

**PH.D. THESIS SUMMARY**

**A POTENTIALLY ANTI-INFLAMMATORY TARGET:  
ENZYMATIC ACTIVITY OF THE MACROPHAGE MIGRATION INHIBITORY  
FACTOR**

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## THEORETICAL BACKGROUND

The macrophage migration inhibitory factor (MIF) was the first, *in vivo* verifiable immunomediator, without immunoglobulin structure. It was originally described in 1966 as a soluble factor expressed by T cells in delayed-type hypersensitivity responses (DTH) and was later shown to be expressed by activated T cells under various conditions (Bloom and Bennett, 1966; David, 1966). Although a lot of mechanisms for the action and site of formation are known today, MIF is hard to classify, because it can act not only as a cytokine, but also as a hormone, moreover, it has enzymatic functions, too.

The human monomer MIF consists of 114 amino acids, with a molecular mass of 12.4 kDa. Its three dimensional structure was identified by X-ray crystallography as a homotrimer molecule, consisting of three homologous subunits (Sun *et al.*, 1996; Suzuki *et al.*, 1996). The MIF structure is unique among cytokines, and no significant sequence homologies have been found between MIF and other known proteins. In spite of this, three-dimensional x-ray crystallographic studies have shown that human MIF exists as a homotrimer and is structurally related to the bacterial enzymes. The sources of MIF are various; it can occur in the whole body ranging from the immune cells across the corticotrop cells of the pituitary gland to the endothelial cells (Calandra and Roger, 2003). Under special stimuli it can emerge from the intracellular vesicles, but it has a standard serum concentration (3–5ng/ml) (Metz and Bucala, 1997).

MIF regulates cell activation through extracellular, receptor-mediated signaling pathways, and intracellular interactions. Although the mechanism of action of MIF is not completely understood, MIF is known to activate mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinase (ERK) and p38 MAPK in synoviocytes similar to other pro-inflammatory cytokines (Onodera *et al.*, 2004; Santos *et al.*, 2004). MIF is the only cytokine known to directly downregulate p53 expression and function (Hudson *et al.*, 1999; Mitchell *et al.*, 2002; Leech *et al.*, 2003; Lacey *et al.*, 2003).

Though classified as a proinflammatory cytokine, MIF had been shown to represent surprising and unique properties, which distinguishes it from other cytokines. A classical cytokine receptor for this protein had not been identified as yet. MIF is endocrine factor also, because it was identified as a major secreted protein released by anterior pituitary cells in response to LPS stimulation (Bernhagen *et al.*, 1993). Serum MIF concentrations show a circadian rhythm, similar to that of cortisol (Petrovsky *et al.*, 2003). In spite of the fact, that cytokines are usually stimulated by glucocorticoids (Wiley *et al.*, 2004), MIF is differentially

influenced by glucocorticoids and the cytokine has the unique ability to override the anti-inflammatory and immunosuppressive effects of glucocorticoids on macrophages and T cells. Uniquely, the MIF molecule comprises two evolutionarily conserved motifs that otherwise have only been identified in bacterial enzymes and catalyze isomerization/tautomerization and oxidation–reduction reactions. It has been proved that the homotrimeric tertiary structure of MIF is required for assembly of the tautomerase catalytic region (Rosengren et al., 1997). Human MIF and *p*-hydroxyphenylpyruvate (OHPP) crystal structures have a substrate-binding hydrophobic cavity that lies between two adjacent subunits of the homotrimer (Lubetsky et al., 2002).

The molecular function *in vivo* of the tautomerase catalytic properties of MIF is not clear and results from different laboratories have provided conflicting evidence for the necessity of the catalytically required residues for biological function. Recently, small-molecule inhibitors interacting with the active tautomerase/isomerase pocket of MIF have been shown to inhibit its cytokine function (Orita et al., 2002). Targeting the catalytic pocket of MIF with small-molecule inhibitors could, therefore, represent a new and selective anti-cytokine approach.

