The Active form of Leflunomide, HMR1726, Facilitates TNF- α and IL-17 Induced MMP-1 and MMP-3 Expression

¹Dorothea Alexander, ²Björn Friedrich ¹Tanja Abruzzese, ³Bernd Gondolph-Zink, ¹Nikolaus Wülker and ¹Wilhelm K. Aicher

¹Center for Orthopaedic Surgery, University Hospital Tuebingen, Research Laboratory, ²Department of Internal Medicine IV, University Hospital Tuebingen, ³Sana-Klinik Zollernalb GmbH, Albstadt

Key Words

Leflunomide • TNF- α • IL-17 • Metallo-proteinases • Synovial fibroblasts • MAP kinases

Abstract

Background/Aims: To elucidate the influence and mode of action of HMR1726 (the active metabolite of leflunomide) on TNF- α and IL-17 activated metalloproteinases expression in synoviocytes. **Methods:** Synovial fibroblasts from RA and OA patients were stimulated with both cytokines and altered gene expression in the presence or absence of leflunomide was detected by microarray analyses and quantitative RT-PCR. Protein expression was detected by western blotting and commercial ELISAs. **Results:** Microarray analyses revealed that the addition of HMR1726 (50 μ M) to TNF- α and IL-17-stimulated synoviocytes induced gene expression of metallo-proteinases, especially MMP-1 and -3 in comparison to activated synoviocytes in the absence of leflunomide. To confirm these data, we examined the influence of different concentrations of HMR1726 in synoviocytes from further 5 OA and 7 RA patients by quantitative PCR. HMR1726 gradually induced MMP-1 and MMP-3 gene expression in a dose-

dependent manner. Similar results were observed on protein levels. Examination of signal transduction pathways participating in the regulation of leflunomide-induced MMPs expression showed that the mechanism underlying activation of MMP-1 is in part p38- and activation of MMP-3 was MEK1/2-dependent. **Conclusion:** Leflunomide was not able to abolish expression of metallo-proteinases in synoviocytes activated with TNF- α and IL-17.

Copyright © 2006 S. Karger AG, Basel

Introduction

Rheumatoid arthritis is characterized by chronic inflammation of the synovium accompanied by destruction of cartilage that results from the release of matrix proteinases produced by the synoviocytes. Inflammation and subsequent destruction of joint tissue are initiated by the influx of mononuclear cells, which produce proinflammatory cytokines such as IL-1 β , IL-17 and TNF- α and activate locally the synovial fibroblasts.

Leflunomide is a disease modifying antirheumatic drug through its ability to slow the progression of RA.

The drug affects the T-cell activation and modulates T-cell progression [1-3] through inhibition of the enzyme dihydroorotate dehydrogenase (DHODH) that prevents activated lymphocytes from accumulating sufficient pyrimidines to support increased DNA synthesis [4-7]. This primary mode of action for the active form of leflunomide has been identified at low, therapeutically applicable doses. At higher doses, A771726 inhibits tyrosine kinases responsible for early T cell and B cell signaling in the G₀/G₁ phase of the cell cycle [8-10].

Since activated synovial fibroblasts are also involved in the pathogenesis of rheumatoid arthritis and leflunomide seems to improve the progression of RA, we investigated whether leflunomide is able to inhibit TNF- α and IL-17-mediated cellular responses by exact quantification of metalloproteinases expression using microarray analyses and quantitative real-time PCR. TNF- α and IL-17 were chosen, because of their simultaneous upregulation in the joint of RA patients [11-15] and because of their known additive cartilage and bone destructive effects [16, 17].

Materials and Methods

Synoviocytes cultures and RNA extraction

Synovial fibroblasts were isolated by collagenase type XI (Sigma) treatment from rheumatoid arthritis (RA, n = 11) and osteoarthritis (OA, n = 13) patients after surgical synovectomy. All RA patients fulfilled the diagnostic criteria of the American College of Rheumatology. The basic therapies of the nine RA patients included in this study are listed in table 1.

Cells were cultured in DMEM supplemented with 10% FCS and 100 U/ml penicillin/streptomycin. Synoviocytes from passages 3-5 were seeded into 250 cm² culture flasks (1x10⁶ cells/flask). After an overnight incubation period, synoviocytes were pre-incubated for two hours with the indicated concentrations of HMR1726 (Aventis Pharma, Frankfurt, Germany) before addition of pro-inflammatory cytokines TNF- α (1ng/ml, Roche Diagnostics, Mannheim, Germany) and IL-17 (10ng/ml, R&D Systems Wiesbaden, Germany).

From activated synoviocytes total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. First-strand cDNA for the real-time PCR experiments was synthesized by reverse transcription at 42°C in a 20 μ l reaction mixture containing 1 μ g of total RNA. Reactions were diluted to 100 μ l with DEPC treated water.

