph (Figure 13B) shows that inhibitor A and B did not change IL-8 mRNA expression.

After induction by pro-inflammatory cytokines gene expression of MMP-1 was not so remarkable increased in RA2 (red) and ACT (pink). For RA18 (blue) and RA5 (green) MMP-1 mRNA expression was increased. After treatment using inhibitor A MMP-1 was not changed noticeably. However expression of MMP-1 in RA5 inhibited by B was up-regulated noticeably in the top graph. As seen in graph inhibitor B increased expression of the gene in only one patient (Figure 13C).

Gene expression of MMP-3 after stimulation in RA ACT (pink) and RA2 (red) was not changed noticeably. However, expression was increased significantly in both RA18 (blue) and RA5 (green). So our inhibitors, both A and B did not have any effect on ACT and RA2. Gene expression level in RA18 was reduced by both inhibitors. In RA5, inhibitor A did not influence too much while inhibitor B increased the level almost more than two times. The bottom graph shows that inhibitor A and B did not have a noticeable effect on the MMP-3 mRNA expression. (Figure 13D)

Gene expression of VEGF was increased in RA18 (blue), RA2 (red) and RA ACT (pink). After treatment using inhibitor A and B gene expression level of VEGF was increased in ACT, RA2 and RA18. RA ACT has been up-regulated almost three times more by both inhibitors (Table 5). The level in RA 18 and RA2 was increased after treatment with two inhibitors. Cells from RA5 were not induced by IL-1β and TNFα, which might possibly lead to no effect by both inhibitors. From graph on the bottom side, inhibitor A increased VEGF mRNA in 2 out of 4 patients and inhibitor B up-regulated 3 out of 4 patients. (Figure 13E)

Induction with IL-1β and TNFα resulted in strong up-regulation of IL-23p19 mRNA expression in SF from different patients. Inhibitor B could suppress effectively gene expression of IL-23 in all patients, while inhibitor A decreased expression level in 2 out of 4 patients and inhibitor B in the cells from all patients. In the bottom graph it

can be seen that both inhibitor blocked IL-23p19. Inhibitor B reduced the gene expression with low probability distribution. (Figure 13F)

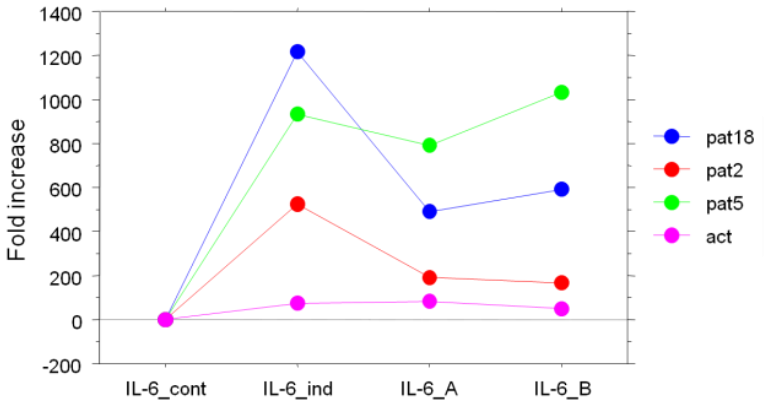
Table 5 shows effect of inhibitor B on each cytokine of different patients. The value is calculated by approximate approach $2^{\Delta\Delta Ct}$, and the control condition is one.

Table 5. Fold increase of each inflammatory cytokines by RA SF from 4 RA patients induced with IL-1β and TNFα and treated with mPGES-1 inhibitor B

		RA ACT	RA 2	RA 5	RA 18
IL-6	Induced	77,9	523	932	1219
	Inhibitor B	50,41	168,66	1030,28	588,63
IL-8	Induced	309	3408,11	765,9	26752,1
	Inhibitor B	335,85	852,12	2036,82	16171,97
MMP-1	Induced	2,17	5,03	61,31	46,31
	Inhibitor B	6,22	4,63	144,12	37,90
MMP-3	Induced	45,93	387,88	1320,93	28688,35
	Inhibitor B	61,41	169,71	2834,01	16044,35
VEGF	Induced	2,403	4,046	1,2	8,503
	Inhibitor B	5,86	5,80	1,58	11,00
IL-23	Induced	1101,02	5365,65	7355,16	2756,13
	Inhibitor B	396,02	3292,08	4210,59	278,55