## **AIMS AND THEMATIC COMPONENTS**

The main purpose of this thesis has been to investigate the phenylpyruvate enzymatic activities of MIF, and to manipulate the effects by different compounds. These experiments were carried out on Sigma tautomerase, human samples, and tubulin. The most effective substance was also tested in an *in vivo* model. Since MIF has a connection with osteoporosis, I am going to deal with that problem at the end of this chapter.

### **The five main topics are as follows:**

- I. Investigation the phenylpyruvate enzymatic activities of MIF
- II. Analysis of quantity and activity of MIF in human samples
- III. *In vivo* testing of caffeic acid, as an effective MIF inhibitor in mouse arthritis model
- IV. Studies on the connection between microtubule function and MIF
- V. Postmenopausal osteoporosis prevention with phytoestrogens

## METHODS

### 1.1. Protein methods, purifications

Protein quantification: with Bradford (1976) photometry.

SDS-polyacrylamide gelelectrophoresis: according to Laemmli (1970).

Western Blot: primer antibody: rabbit anti-rat MIF (1:2.000), rabbit anti- $\alpha$ Tubulin (1:200), secondary antibody: goat anti-rabbit-IgG-HRP conjugated (1:2.000).

Silver stain: the gels were fixed 2 hours, then overnight. After washing they were incubated in equilibrating solution, finally the excess amount of silver was clean with distilled water.

Tubulin isolation: according to Shelanski (1973).

The porcine brain was homogenized in ice, with 1ml/400mg homogenization buffer. The tubule obtained after the repeated spinning of the cytosolic fraction, was polymerized in 37°C water bath. GTP, MgCl<sub>2</sub> and glycerin was also added. The polymerized tubule was separated with spinning and the pellet was resuspended. To depolymerize it was kept on ice cold water. This tubule has big microtubule associated protein (MAP) content.

HM (high molar)- tubulin purifying: according to Castoldi and Popov (2003).

The brain was homogenized in 1 ml/g depolymerizing buffer. After centrifugation the supernatant was completed with HM-PIPES buffer, GTP, ATP and glycerol. The polymerization was achieved in 37°C water bath. After the spinning, the gel, localized at the bottom of the tube, was resuspended with 20 ml depolymerising buffer. After it, the sample was depolymerized in 0°C. the polymerization- depolymerization cycles were carried out two more times. The depolymerized tubule was the frozen immediately.

### Joint homogenization

The tibiotarsal joints of mice were amputate, and homogenized in 1ml/10mg buffer. After spinning, the supernatant was used for ELISA analysis.

### 1.2. Production of chemical compounds

These experiments were planned and carried out by Tamás Lóránd (Institute of Organic and Medicinal Chemistry). Structural verification of the compounds was made with the help of FT IR and NMR spectroscopy.

### **1.3. Immunological methods**

#### ELISA (MIF ELISA)

Enzyme-Linked Immunosorbent Assay. A 96-well micro plate was coated with the diluted capture antibody, and incubates overnight at room temperature. Next day, after washing and blocking the samples (diluted 1x, 5x, 10x) and standards were added. Then the detection antibody, and Streptavidin-HRP, finally the substrate solution were added. The reaction was stopped with H<sub>2</sub>SO<sub>4</sub> stop solution. The optical densities of each well were determined immediately, using a micro plate reader set to 450 nm (wavelength correction: 570 nm).

The IL-1 $\beta$  (interleukin) ELISA was measures also in 450 nm, but the wavelength correction: was 620 nm.

#### TR-FIA, DELFIA methods

With Time Resolved Fluoroimmunoassay, Dissociation-Enhanced Lanthanide Fluorescent Immunoassay the phytoestrogen contents of sera was investigated in Prof. Dr. Hermann Adlercreutz laboratory (Folkhälsan Research Center; Inst. for Preventive Medicine, Nutrition and Cancer; Department of Clinical Chemistry University of Helsinki; Finland).