Microarray analyses

Microarray analyses were performed from total RNA extracted from cells activated for 48 hours with TNF- α and IL-17 in the absence (control) or in the presence of 50 μ M HMR1726 (treated sample). One microgram of total RNA was subjected to a linear amplification protocol. The amplified RNA from the

treated sample (TNF/IL-17/LEF 50) was labelled by reverse transcription with Cy5 fluorescence dye and the amplified RNA from the control (TNF/IL-17) with Cy3, respectively. The labelled samples were then hybridised to a human PIQORTM Immunology microarray (Memorec Biotec GmbH, Cologne, Germany). Each cDNA was spotted as quadruplicates on different positions of the array. In total two microarrays (1 RA, 1 OA) were performed in order to analyse the effect of HMR1726 on the gene expression profile. Local background was subtracted from the signal to obtain the net signal intensity and the ratio of Cy5/Cy3. The negative control for each array was computed as the mean of signal intensity of 4 spots representing hybridisation of salmon sperm and 4 spots representing buffer only. Normalized ratios are shown as Cy5 signal intensity divided by Cy3 signal intensity of the respective gene.

Quantitative real-time PCR

To quantitate mRNA expression, real-time PCR with the LightCycler System (Roche Diagnostics, Mannheim, Germany) was established. PCRs for GAPDH, MMP-1 and MMP-3 were performed in a final volume of 20 μ l containing 2 μ l cDNA, 2 μ l primer mix (commercial primer kits from Search LC, Heidelberg, Germany), 2 μ l DNA Master Sybr Green I mix (Roche Molecular Biochemicals, Mannheim, Germany) and 14 μ l DEPC treated water. Amplification of the target DNA was performed during 35 cycles of 95°C for 10s, 68°C for 10s and 72°C for 16s, each with a temperature transition rate of 20°C/s and a secondary target temperature of 58°C with a step size of 0.5°C. Melting curve analysis was performed at 95°C 0s, 58°C 10s, 95°C 0s to allow discrimination between unspecific primer dimers and specific PCR products. The amplified products were then separated on 1.5% agarose gels to confirm the expected size. The transcript levels of the housekeeping gene GAPDH were also determined for each sample using a commercial primer kit (Search LC, Heidelberg, Germany). Finally, results were calculated as a ratio of the target versus house keeping gene transcripts.

Enzyme immunoassay (ELISA)

The release of MMP-1 and MMP-3 in supernatants collected from TNF- α and IL-17 activated synovial fibroblasts in the presence or absence of leflunomide was measured by sandwich ELISAs (Amersham Pharmacia, Freiburg, Germany) according to the manufacturer's instructions.

MAP kinases inhibition assays

For MAP kinases inhibition assays, SB203580 (inhibitor of p38 kinase), U0126 (inhibitor of MEK1/2) and the inactive inhibitor SB202474 (Calbiochem, Darmstadt, Germany) were added in a concentration of 20 μ M to TNF- α and IL-17 activated synoviocytes in the absence or presence of 50 μ M HMR1726. Leflunomide and the kinase inhibitors were simultaneously given to the cell cultures two hours prior stimulation with both pro-inflammatory cytokines. Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. First-strand cDNA for the quantitative PCR was synthesized by reverse transcription at 42°C in a 20 μ l reaction mixture containing 1 μ g of total RNA.

Table 1. Age (years), rheumatoid factor (RF), sex and basic therapy (DMARD = disease modifying anti-rheumatic drugs) from the eleven rheumatoid arthritis (RA) patients included in this study. ne = not examined, NSAR = non-steroidal antirheumatics.

Patient	Age	RF	Sex	DMARD
RA 1	40	-	female	Hydroxychloroquin, Methotrexat
RA 2	56	+	female	Prednisolone
RA 3	56	+	male	Prednisolone, Leflunomide
RA 4	28	-	female	Hydroxychloroquin, Prednisolone, Sulphasalazin
RA 5	28	+	female	No basic therapy
RA 6	59	+	female	Prednisolone, Methotrexat
RA 7	61	+	female	Prednisolone
RA 8	67	+	female	Methotrexat, Leflunomide
RA 9	83	ne	female	Prednisolone
RA 10	69	-	female	NSAR
RA 11	75	-	male	NSAR, Corticosteroid

Western blot

For western blot analysis 2×10^6 cells were washed with ice-cold PBS and detached from flasks on ice using a cell scraper. After centrifugation (1400 rpm at 4°C) pelleted cells were dissolved in 80 µl lysis buffer (1% NP40; 150 mM NaCl; 50 mM Tris/HCL, pH 8 plus protease inhibitors (Complete, EDTA-free, Roche)) and were incubated overnight at -70°C. Cell debris were removed by centrifugation (Avanti™ 30 Beckman centrifuge, 20627g, 4°C, 20 min.) and protein concentrations in the supernatants were measured by a colorimetric assay (RD DC Protein Assay, Bio Rad) according to the manufacturer instructions. After addition of Laemmli sample buffer and denaturation at 95°C, a total amount of 50 µg of cellular extracts from the samples were separated by SDS-PAGE (10%) and transferred onto nitrocellulose membranes. The membranes were analyzed by immunoblotting using the anti phospho-p38 MAP kinase (Thr180/Tyr182) and the anti phospho-p44/42 MAP kinase (Thr202/Tyr204) specific primary antibodies (Cell Signaling Technology, Beverly, USA) overnight by 4°C. Binding of the primary antibodies was detected with peroxidase conjugated goat-anti rabbit secondary antibodies (Dianova, Hamburg, Germany) and visualized by the enhanced chemiluminescence method (Amersham Pharmacia Biotech, Freiburg, Germany).