A. IL-6

A-1



A-2

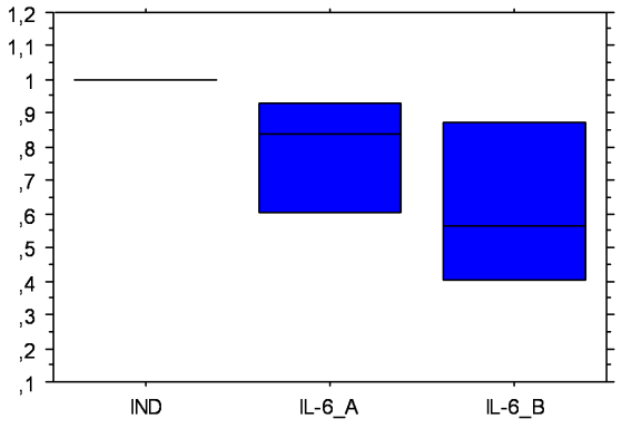
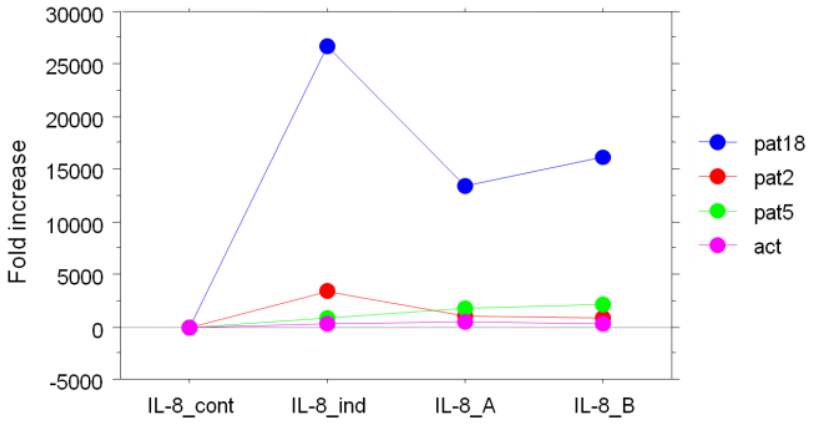


Figure 13 A A-1 presents the fold increase of IL-6 mRNA expression for each patient. A-2 presents the normalized levels of mRNA in stimulated cells and relative increase and decrease of mRNA expression by the inhibitors. Inhibitor A reduced the expression of IL-6 mRNA, although to a different extent. The most pronounced suppression of IL-6mRNA was observed in the cells from patient RA18 (blue one). The inhibitor B reduced mRNA expression of IL-6 RA ACR, RA 2 and RA18 while in the cells from patient RA 5 the expression was increased.

B. IL-8

B-1



B-2

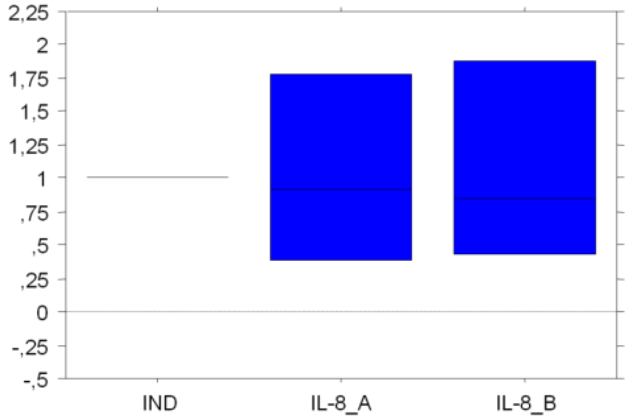
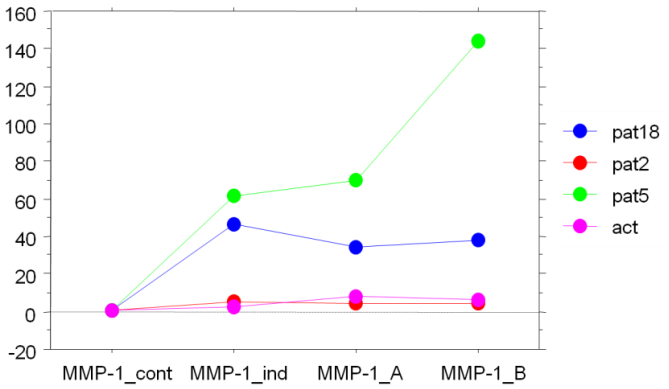


Figure 13B B-1 presents the fold increase of IL-8 mRNA expression for each patient. B-2 presents the normalized levels of mRNA in stimulated cells and relative increase and decrease of mRNA expression by the inhibitors. Both inhibitor A and B suppressed expression of IL-8 in RA2 (red) and RA18, but they have no effect on gene expression of IL-8 in RA ACT (pink) and increased gene expression of RA5 (green) significantly.