The plasma was mixed with  $\beta$ -glucuronidase and sulphatase in acetate buffer and incubated overnight at 37°C. Diethyl ether was used to extract unconjugated enterolactone after hydrolysis. The hydrolyzed extract in buffer was then pipetted into prewashed goat anti-rabbit IgG microstrips, simultaneously, antiserum (dilution 1:250,000) and europium-labeled enterolactone (dilution 1:400,000) were added to the microstrips.

After incubation and shaking of the strips slowly at room temperature for 90 minutes, the strips were washed. Subsequently, DELFIA enhancement solution was added to each well, finally the fluorescence was read in a DELFIA Victor multilabel counter.

Duplicate control plasma and triplicate samples were processed through the procedure. Results were calculated according to the formula: Plasma ENL  $\hat{=}$  concentration (read)  $\times$  1/recovery  $\times$  dilution factor.

### **1.4. Bioactivity tests**

#### Phenylpyruvate tautomerase activity (ketonase and enolase reaction)

The measurements were carried out in the Department of Biophysics, and in the Institute of Organic and Medical Chemistry. As a model of MIF, tautomerase from bovine kidney (Sigma) was used.

The reactions were measured at 20°C, in double well UV spectrophotometer. The ketonase reaction was followed in 288nm, till 75 second, the enolase was measured in 300nm till 900 sec. The reaction mixture contained buffer, enzyme and substrate. The substrate was phenylpyruvate in the ketonase, and Na-phenylpyruvate in the enolase reaction. The enolase mixture contained boric acid too. In the control cuvette the enzyme was missing. The inhibitors were diluted freshly (1, 5, 20, 50, 100, 200, 400µM final concentrations).

#### Tubulin polymerization

The polymerization of the tubulin was followed at 350nm, with double way UV spectrophotometer at 33°C, in the Department of Biophysics. The cuvette contained 1mg/ml tubulin MgCl<sub>2</sub> and GTP. In the control cuvette instead of tubulin buffer was used. The measurement was stopped after the plateau phase.

### **1.5.Immunohistochemical methods**

#### Light microscopic immunohistochemistry on paraffin embedded sections

The paraffin was removed from the 10 µm sections, and then the sections were rehydrated, and washed with Tris buffer. After three heating cycles in a microwave oven endogenous peroxidase activity was blocked in 1% H<sub>2</sub>O<sub>2</sub>. The sections were preincubated in normal horse serum; this step was followed by incubation in the primary rabbit anti-MIF antibody (1:5.000, ill. 1:10.000) and anti-αTubulin: (1:200). Binding sites were visualized with a biotinylated anti-rabbit IgG (1:100) and the avidin–biotin–peroxidase (ABC) detection system. The peroxidase substrate medium was 3,3'-diamino-benzidine and H<sub>2</sub>O<sub>2</sub>. The sections were then washed, dehydrated, cleared in xylene and covered with DEPEX. Controls included the omission of the primary or secondary antibodies.

#### Light microscopic immunohistochemistry on floating sections

60 µm sections were cut using a vibrating microtome. Endogenous peroxidase activity was blocked in H<sub>2</sub>O<sub>2</sub>. Following a 30min treatment in Triton-X-100 the sections were preincubated in normal horse serum. The rest of the steps applied were similar to those of the previous method. The sections were then washed, mounted on glass slides, counterstained, dehydrated, cleared in xylene and covered with DEPEX.