Apoptosis and proliferation assays

Apoptosis was detected using a commercial Cell Death Detection ELISA (Roche, Mannheim, Germany). This photometric enzyme immunoassay is based on quantification of cytoplasmatic histone-associated DNA fragments after induced cell death. This allows specific determination of mono- and oligonucleosomes in culture supernatants or cell lysates.

For the examination of cell viability and proliferation, a commercially available kit was used (Biozol, Eching, Germany). This test is based on the reduction of tetrazolium salts into formazan derivatives by intact mitochondria.

Statistical analyses

Statistical analysis was performed using parametric *t*-tests for data with a normal distribution. A value of $p < 0.05$ was considered as significant (*), a value of $p < 0.01$ was considered as high significant (**). Data obtained were expressed as means $\pm$ standard deviation (SD).

Results

Microarray analysis - effects of HMR1726 on MMP mRNA expression

Microarray analyses were performed from TNF- α and IL-17 activated cells in the presence (treated sample, 1 RA/1 OA) or the absence (control, 1 RA/1 OA) of HMR1726 50µM (Fig. 1) on purpose to find genes which are influenced by leflunomide. Surprisingly, we found elevated levels of MMPs in the leflunomide treated sample.

In synoviocytes from one RA patient incubated with leflunomide MMP-1 gene expression was 4.7 ± 0.47 fold higher in comparison to those without HMR1726. Likewise, MMP-3 expression showed higher levels (2.75 ± 0.25) under the influence of leflunomide than the untreated control. Additionally, slightly elevated levels of MMP-2 (1.84 ± 0.17) and MMP-14 (2.01 ± 0.22) could be observed in the leflunomide-treated RA sample in comparison to the control. Microarray analyses in activated synoviocytes from one OA patient revealed a 4.05 ± 0.25 fold induction of MMP-1 and a 3.47 ± 0.3 fold elevation of MMP-3 m-RNA expression in the presence of HMR1726 50µM. No elevation of MMP-2

Fig. 1. Microarray analyses were performed from cells (1 RA and 1 OA patient) activated with TNF- α (1 ng/ml) and IL-17 (10 ng/ml) for 48 h (control) in comparison to TNF- α /IL-17 activated cells cultured in the presence of HMR1726 50 μ M (treated sample). Only one control is illustrated because both controls (1 RA and 1 OA patient) have the same value (=1). Each cDNA is spotted as quadruplicates on different positions on the microarray consequently the standard deviation of four ratios for the respective gene is shown.

Fig. 2. 2.1 Synoviocytes from 7 RA and 5 OA patients were activated with TNF- α (1 ng/ml) and IL-17 (10 ng/ml) in presence of different concentrations of the active form of leflunomide, HMR1726 (5-100 μ M). As controls we used RA/OA cells activated with both cytokines in the absence of leflunomide. Gene expression of MMP-1 and MMP-3 from RA patients (A, C) and OA patients (B, D) were quantified in comparison to control cells by quantitative real-time PCR. The ratios target gene/GAPDH (x-fold) in comparison to controls (=1) are denoted on the Y-axis. *p < 0.05, **p < 0.01 2.2 TIMP-1 gene expression in cells from the same patients mentioned in 2.1 was quantified by real-time PCR. *p < 0.05, **p < 0.01

and MMP-14 gene expression could be observed in activated synoviocytes from the OA patient. Transcript levels of the tissue inhibitor of metallo-proteinases-1 (TIMP-1) were found to be increased both in activated cells from the RA and OA patient (2.24 ± 0.11 and 2.52 ± 0.11) in the presence of leflunomide.

Quantitative real-time PCR - effects of different HMR1726 concentrations on MMP-1, -3 and TIMP-1 mRNA expression

To validate the data from microarray analyses activated synoviocytes from additional 7 RA and 5 OA

patients were examined for the quantification of MMPs gene expressions. In this study examined RA and OA patients showed almost the same baselines of MMP-1 and -3 expressions. Stimulation of synoviocytes with TNF- α and IL-17 strongly induced basic levels of MMP-1 and MMP-3. In average, MMP-1 gene expression increased 14-fold and MMP-3 mRNA levels 18-fold after addition of both cytokines to cells from RA patients (data not shown). A 21-fold and 61-fold enhancement of the mentioned proteinases was detected after stimulation of cells from OA patients (data not shown).