C. MMP-1

C-1



C-2

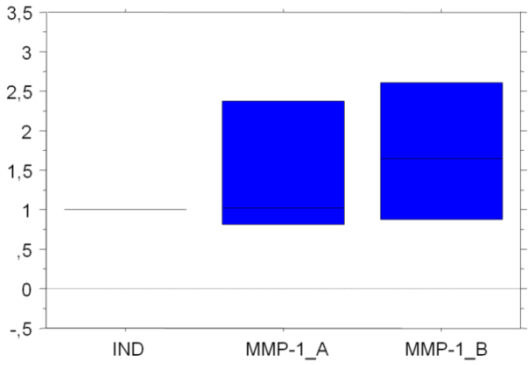
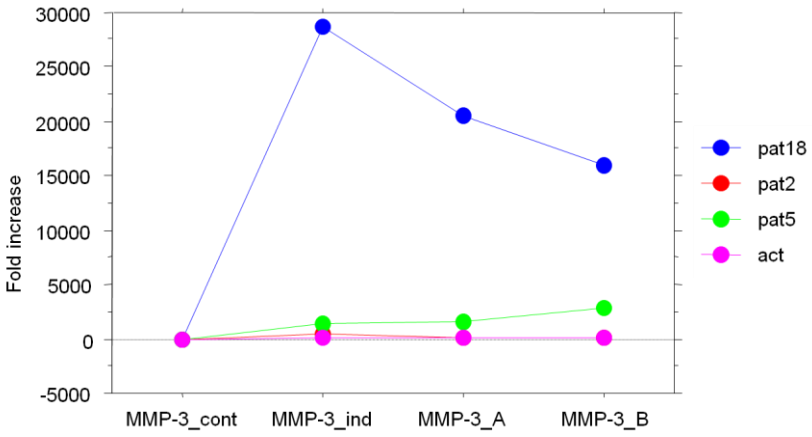


Figure 13 C C-1 presents the fold increase of MMP-1 mRNA expression for each patient. C-2 presents the normalized levels of mRNA in stimulated cells and relative increase and decrease of mRNA expression by the inhibitors. After induction by pro-inflammatory cytokines gene expression of MMP-1 was not so remarkable increased in RA2 and ACT. For RA18 and RA5 MMP-1 mRNA expression was increased. MMP-1 was not changed inhibitor A noticeably. However expression of MMP-1 in RA5 inhibited by B was up-regulated noticeably in C-1. As seen in C-2 inhibitor B increased expression of the gene in only one patient

D. MMP-3

D-1



D-2

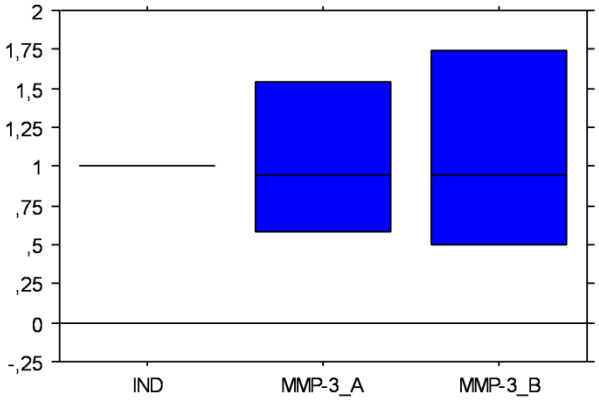
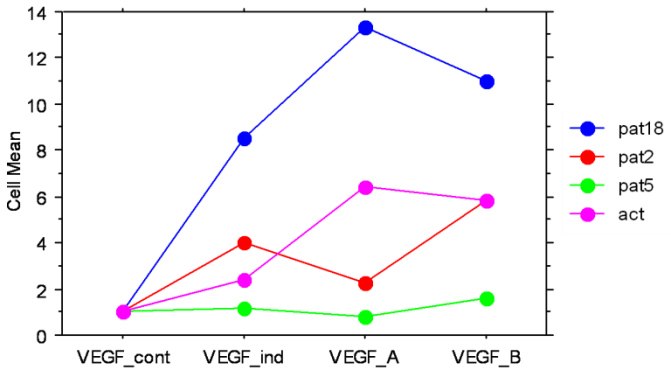


Figure 13D D-1 presents the fold increase of MMP-3 mRNA expression for each patient. D-2 presents the normalized levels of mRNA in stimulated cells and relative increase and decrease of mRNA expression by the inhibitors. In D-1, both A and B did not have any effect on ACT and RA2. Gene expression level in RA18 was reduced by both inhibitors. In RA5, inhibitor A did not influence too much while inhibitor B increased the level almost more than two times. In D-2 inhibitor A and B did not have a noticeable effect on the MMP-3 mRNA expression.