### Electron microscopic immunohistochemistry

The tissue containing the hippocampal formation, the parahippocampal cortex and the temporal neocortex was fixed in a solution containing paraformaldehyde and glutaraldehyde in phosphate buffer. Sections, 60µm thick, were cut with a vibrating microtome and collected in PBS. To enhance penetration of immunoreagents, the sections were equilibrated in a cryoprotectant solution, frozen briefly in liquid nitrogen, and thawed in the same cryoprotectant solution. After washing the sections were incubated in H<sub>2</sub>O<sub>2</sub>. Primary and secondary antibodies and ABC complex were added as described above. Further processing for electron microscopic evaluation involved flattening the sections onto a watch glass, followed by immersion in a solution of osmium tetroxide, followed by a wash and dehydration. After the final incubation in 100% ethanol, the sections were transferred to propylene oxide before being placed into aluminum foil boats containing Durcupan resin overnight. Sections were mounted on cleaned slides, a cover slip applied, and the resin cured for 24 h at 56°C. Areas of interest were chosen with light microscope, then cut and reblocked in Durcupan resin. Serial ultra thin sections were contrasted by uranyl acetate and lead citrate according to a standard procedure. The sections were examined with JEOL electron microscope.

## **1.6. Animal experiments**

### Animals

Experiments were performed on male Balb/c mice (n=21) weighing 20 to 25 g. They were obtained for the experiments, and held under standard pathogen-free conditions at 24–25°C. Mice were provided with standard chow and water *ad libitum*. The studies were approved by the Ethics Committee on Animal Research of Pécs University according to the Ethical Codex of Animal Experiments.

### Complete Freund's adjuvant (CFA) induced arthritis

Arthritis of the left tibiotarsal joint of the mice was evoked by *s.c.* injection of CFA (killed mycobacteria suspended in paraffin oil) into the plantar surface of the left hind paw and the root of the tail. To enhance systemic effects, an additional injection was given into the tail on the following day (Helyes et al., 2001). The paw changes were measured before the experiment and every other day after CFA administration.

### Measurement of Paw Edema

The volume of the paws was measured by plethysmometry, which function on the principle of communicating vessels. The paw volume is expressed in cubic centimeters. Edema was expressed as percentage of initial control values.

### Measurement of the mechanonociceptive threshold

The aesthesiometer was used to assess touch sensitivity on the plantar surface of the paw. After acclimation and cessation of exploratory behavior, the operator placed the touch stimulator unit under the animal's paw. After pressing the start key, an electrodynamic actuator of proprietary design lifts a straight metal filament, which begins to exert an increasing upward force at a preset rate of application until a stop signal (when the animal removes the paw) is attained. The paw withdrawal threshold is numerically shown in grams on the digital screen. Mechanonociceptive threshold of the paws was expressed in % compared to the initial control values.

## **I. INVESTIGATION THE PHENYLPYRUVATE ENZYMATIC ACTIVITIES OF MIF**

### **Introductions**

To inhibit the proinflammatory activity of MIF has two alternatives: inhibition of MIF by neutralizing antibodies / anti-sense mRNS or using small molecule inhibitors of its catalytic site. In spite of that, no one knows the biologic function of MIF tautomerase activity, a lot of laboratories tried to develop small inhibitor molecules. With computer models, some potent molecules were found, e.g. derivatives of (E)-2-fluoro-p- hydroxycinnamate (Taylor et al., 1999), dopachrome analogues (Zhang and Bucala, 1999), tryptophan and tyrosine- (Dios et al., 2002), or coumarin and chromerone analogues (Orita et al., 2001).

A purified form of MIF tautomerase commercially available from Sigma give the same 12.4 kDa band as the recombinant human MIF (rMIF), and possess the same tautomerase activity as well (Rosengren et al., 1997). Furthermore the flavonoids luteolin and quercetin affect phenylpyruvate tautomerase activity of bovine kidney phenylpyruvate tautomerase and of recombinant human MIF in a similar manner (Garai and Adlercreutz, 2004). Based on these facts it is presumably, that the small molecular inhibitors could achieve the same effect on both enzyme. Therefore the Sigma tautomerase was used instead of the expensive rMIF.