Fig. 3. A. Synoviocytes from 4 RA and 2 OA patients were activated with TNF- α (1 ng/ml) and IL-17 (10 ng/ml) in the presence of different concentrations of the active form of leflunomide, HMR1726 (5-100 μ M), and MMP-1 protein release in supernatants was measured by ELISA. RA and OA control cells were stimulated with both cytokines in absence of leflunomide. The induction indices (x-fold) in comparison to controls (=1) are denoted on the Y-axis. * $p < 0.05$, ** $p < 0.01$

B. MMP-3 protein release in supernatants from the same cells mentioned in A. was detected by ELISA. No significant differences were found in comparison to protein release in control cells. C. TIMP-1 protein release in supernatants from the same cells mentioned in A. was detected by ELISA. No significant differences were found in comparison to protein release in control cells.

The induction indices caused by HMR1726 are described as fold-increase $\pm$ SD in comparison to the control (TNF- α /IL-17 activated cells). The mean value of gene expression of the control cells was set in the figure 2 as one.

Incubation of TNF- α /IL-17 activated cells in the presence of HMR1726 (5-100 μ M) enhanced the cytokine-induced MMP-1 and MMP-3 gene expression in a dose-dependent manner and reached statistically significant values in all experiments from a HMR1726 concentration of 25 μ M (Fig. 2.1).

MMP-1 m-RNA expression was enhanced between 1.12 ± 0.19 fold in the presence of 5 μ M HMR1726 to 6.2 ± 1.8 ($p < 0.01$) fold at a concentration of 100 μ M HMR1726 in activated synoviocytes from 7 RA patients (Fig 2.1 A). In the same cells MMP-3 expression increased significantly between 1.4 ± 0.3 ($p < 0.05$) fold at 5 μ M HMR1726 and 4.6 ± 2.1 ($p < 0.01$) fold at 100 μ M HMR1726 (Fig 2.1 C).

Similar significant results are shown in Fig. 2.1 B and 2.1 D regarding MMP-1 (1.4-, 2.3- and 4.6-fold induction at a concentration of 25, 50 and 100 μ M HMR1726 respectively) and MMP-3 gene expression (1.4-, 1.5- and 2-fold induction at a concentration of 25, 50 and 100 μ M HMR1726 respectively) in activated synoviocytes from 5 OA patients. However, induction

Fig. 4. Apoptosis induction in cell lysates and supernatants from activated synoviocytes (1 RA and 2 OA patients) in the presence of different concentrations of HMR1726 was detected by cell death detection ELISA. The enrichment of nucleosomes (mU) is denoted on the Y-axis.

indices caused by HMR1726 seemed to be higher in synoviocytes derived from RA patients in comparison to synoviocytes derived from OA patients.

Analysis of TIMP-1 gene expression in activated fibroblasts from 7 RA and 5 OA patients showed an

Fig. 5. Synoviocytes from 3 RA and 2 OA patients were activated with TNF- α and IL-17 in the presence of 50 μ M HMR1726. A. and B. The p-38-specific inhibitor (SB203580) and the specific inhibitor of MEK1/2 (U0126) were added (20 μ M) to the cells and gene expression of MMP-1 and MMP-3 was quantified by real-time PCR. C. and D. Release of MMP-1 and -3 in supernatants from cells cultured under in (A) mentioned conditions (1RA and 20A) was measured by ELISA.

inductive tendency in the presence of HMR1726 (Fig. 2.2 A and B). The induction indices reached significant values in a dose-dependent manner from a HMR1726 concentration of 25 μ M in activated synoviocytes from 7 RA patients incubated with leflunomide ($p < 0.05$ in the presence of 25 μ M HMR1726 and $p < 0.01$ in the presence of 50 and 100 μ M HMR1726). A similar tendency was found in stimulated cells from OA patients however, the induction indices were not significant higher in presence than in absence of leflunomide.

Effects of different HMR1726 concentrations on MMP-1, -3 and TIMP-1 protein expression

MMP-1 protein expression in supernatants from TNF- α /IL-17 activated synoviocytes in the presence of different HMR1726 concentrations was examined by ELISA. Results from 6 patients (4 RA and 2 OA) revealed the same increasing tendency in dependence of leflunomide concentration. However, the induction indices remained lower than on transcription levels, respectively. MMP-1 protein expression increased between 1.25 ± 0.21

fold ($p < 0.05$) in the presence of 25 μ M HMR1726 to 1.87 ± 0.33 fold ($p < 0.001$) at a concentration of 100 μ M HMR1726 in activated synoviocytes from 4 RA and 2 OA patients (Fig. 3 A). However, neither an induction nor a significant reduction of MMP-3 protein levels through different concentrations of HMR1726 could be observed using the mentioned commercial ELISA (Fig. 3 B). Likewise, TIMP-1 protein release reached no significant alterations on dependence to different leflunomide concentrations (Fig. 3C).