E. VEGF

VEGF-1



VEGF-2

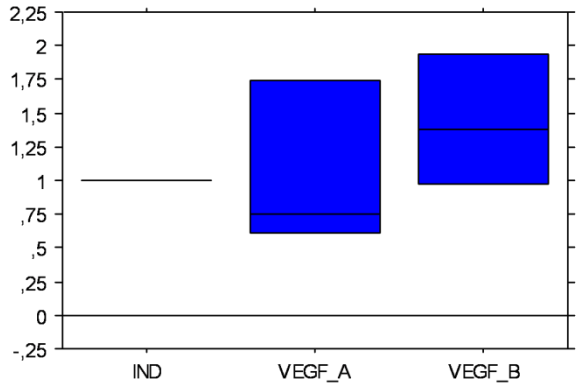
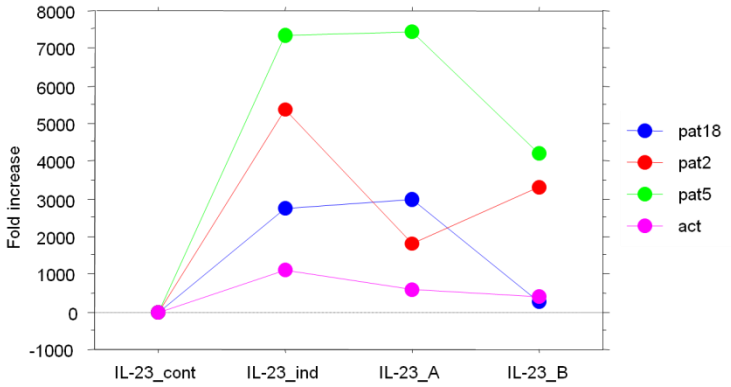


Figure 13E E-1 presents the fold increase of VEGF mRNA expression for each patient. E-2 presents the normalized levels of mRNA in stimulated cells and relative increase and decrease of mRNA expression by the inhibitors. Cells from RA5 were not induced by IL-1β and TNFα, which might possibly lead to no effect by both inhibitors. After treatment using inhibitor A and B gene expression level of VEGF was increased in ACT, RA2 and RA18. In E-2 inhibitor A increased VEGF mRNA in 2 out of 4 patients and inhibitor B up-regulated 3 out of 4 patients.

F. IL-23p19

IL-23p19-1



IL-23p19-2

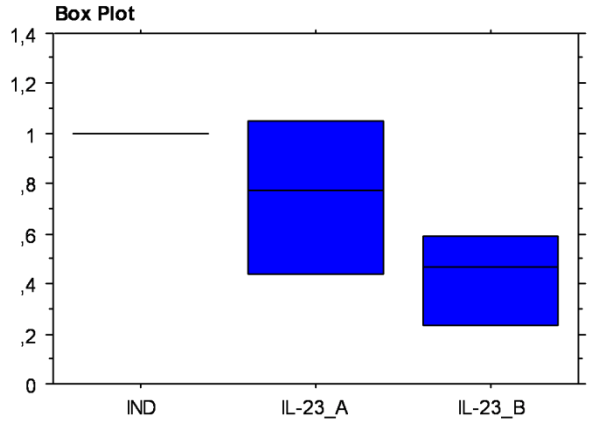


Figure 13F F-1 presents the fold increase of IL-23p19 mRNA expression for each patient. F-2 presents the normalized levels of mRNA in stimulated cells and relative increase and decrease of mRNA expression by the inhibitors. Inhibitor A decreased expression level in 2 out of 4 patients and inhibitor B in the cells from all patients. In F-2 it can be seen that both inhibitor blocked IL-23p19

Figure 13 Two graphs are presented for each mediator. **In the top one the results were plotted on each inflammatory mediator for all patients using the two inhibitors. Each line is one patient. In bottom graphs, the data was normalized with respect to the induced results, and the distribution of relative increase and decrease of the two inhibitors was plotted. Cont and IND present Control resp. Induced.**

Inhibitor B suppressed inflammatory PGE2 production more effectively than inhibitor A (Table 4 and Figure 6). From the experiments, inhibitor B more efficiently suppressed PGE2 with less increase in PGI2 (Figure 6 and Figure 7), compared to inhibitor A. Inhibitor B suppressed IL-23p19 in all patients and IL-6 in three out of four patients. Therefore, inhibitor B looks more promising. Effects of NS-398 on these important inflammatory cytokines and mediators were included in this study. The effects on mRNA expression of biomarkers by either inhibitor B or NS-398 are shown in the graphs (Figure 14). The graphs show the average values and variations (SD) of fold increase after normalization based on biomarkers without inhibitor treatment. The induced items in the graph are one. From the graphs, we can see that gene expressions of VEGF and MMP-1 were not changed by both inhibitor B and NS-398, (Figure 14). NS-398 decreased MMP-3, IL-8, IL-23p19 and IL-6 mRNA expression. Inhibitor B decreased only IL-23p19 and IL-6 mRNA expression while it did not change expression of IL-8 and MMP-3. While inhibitor B seems to be less efficient in the inflammatory biomarker suppression compared to NS-398. The lower efficiency of mPGES-1 inhibitor B compared to COX-2 inhibitor might be explained by several reasons. The inhibitor B that we used in this study is still under development. It is not as mature as known COX-2 inhibitor. Firstly the stability of mPGES-1 inhibitor B is low, especially when it is solved in the room temperature. And solubility in the room temperature is not good enough. Secondly NS-398 could inhibit both PGE2 and PGI2. Meanwhile the increase of PGI2 production by mPGES-1 inhibitor B might contribute to cytokine production.