## **I.1. Anti-inflammatory compounds can affect the phenylpyruvate activity of MIF**

Among inhibitors acetaminophen was tested first, because this and its metabolites inhibit the dopachrome tautomerase activity (Senter et al., 2002), but the effect of these molecules on the phenylpyruvate enol–keto conversion mediated by MIF has not yet been analyzed. In the next step some plant derivatives with anti-inflammatory activity were tested, finally some non-steroidal anti-inflammatory drugs (NSAID) have been tested. The mechanism of inhibition was determined by Lineweaver–Burk plot. The  $IC_{50}$  values (micromolar concentration producing 50% inhibition) of the inhibitors were also calculated.

### **Results**

1. Acetaminophen could inhibit not only the dopachrome but also the phenylpyruvate activity of MIF ( $IC_{50}=1.0\mu\text{M}$ ).
2. The tested small anti-inflammatory molecules inhibited the ketonase and enolase reaction in a concentration dependent manner. In the MIF ketonase reaction caffeic acid (a phenylpropane) ( $IC_{50}=0.55\mu\text{M}$ ) and curcumin ( $IC_{50}=0.7\mu\text{M}$ ) were the most effective. In the coumarin group umbelliferon (7-hydroxycoumarin) ( $IC_{50}=2.6\mu\text{M}$ ), among the miscellaneous group resveratrol ( $IC_{50}=1.9\mu\text{M}$ ) were the most potent. Concerning the enolase reaction likewise caffeic acid had the best inhibitory potential.
3. Among the NSAIDs piroxicam was the best, but its inhibitor activity ( $IC_{50}=120.9\mu\text{M}$ ) still lagged behind most of the plant-derived agents investigated. Phenylbutazone and ibuprofen exhibited inhibitor activity only at millimolar concentrations.
4. The Lineweaver–Burk plot of caffeic acid suggests that the mechanism of inhibition is competition with substrate.

### **Conclusions**

Most of the investigated anti-inflammatory plant compounds approved really good inhibitor. Although the NSAIDs are known to exist in tautomeric forms, none of them was effective in our system. It may be assumed that the plant derived compound's strong antioxidant character might have also contributed to the effects observed in our *in vitro* system. The mechanism of this inhibition needs to be further analyzed regarding each compound.

Concerning MIF's glucocorticoid counteracting pro-inflammatory effect, the effect of these plant-derived agents on MIF tautomerase might lead to better exploitation of their putative glucocorticoid-sparing potency in the chronic, autoimmune diseases therapy.

## **I.2. The effect of chemically synthesized small molecules on MIF's tautomerase**

Such compounds were sought in this section, which structure is in part similar to the most potent plant compounds, or to the MIF's substrate. To enhance the inhibitory potential many compounds were synthesized by Tamás Lóránd (Department of Biochemistry, Institute of Organic and Medicinal Chemistry). These were principally tested in the ketonase reaction.

### **Results**

1. Estimation of the structure-effect connection could analyze only as family compounds. The steric and electronic character of the compounds could also play a role in the structure-effect correlations.
2. Among the 2-arylidencyclohexanones studied, para-bromo substituted derivative and piperonilidene showed the highest activity. The effects of the six-member 7-8 compounds were generally smaller than those of the cyclopentanones.
3. The 2-arylidenebenzocycloalkanones showed a very low inhibition, except the 2-pyridyl compound.

### **Conclusions**

Although some synthesized compounds could achieve almost the same inhibitory concentrations as the plant phenolics, we could not synthesize better compounds so far. To find an effective lead molecule in our further studies, the QSAR (Quantitative Structure-Activity Relationships) analysis technique may also be applied.