Effect of different concentrations of HMR1726 on the viability and apoptosis induction in synoviocytes

Without stimulation by cytokines, the incubation of HMR1726 up to a concentration of 100 μ M HMR1726 showed no impairment of cell viability in synoviocytes after an incubation period of 24 and 48 hours respectively, as detected by the viability test (data not shown). Analysis of apoptosis induction revealed a strong enrichment of nucleosomes in cell lysates and supernatants from

Fig. 6. To investigate whether phosphorylation of p-38 and p42/44 MAPK is mediated by leflunomide, specific antibodies against the phosphorylated forms of p-38 and p42/44 MAPK were used for western blotting. Representative results from three independent experiments (from 1 RA, 2 OA patients) are shown. A. p-p38 expression in synoviocytes incubated with TNF- α , IL-17 and HMR1726 (50 μ M) alone or in combination as indicated in the table for 48 h. B. time course of p-p38 expression (30', 1h, 3h, 24 h) in synoviocytes incubated with TNF- α and IL-17 in presence or absence of HMR1726 (50 μ M) as indicated in the table for 48 h. C. p-Erk1/2 expression in synoviocytes incubated with TNF- α and IL-17 in presence or absence of HMR1726 (50 μ M) for 3 and 48 h.

TNF- α /IL-17 activated synoviocytes from one RA and 2 OA patients in the presence of 250 μ M HMR1726 ($p < 0.001$, $n = 3$) in comparison to the activated control in absence of leflunomide (Fig. 4).

Signal transduction pathways involved in the HMR1726-induced MMP-1 and MMP-3 expression

Quantitative real-time PCR

To investigate the involvement of signal transduction pathways participating in the regulation of the HMR1726-induced MMP-1 and -3 gene expression, TNF/IL-17 activated synovial fibroblasts were treated with MAP-kinase inhibitors. In synoviocytes from 3 RA and 2 OA patients ($n = 5$), TNF- α and IL-17 induced MMP-1 gene

expression increased 3.2 ± 0.97 fold in the presence of 50 μ M HMR1726 after 48 h. The p-38-specific inhibitor SB203580 (20 μ M) significantly reduced ($p < 0.001$) leflunomide-induced MMP-1 transcript levels to 21.25 % (0.68 ± 0.36 fold) (Fig. 5A). The specific inhibitor of MEK1/2 (U0126 20 μ M) showed no significant reduction of leflunomide-induced MMP-1 gene expression. In the same activated cells MMP-3 gene expression increased 2.2 ± 1 fold in the presence of 50 μ M HMR1726 and this activation could be significantly abolished ($p < 0.005$) by the addition of the specific inhibitor of MEK1/2 (U0126 20 μ M) (Fig. 5B). Addition of the p-38-specific inhibitor SB203580 (20 μ M) showed a slight but not significant reduction of leflunomide-induced MMP-3 gene expression.

The use of an inactive inhibitor (SB202474) showed no significant reduction of HMR1726-induced MMP-1 and -3 expression (n=3, data not shown).

ELISA

In supernatants from 1 RA and 2 OA patients, HMR1726 induced MMP-1 protein levels 1.4 ± 0.05 fold (n = 3). The p-38-specific inhibitor SB203580 (20 μ M) could completely diminish leflunomide-induced MMP-1 protein expression (p < 0.01, Fig. 5C). Application of the specific inhibitor of MEK1/2 (U0126 20 μ M) showed no reduction of leflunomide-induced MMP-1 protein levels.

With regard to MMP-3 protein expression, the use of the MEK1/2 inhibitor U0126 could completely reduce leflunomide- as well as TNF- α /IL-17-induced MMP-3 levels (Fig. 5D). However, only the RA patient showed an induction of MMP-3 protein expression after addition of HMR1726, not the OA patients.

Western Blotting

To determine whether phosphorylation of p-38 and p42/44 MAPK is mediated by leflunomide, activation of these kinases was assayed by using specific antibodies for the phosphorylated forms of p-38 and p42/44 MAPK by western blotting. Representative results from three independent experiments (from 1 RA, 2 OA patients) are shown in figure 6.

The incubation of synoviocytes for 48 h with leflunomide alone slightly reduced the signal of the phosphorylated p-38 kinase (Fig. 6A, left panel) but addition of TNF alone or in combination with 50 μ M HMR1726 resulted in reduced p-p38 protein levels. Of note, IL-17 alone caused no stronger signals of p-p38 in comparison to the respective untreated control. However, synoviocytes treated with IL-17 in combination with 50 μ M HMR1726 and/or TNF- α showed strongly induced protein levels of phosphorylated p-38 after 48 h (Fig. 6A right panel).