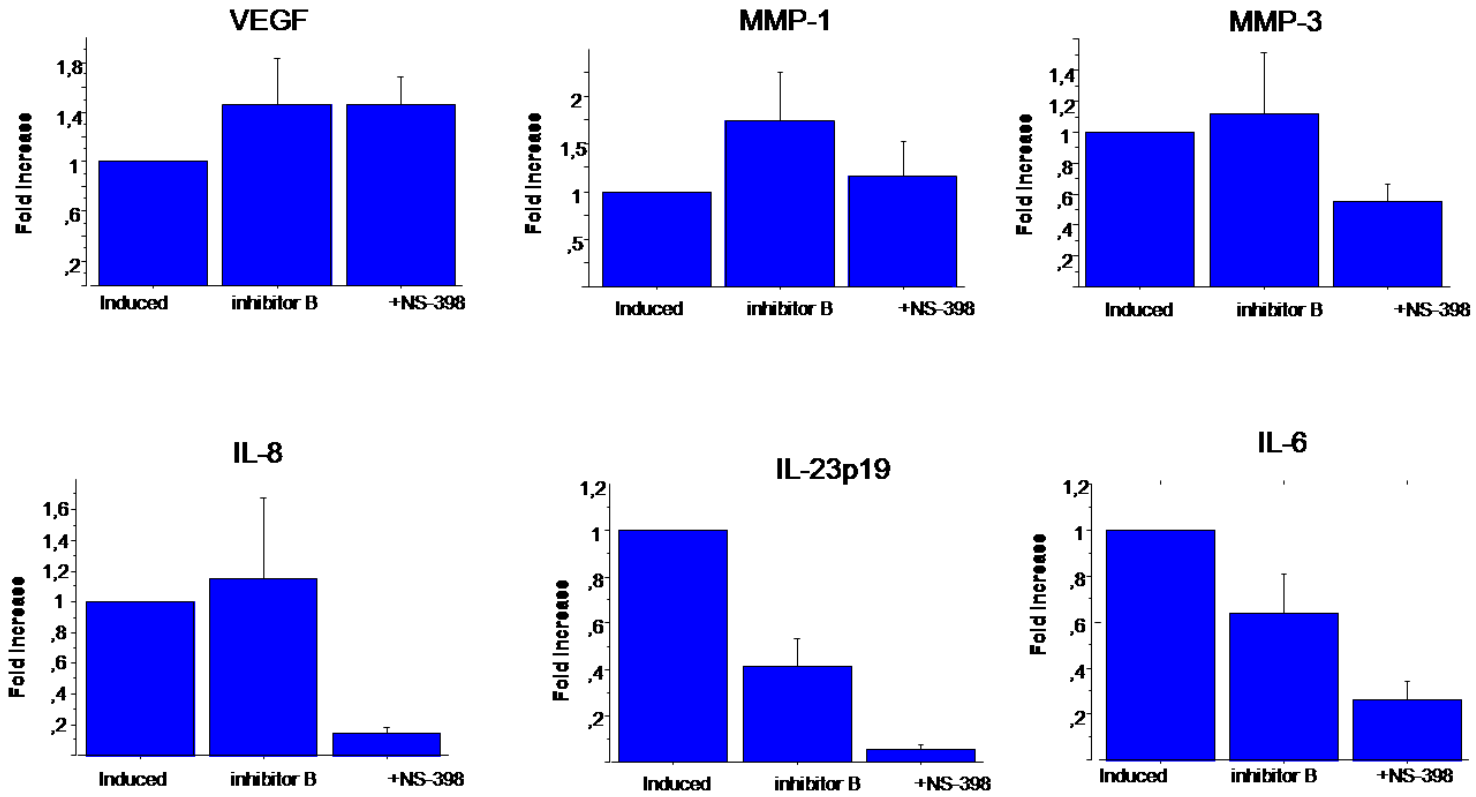


Figure 14: The graphs show the average values and variations of fold increase after normalization based on biomarkers without inhibitor treatment. The induced items in the graph are one.