## **I.3. The effect of ketone bodies on MIF's phenylpyruvate activity**

While screening the effects of experimental-phase anti-inflammatory agents on MIF phenylpyruvate tautomerase, we have identified acidic CH groups as markers of inhibitory enzymatic activity. Guided by this experience we have sought simple model molecules with strong acidic CH groups to test their potential inhibitor activity. Among these substances acetylacetone was of particular interest, because this substance is known to undergo tautomerisation (Watarai and Suzuki, 1974). Akin to acetylacetone, other ketone bodies – acetoacetate,  $\beta$ -hydroxybutyrate and acetone – are also CH acidic, and affect migration of bovine leukocytes *in vitro* (Suriyasathaporn et al., 1999), hence they might curb immune

competence and possess anti-inflammatory activity, too (Sato et al., 1992; Sjögren et al., 1999). Therefore, we have studied the effects of ketone bodies on MIF's enzymatic activity.

## Results

1. Ketone bodies are able to differentially inhibit MIF phenylpyruvate tautomerase *in vitro*. Against phenylpyruvate ketonase activity of MIF, acetylacetone and 2-propionyl cyclohexanone exhibited the strongest inhibitor potency, benzylideneacetone, acetoacetate,  $\beta$ -hydroxybutyrate showed moderate inhibition, while acetone and ethyl acetoacetate exhibited a still weaker inhibitory activity.
2. The phenylpyruvate enolase activity of MIF has not been affected up to 10 mM of the ketone bodies.

## Conclusions

Normal serum levels of acetoacetate and  $\beta$ -hydroxybutyrate are in the 70–150  $\mu$ M range (Kalapos et al., 2003), hence their „*in vivo* inhibitory action on the ketonase of MIF tautomerase” could be postulated concerning the IC<sub>50</sub> levels of 14 and 70  $\mu$ M obtained *in vitro* here for acetoacetate and  $\beta$ -hydroxybutyrate, respectively. This phenomenon together with MIF's action as a cytokine could have a role in bacterial infection, a situation counteracting the elevation of ketone body levels characteristic of fasting animals (Neufeld et al., 1976).

### **I.4. Certain lipopolysaccharides can affect the MIF's phenylpyruvate activity**

The LPS bind in monocytes, neutrophils and to macrophages across LPS binding protein (LBP) to the CD14 molecule (Haziot et al., 1997). This binding represents a signal to the Toll-like receptor-4 (TLR4), which has an effect to enhance the expression of MIF (Roger et al., 2001). With regard to the MIF-LPS relationship, we have assumed that LPS might have a direct influence on MIF's tautomerase activity.

Therefore, whole LPS and certain parts of it derived from different bacteria were tested in our *in vitro* system. LPS prepartes were donated by Béla Kocsis (Department of Medical Microbiology and Immunology). The used LPS concentrations were: 40, 20 and 2pg/l which correspond to the toxic, intermediate and subclinic concentrations, respectively in the blood.

## Results

1. LPS can stimulate or inhibit the tested reaction in a concentration dependent manner.
2. Whole LPS of *E. coli* and *Salmonella sonnei II* can stimulate the MIF tautomerase reaction at 39°C.
3. Whole LPS of *Salmonella adelaide* inhibited both the ketonase and the enolase reactions. Nevertheless the Lipid-A compounds, responsible for the toxic effect, stimulated the enzyme reactions.

## Conclusions

LPSs with different structures brought influence to bear on MIF tautomerase. The direction of the effects depends on the LPS structure.

### I.5. Flavonoids and phytoestrogens affect the phenylpyruvate activity of MIF

The type II estrogen binding site discovered in 1978 from rat uteri can bind not only the estrogen (Eriksson et al., 1978), but also has an ability to bind some flavonoids (Markaverich et al., 1988). This binding site reportedly co-purifies with a tyrosinase-like enzymatic activity from rat uterine nuclear fraction (Garai and Clark, 1992). Its substrate specificity was akin to MIF's tautomerase. In rat uterine nuclear extract, featuring type II sites, a tautomerase activity resembling to MIF has been detected (Garai et al., 2001), which flavonoid ligands inhibited MIF tautomerase also (Garai and Adlercreutz, 2004). These data support the possible participation of MIF in type II estrogen binding phenomena.