Examination of phosphorylated p38 in course of time (Fig. 6B) revealed a reduced signal intensity of phosphorylated p38 upon addition of leflunomide after 30 min and 1 h. However, after 3 and 24 h, p38 phosphorylation increased through leflunomide compared to the respective control. Additionally, analysis of phosphorylated p42/44 MAPK (Fig. 6C) clearly showed elevated protein levels after treatment of synoviocytes with both cytokines in the presence of leflunomide after 3 and 48 h of incubation in comparison to protein levels in stimulated cells in absence of HMR1726.

Discussion

These are the first published data describing the effects of the active metabolite of leflunomide, HMR1726. The data obtained in this study showed that HMR1726, the active metabolite of leflunomide, may induce MMP-1 and MMP-3 gene expression in TNF- α and IL-17 activated synoviocytes from RA and OA donors. The effects are dose-dependent and independent on the therapeutic regiment used for treatment of the RA patients analysed in this report. Leflunomide-induced MMP-1 protein expression seemed to be less prominent when compared with effects obtained on transcript levels. However, from a HMR1726 concentration of 25 μ M leflunomide-induced MMP-1 protein levels reached significant values (n = 6). In contrast to gene expression results, which clearly show significantly induced MMP-3 transcript levels through HMR1726, no significant induction or reduction of MMP-3 protein expression could be obtained by the addition of HMR1726, as detected by ELISA. This might be based on the slight induction of TIMP-1 through leflunomide (Fig. 2.2) which probably blocks leflunomide-induced MMPs release. However, analysis of TIMP-1 protein release by ELISA showed no significant alterations through HMR1726 (Fig. 3C). However, exact quantification of protein expression by ELISA is questionable because of the abundant amounts of these proteins in supernatants from synoviocytes. Taken together, in our experiments HMR1726 was not able to abolish proteinases expression in TNF- α and IL-17 activated synoviocytes.

Interestingly, MMPs induction all alone through the cytokines TNF- α /IL-17 was stronger in synoviocytes from OA patients than in cells derived from RA patients. Probably, DMARDs (disease modifying anti-rheumatic drugs) treatment of RA patients for a long period of time has a suppressive effect on sensitivity of cells for pro-inflammatory cytokines. The different inductivity of both cell types does not correlate with the response of these cells to leflunomide. The influence of HMR1726 on MMPs induction is independent on lower sensitivity of RA cells. In contrary, a more prominent inductive effect of HMR1726 on proteinases gene expression was found in activated synoviocytes from RA in comparison to those from OA patients, as shown in Fig. 2. Probably, RA synoviocytes differ from OA specimens with regard to their reactivities to HMR1726. Recently, we could show some differences between RA and OA fibroblasts with respect to their sensitivities [18].

Previous studies revealed that leflunomide shows protective effects on T-cells through suppression of TNF-induced NF- κ B and AP-1 activation [19, 20]. Cutolo et al. could demonstrate that the active metabolite of leflunomide, A771726, showed anti-inflammatory effects on LPS activated cultured macrophages derived from RA patients [21]. Grisar et al. reported that only a high concentration of 100 μ M A771726 inhibited the transendothelial migration of inflammatory cells by reducing adhesion and transmigration capacity [22].

Contrary to the data published by Burger *et al.* [23], HMR1726 induced in our study in activated synoviocytes expression of metalloproteinases both on gene and protein level (especially MMP-1) in a dose-dependent way. These contradictory results might be based on differences of methods used for quantification of m-RNA by microarray analyses and real-time PCR in comparison to northern blot analysis. Furthermore, we used for our experiments HMR1726 as the free acid amide of A771726, a very stable form of this drug optimized for tissue cultures. Its activity can be detected after several days of incubation at 37°C.

Migita *et al.* [24] report on inhibitory effects of A771726 using IL-1 β -stimulated synoviocytes and showed a reduced MMP-1 and -3 expression associated with suppression of ERK1/2, p-38 and JNK1/2 kinase activity. Using HMR1726, we observed a suppression of p-38 only after a short incubation period of 30' and 1h, a longer incubation period of 3, 24 and 48 h revealed in contrast to these results an induction of p-38. Western blotting results showed after 48h of incubation with HMR1726 also a stronger phosphorylation of Erk1/2. Addition of specific kinase inhibitors diminished leflunomide-induced proteinases gene- and protein expression as shown in Fig. 5A-D.

Clinical studies clearly show that the net return of leflunomide has a positive effect on RA disease progression and bone erosions [25-27]. Nevertheless, several drug-related adverse events are known to be associated with leflunomide treatment including diarrhoea, abnormalities in liver enzymes, rash, and hypertension. But also induction of interstitial nephritis, acute hepatitis, pancytopenia, peripheral neuropathy, and development of lung nodulosis and increase of rheumatoid nodules are described as drug-associated side effects [28-33]. Leflunomide-induced proteinases expression described in this work might be involved in development of adverse events mentioned above.