Interestingly, mPGES-1 inhibitor B decreased IL-23p19 and IL-6 mRNA expression with low variation. IL-23 is a heterodimeric cytokine composed of two subunits, a p40 subunit also found in IL-12 and a specific p19 subunit. In this study we focus on the mRNA expression of IL-23p19. Subunit p40 was used to assay IL- 12. The receptor of IL-23 (IL-23R) is also heterodimer. One subunit is shared with IL-12 receptor (IL-12RB1). Thus many effects on IL-12 may be mediated by IL-23. The expression of IL-23R receptor on Th17 cells is induced by IL-6 and TGF-b. Th17 cells produce IL-17,a pro-inflammatory cytokine that stimulate the production of molecules such as IL-1, IL-6, TNFα and some chemokines contributing to inflammation [34] (Figure 15). Inhibitor B reduces IL-23 and IL-6 mRNA expression. That suggests IL-17 might be suppressed by the inhibitor as well.

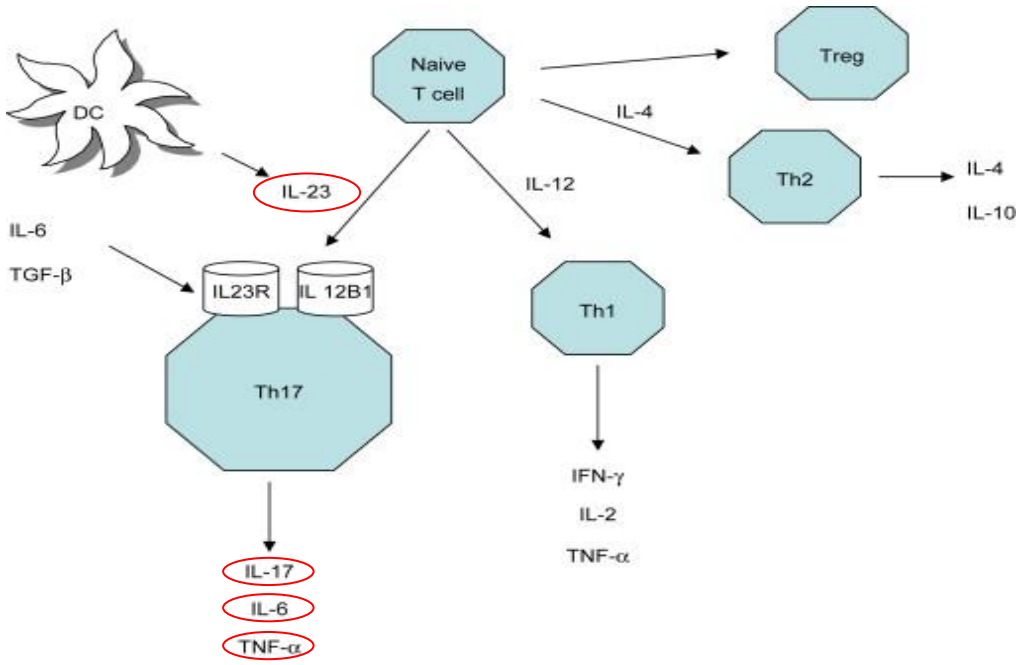


Figure 15: Role for cytokines in T-cell differentiation. DC, dendritic cell, and Treg, regulatory T cell. Modified from [35]

A possible future study is to look into the effect of selective inhibition of mPGES-1 on the protein level of inflammatory mediators and prostanoid profile. Furthermore, experiments should include more patients to increase the confidence level of the results.

Conclusions

MPGES-1 inhibitor B efficiently suppressed mPGES-1 activity and induced PGE2 production by RA synovial fibroblasts. Inhibition of mPGES-1 was followed by redirection of prostanoid production towards PGI2 biosynthesis. MPGES-1 inhibitor B did not affect mRNA expression of IL-8, MMP-1, MMP-3 and VEGF in induced RA synovial fibroblasts. In contrast, mRNA expression of IL-23p19 was down-regulated by mPGES-1 inhibitor B in all patients, and IL-6 mRNA expression was suppressed in three out of four patients. These results indicate that mPGES-1 inhibition may have beneficial effect on inflammation and joint destruction in RA patients.

References

1 Jakobsson, P.-J., Thorén, S., Morgenstern, R., and Samuelsson, B. Identification of human prostaglandin e synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proc. Natl. Acad. Sci. U.S.A.*, 96 (1999), 7220-7225.

2 Gilmour, R.S and Mitchell, M.D. Nuclear lipid signalling: novel role of eicosanoids. *Exp. Biol. Med* (2001), 226: 1-4.

3 Masahide, M. and Rikio, Y. Study of arachidonic acid pathway in human bladder tumor. *Substance Abuse: Research and Treatment* (2009:3).

4 Coleman, R.A., Smith, W.L., and Narumiya, S. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev.* (1994), 46:205-229.

5 Negishi, M., Sugimoto, Y., and Ichikawa, A. Prostaglandin E receptors. *J Lipid Mediat Cell Signal* (1995), 12:379-391.