Normally, it is hypothesized that a drug with beneficial effects should decrease the production of catabolic enzymes. Surprisingly, the data presented in our study demonstrate the opposite. On the other hand, MMPs are possibly not such important for the progression of the rheumatoid arthritis as expected in this process. Probably, activated lymphocytes play a more important role in boosting and maintaining the inflammation process in the joint of rheumatoid arthritis patients. Further investigations are required to elucidate the molecular mechanism of this drug.

Taken together, in our study leflunomide was not able to abolish expression of matrixproteinases in synoviocytes activated with TNF- α and IL-17.

Acknowledgements

This project was supported by the Fortune Program of the University Tübingen (1218-0-0) to D.A. We thank Dr. Pünter (Aventis Pharma, Germany) for kindly providing us with HMR1726. No competing interests declared.

References

- 1 Brazleton TR, Morris RE: Molecular mechanism of action of new xenobiotic immunosuppressive drugs: tacrolimus (FK506), sirolimus (rapamycin), mycophenolate mofetil and leflunomide. *Curr Opin Immunol* 1996;8:710-720.
- 2 Silva HT, Morris RE: Leflunomide and Malononitrilamides. *Expert Opin Investig Drugs* 1997;6:51-64.
- 3 Cao W, Kao P, Chao A, Gardner P, Ng J, Morris R: Mechanism of anti-proliferative action of leflunomide. *J Heart Lung Transplant* 1995;14:1016-1030.
- 4 Cherwinski HM, Cohn RG, Cheung P, Webster DJ, Xu Y-Z, Caulfield JP, Young JM, Nakano G, Ransom JT: The immunosuppressant leflunomide inhibits lymphocytes proliferation by inhibiting pyrimidine biosynthesis. *J Pharmacol Exp Ther* 1995;275:1043-1049.

- 5 Williamson RA, Yea CM, Robson PA, Curnock AP, Gadher S, Hambleton AB, Woodward K, Bruneau JM, Hambleton P, Moss D, Thomson TA, Spinella-Jaegle S, Morand P, Courtin O, Sautes C, Westwood R, Hercend T, Kuo EA, Ruuth E: Dihydroorotate dehydrogenase is a high affinity binding protein for A77 1726 and mediator of a range of biological effects of the immunomodulatory compound. *J Biol Chem* 1995;270:22467-22472.
- 6 Williamson RA, Yea CM, Robson PA, Curnock AP, Gadher S, Hambleton AB, Woodward K, Bruneau JM, Hambleton P, Spinella-Jaegle S, Morand P, Courtin O, Sautes C, Westwood R, Hercend T, Kuo EA, Ruuth E: Dihydroorotate dehydrogenase is a target for the biological effects of leflunomide. *Transplant Proc* 1996;28:3082-3084.
- 7 Fairbanks LD, Bofil M, Ruckemann K, Simmonds HA: Importance of ribonucleotide availability to proliferating T-lymphocytes from healthy humans. *J Biol Chem* 1995;270:29682-29691.
- 8 Elder RT, Xu X, Williams JW, Gong H, Finnegan A, Chong ASF: The immunosuppressive metabolite of leflunomide, A77 1726, affects murine T cells through two biochemical mechanism. *J Immunol* 1997;159:22-27.
- 9 Siemasko K, Chong ASF, Jack HM, Gong H, Williams JW, Finne A: Inhibition of JAK3 and STAT6 tyrosine phosphorylation by the immunosuppressive drug leflunomide leads to a block in IgG1 production. *J Immunol* 1998;160:1581-1588.
- 10 Xu X, Williams JW, Gong H, Finnegan A, Chong ASF: Two activities of the immunosuppressive metabolite of leflunomide, A77 1726: inhibition of pyrimidine nucleotide synthesis and protein tyrosine phosphorylation. *Biochem Pharmacol* 1996;52:527-534.
- 11 Chaubaud M, Durand JM, Buchs N, Fossiez F, Page G, Frappart L, Miossec P: Human interleukin-17: a T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. *Arthritis Rheum* 1999;42:963-970.
- 12 Chaubaud M, Garnero P, Dayer JM, Guerne PA, Fossiez F, Miossec P: Contribution of IL-17 to synovium matrix destruction in rheumatoid arthritis. *Cytokine* 2000;12:1092-1099.
- 13 Ziolkowska M, Koc A, Luszczkiewicz G, Ksiezopolska-Pietrzak K, Klimzak E, Chwalinska-Sadowska H, Maslinski W: High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism. *J Immunol* 2000;164:2832-2838.
- 14 Yao Z, Painter SL, Fanslow WC, Ulrich D, Macduff M, Spriggs MK, Armitage RJ: Human IL-17: a novel cytokine derived from T cells. *J Immunol* 1995;155:5483-5486.
- 15 Lubberts E, Koenders MI, van den Berg WB: The role of T cell interleukin-17 in conducting destructive arthritis: lessons from animal models. *Arthritis Res Ther* 2005;7:29-37.
- 16 Kehlen A, Pachnio A, Thiele K, Langner J: Gene expression induced by interleukin-17 in fibroblast-like synoviocytes of patients with rheumatoid arthritis: upregulation of hyaluronan-binding protein TSG-6. *Arthritis Res Ther* 2003;5:R186-R192.
- 17 Koshy PJ, Henderson N, Logan C, Life PF, Cawston TE, Rowan AD: Interleukin 17 induces cartilage collagen breakdown: novel synergistic effects in combination with proinflammatory cytokines. *Ann Rheum Dis* 2002;61:704-713.
- 18 Weis-Klemm M, Alexander D, Pap T, Schuetzle H, Reyher D, Franz JK, Aicher WK: Synovial fibroblasts from rheumatoid arthritis patients differ in their regulation of IL-16 gene activity in comparison to osteoarthritis fibroblasts. *Cell Physiol Biochem* 2004;14:293-300.
- 19 Manna S, Aggarwal BB: Immunosuppressive leflunomide metabolite (A77 1726) blocks TNF-dependent nuclear factor- κ B activation and gene expression. *J Immunol* 1999;162:2095-2102.
- 20 Manna S, Mukhopadhyay A, Aggarwal BB: Leflunomide suppresses TNF-induced cellular responses: effects on NF- κ B, activator protein-1, c-Jun N-terminal protein kinase, and apoptosis. *J Immunol* 2000;165:5962-5969.
- 21 Cutolo M, Sulli A, Ghiorzo P, Pizzorni C, Cravittio C, Villaggio B: Anti-inflammatory effects of leflunomide on cultured synovial macrophages from patients with rheumatoid arthritis. *Ann Rheum Dis* 2003;62:297-302.
- 22 Grisar J, Aringer M, Köller MD, Stummvoll GH, Eselböck D, Zwölfer B: Leflunomide inhibits transendothelial migration of peripheral blood mononuclear cells. *Ann Rheum Dis* 2004;63:1632-1637.
- 23 Burger D, Begue-Pastor N, Benavent S, Gruaz L, Kaufmann MT, Chicheportiere R, Dayer JM: The active metabolite of leflunomide, A77 1726, inhibits the production of prostaglandin E(2), matrix metalloproteinase 1 and interleukin 6 in human fibroblast-like synoviocytes. *Rheumatol (Oxford)* 2003;42:89-96.
- 24 Migita K, Miyashita T, Ishibashi H, Maeda Y, Nakamura M, Yatsushashi H, Ida H, Kawakami A, Aoyagi T, Kawabe Y, Eguchi K: Suppressive effect of leflunomide metabolite (A77 1726) on metalloproteinase production in IL-1 β stimulated rheumatoid synovial fibroblasts. *Clin Exp Immunol* 2004;137:612-616.
- 25 Schorlemmer HU, Kurrle R, Schleyerbach R, Barlett RR: Disease-modifying activity of malononitrilamides, derivatives of leflunomide's active metabolite, on models of rheumatoid arthritis. *Inflamm Res* 1999;48:113-114.
- 26 Smolen JS, Kalden JR, Scott DL, Rozman B, Kvien TK, Larsen A, Loew-Friedrich I, Oed C, Rosenberg R: Efficacy and safety of leflunomide compared with placebo and sulphasalazine in active rheumatoid arthritis: a double-blind, randomised, multicenter trial: European Leflunomide study group. *Lancet* 1999;353:259-266.
- 27 Alastair JJ, Wood MD: New drugs for rheumatoid arthritis. *N Engl J Med* 2004;350:2167-2179.
- 28 Haydar AA, Hujairi N, Kirkham B, Hangartner R, Goldschmidt DJ: Chronic overdose of leflunomide inducing interstitial nephritis. *Nephrol Dial Transplant* 2004;19:1334-1335.
- 29 Sevilla-Mantilla C, Ortega L, Agundez JA, Fernandez-Gutierrez B, Ladero JM, Diaz-Rubio M: Leflunomide-induced acute hepatitis. *Dig Liver Dis* 2004;36:82-84.
- 30 Hill RL, Topliss DJ, Purcell PM: Pancytopenia associated with leflunomide and methotrexate. *Ann Pharmacother* 2003;37:149.
- 31 Bharadwaj A, Haroon N: Peripheral neuropathy in patients on leflunomide. *Rheumatol (Oxford)* 2004;43:934.
- 32 Rozin A, Yigla M, Guralnik L, Keidar Z, Vlodavsky E, Rozenbaum M, Nahir AM, Balbir-Gurman A: Rheumatoid lung nodulosis and osteopathy associated with leflunomide therapy. *Clin Rheumatol* 2005;1-5: [Epub ahead of print]
- 33 Braun MG, Van Rhee R, Becker-Capeller D: Development and/or increase of rheumatoid nodules in RA patients following leflunomide therapy. *Z Rheumatol* 2004;63:84-87.