6 Kobayashi, T. and Narumiya, S. Function of prostanoid receptors: studies on knockout mice. *Prostaglandins Other Lipid Mediat* (2002), 68-69: 557-573.

7 Samuelsson, B., Morgenstern, R., and Jakobsson, P.-J. Membrane Prostaglandin E Synthase-1: A Novel Therapeutic Target. *Pharmacol Rev* (2007), 59:207-224.

8 Agro, A., Langdon, C., Smith, F., and Richards, C.D. Prostaglandin E2 enhances interleukin 8 (IL-8) and IL-6 but inhibits GMCSF production by IL-1 stimulated human synovial ®broblasts in vitro. *J. Rheumatol.* (1996), 23: 862- 868.

9 Sheibanie, A.F., Tadmori, I., Jing, H., Vassiliou, E., and Ganea, D. Prostaglandin E2 induces IL-23 production in bone marrow-derived dendritic cells. *FASEB J* (2004), 8(11):1318-20.

10 Ben-AV, P., Crofford, L.J., Wilder, R.L., and Hla, T. Induction of vascular endothelial growth factor expression in synovial ®broblasts by prostaglandin E and interleukin-1: a potential mechanism for in¯ammatory angiogenesis. *FEBS Lett.* (1995), 372: 83- 87.

11 Attur, M.,Al-Mussawir, H.E., Patel, J., Kitay, A. Dave, M., Palmer, G.,Pillinger, M.H.,Abramson, S.B.. Prostaglandin E2 exerts catabolic effects in osteoarthritis cartilage: evidence for signaling via the EP4 receptor. *J Immunol* (2008), 181:5082-5088.

12 Molloy, E.S., Morgan, M.P., Doherty, G.A., McDonnell, B., O'Byrne, J., Fitzgerald, D.J., and McCarthy, G.M. Mechanism of basic calcium phosphate crystal-stimulated matrix metalloproteinase-13 expression by osteoarthritic synovial fibroblasts: inhibition by prostaglandin E2. *Ann Rheum Dis* (2008), 67: 1773-1779.

13 Aryeh, M. A. and Michael, H. P. The Role of the Synovial Fibroblast in Rheumatoid Arthritis. *Bulletin of the NYU Hospital for Joint Diseases* (2006), Volume 64, Numbers 1 & 2;20-24.

14 Friesen, R.W. and Mancini, J.A. Microsomal prostaglandin E2 synthase-1 (mPGES-1): a novel anti-inflammatory therapeutic target. *J Med Chem* (2008), 51(14):4059-67.

15 A. V. Sampey, S. Monrad, L. J. Crofford. Microsomal prostaglandin E synthase-1: the inducible synthase for prostaglandin E2. *Arthritis Research & Therapy* (2005), 7:114-117.

16 Jeehee, Y.,CHO, M.-L.,KIM, Y.-J.,HYE, S.Y.,PARK, S.-H.,JIN, C.-Z.,PAIK, D.-J., KIM, H.-Y.. New cyclooxygenase-2 inhibitor DFU regulates vascular endothelial growth factor expression in rheumatoid synoviocytes. *Immunology letters* (2005), vol. 96, no2, pp. 219-224.

17 Brentano, F., Ospelt, C., Stanczyk, J., Gay, R.E., Gay, S., and Kyburz, D. Abundant expression of the interleukin (IL)23 subunit p19, but low levels of bioactive IL23 in the rheumatoid synovium: differential expression and Toll-like receptor-(TLR) dependent regulation of the IL23 subunits, p19 and p40, in rheumatoid arthritis. *Ann Rheum Dis* (2009), 68:143-150.

18 Amir, F.S., Iman, T., Jing, H., E., Vassiliou, and Ganea, G. Prostaglandin E2 induces IL-23 production in bone marrow-derived dendritic cells. *The FASEB Journal* (2004), 18:1318-1320.

19 Nagase, H. and Woessner, J.F. Matrix metalloproteinases. *J. Biol. Chem* (1999),

274: 21491–21494.

20 Vincenti, M.P. and Brinckerhoff, C.E. Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors. *Arthritis Res.* (2002), 157–164.

21 Pillinger, M. H., Rosenthal, P. B., Tolani, S. N., Apsel, B., Dinsell, V., and Greenberg, J. Cyclooxygenase-2-derived E prostaglandins down-regulate matrix metalloproteinase-1 expression in fibroblast-like synoviocytes via inhibition of extracellular signal-regulated kinase activation. *J. Immunol.* (2003), 171: 6080–6089.

22 DeWitt, D.L. Prostaglandin endoperoxide synthase: regulation of enzyme expression. *Biochim Biophys Acta* (1991), 1083:121–134.

23 Crofford, L.J., Wilder, R.L., Ristimäki, A.P., Sano, H., Remmers, E.F., Epps, H.R., and Hla, T. Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1 beta, phorbol ester, and corticosteroids. *J Clin Invest* (1994), 93:1095–1101.

24 Kojima, F., Naraba, H., Sasaki, Y., and Okamoto R., Koshino T., Kawai S. Coexpression of microsomal prostaglandin E synthase with cyclooxygenase-2 in human rheumatoid synovial cells. *J Rheumatol.* (2002 Sep), 29(9):1836-42.

25 Dickman, A. and Ellershaw, J. NSAIDS: gastroprotection or selective COX-2 inhibitor? *PalliatiVe Med.* (2004), 18:275-286.

26 Grosser, T., Fries, S., and Fitzgerald, G.A. Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *J Clin Invest.* (2006), 116: 4-15.

27 Caldwell, B., Aldington, S., Weatherall, M., Shirtcliffe, P., and Beasley, R. Risk of cardiovascular events and celecoxib: a systematic review and meta-analysis. *J. R. Soc. Med* (2006), 99:132-140.

28 Trebino, C.E., Stock, J.L., Gibbons, C.P., Naiman, B.N., Wachtmann, T.S., Umland, J. P., Pandher, K., Lapointe, J.-M., Saha, S., Roach, M.L., Carter, D., Thomas, N. A., Durtschi, B.A., McNeish, J.D., Hambor, J.E., Jakobsson, P.-J., Carty, T.J.. Impaired inflammatory and pain responses in mice lacking an

inducible prostaglandin E synthase. *Proc. Natl. Acad. Sci. U. S. A. 100* (2003), 9044–9049.

29 Westman, M., Korotkova, M., af, Klint, E., Stark, A., Audoly, L.P., Klareskog, L., Ulfgren, A.K., Jakobsson, P.-J.. Expression of Microsomal Prostaglandin E Synthase in Rheumatoid Arthritis Synovium. *Arthritis Rheum.* (2004), 50;1774-1780.

30 Korotkova, M., Westman, M., Gheorghe, K. R., Klint, E.af., Trollmo, C., Ulfgren, A. K., Klareskog, L., Jakobsson, P.-J. Effects of antirheumatic treatments on the prostaglandin E2 biosynthetic pathway. *Arthritis & Rheumatism*, 52, 11 (2005), 3439 - 3447.

31 Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. Basic local alignment search tool. *Journal of molecular biology* (1990), 215(3):403-10.

32 Wang, C.T., Lin, Y.T., Chiang, B.L., Lin, Y.H., Hou, S.M. High molecular weight hyaluronic acid down-regulates the gene expression of osteoarthritis-associated cytokines and enzymes in fibroblast-like synoviocytes from patients with early osteoarthritis. *Osteoarthritis Cartilage* (2006), 14(12), 1237-1247.

33 Dussault, A.-A. and Pouliot, M. Rapid and simple comparison of messenger RNA levels using real-time PCR. *Biol Proced Online* (2006), 8: 1–10.

34 Fitch, E., Harper, E., and Skorcheva, I. Pathophysiology of psoriasis: recent advances on IL-23 and Th17 cytokines. *Curr Rheumatol Rep* (2007), 9:461e7.

35 Wendling, D. Interleukin 23: A key cytokine in chronic inflammatory disease. *Editorial / Joint Bone Spine 75* (2008), 517-519.

36 Ben-Av, P., Crofford, L.J., Wilder, R.L., and Hla, T. Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for in prostaglandin E and interleukin-1: a potential mechanism for inflammatory. *FEBS Lett* (1995), 372:83–7.

37 Probert, L., Plows, D., Kontogeorgos, G., and Kollias, G. The type I interleukin-1 receptor acts in series with tumor necrosis factor (TNF) to induce arthritis in TNF-transgenic mice. *Eur J Immunol* (1995), 25(6):1794-7.

38 Tamura, T., Shirai, T., Kosaka, N., Ohmori, K., and Takafumi, N.

Pharmacological studies of diacerein in animal models of inflammation, arthritis and bone resorption. *Eur J Pharmacol* (2002), 448:81–87.

39 Kamei, D., Yamakawa, K., Takegoshi, Y., Mikami-Nakanishi, M., Nakatani, Y., Oh-Ishi, S., Yasui, H., Azuma, Y., Hirasawa, N., Ohuchi, K., Kawaguchi, H., Ishikawa, Y., Ishii, T., Uematsu, S., Akira, S., Murakami, M., Kudo, I.. Reduced pain hypersensitivity and inflammation in mice lacking microsomal prostaglandin e synthase-1. *J Biol Chem.* , 279(32) (August 2004), 33684-33695.

40 Saiki, R.K., Gelfand, D.H., Stoffel, S.,Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A.. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* (1988), 239(4839):487-91.