Therefore some ligand compounds of the type II estrogen binding site were investigated on MIF phenylpyruvate tautomerase. Furthermore some phytoestrogen known to have beneficial influence on bone mineral density (BMD) were also tested (Fanti et al., 1998). Some further flavonoids (without phytoestrogenic effect) were also tested, whose chemical structure resemble either to the phytoestrogens (eg. naringenin to genistein), or the earlier tested plant phenolics.

## Results

1. Among the type II estrogen binding site both the synthetic estrogen (diethylstilbestrol (DES)) and the estrogen metabolite 2-methoxy-estradiol (2MeOHE2) showed partial inhibition.

2. Among the phytoestrogens the best inhibitor was daidzein ( $IC_{50}=16,33 \mu M$ ), then its metabolite, equol, finally the genistein.
3. The tested flavonoids showed different  $IC_{50}$  in the ketonase and in the enolase reaction. For instance the inhibitory capacity of morin, appeared in *Morus alba*, was two time better in the enolase than in the ketonase reaction. In spite of that, naringenin, founded in citrus fruits, has weaker capability in the enolase.

## **Conclusions**

The tested phytoestrogens and flavonoids are also capable to inhibit the MIF phenylpyruvate activity, but their potency has lagged behind those of anti-inflammatory plant agents.

Although the biological role of MIF enzyme activity is obscure, it can be important, that equol, which play an important role in maintaining the BMD, was more effective in the enolase reaction than the other compounds.

## **II. ANALYSIS OF QUANTITY AND ACTIVITY OF MIF IN HUMAN SAMPLES**

The concentration of MIF is elevated both in the synovial fluid-derived from rheumatoid arthritis, and in the peritoneal fluid- derived from patient suffered in endometriosis, but the enzymatic activity of MIF has not tested yet.

### **II.1. The quantity and activity of MIF in sera and in synovial fluids of patients with arthritis**

MIF is 5-10 time elevated in the serum and synovial fluids of patients with RA, compared to healthy patients (Leech et al., 1999; Onodera et al., 1999). An association between RA disease activity and synovial MIF content in patients with RA was also reported (Morand et al., 2002).

Volunteers suffering from arthritis were investigated in the Department of Immunology and Rheumatology. Fluid samples were taking by joint puncture and parallel to this blood was also taken. The samples were sorted as follows: Rheumatoid arthritis (RA): n=13; Seronegative Spondylarthritis (SNSA): n=10; Arthrosis n=8; Arthritis urica n=4. The MIF immunoreactivity and tautomerase activities were tested.

## Results

1. MIF levels were elevated both in the sera and in the synovial fluids. Comparison the synovial fluids of different diseases, the highest MIF concentration was measured in SNSA. In case of SNSA the serum MIF content was significant higher in relation to RA (\*:p<0.05), or arthrosis (\*\*:p<0.01). MIF content pro protein gave similar results.
2. Despite of the elevated MIF levels only the phenylpyruvate enolase activity was detectable. To see decrease in absorbance in case of ketonase reaction we had to dilute the samples.
3. MIF inhibitors were effective on synovial fluid's enolase activity.
4. MIF was confirmed with Western blot also, but immunoreactivity. MIF Western blots showed not only dimer (25 kDa) and trimer (37 kDa) MIF, but a high molecular weight band in 130 kDa also.

### II.2. The quantity and activity of MIF in peritoneal fluids (PFs) in endometriosis

Elevated level of MIF immunoreactivity was found in PF of women with endometriosis especially with active disease at the early I and II stages (Kats et al., 2002). MIF were detected by immunohistochemistry in the endometrium of women with endometriosis and its cycle dependence has been noted (Akoum et al., 2002). The levels and enzymatic activities of MIF were examined from PFs, gained during diagnostic laparoscopy from volunteers in the Baranya Country Hospital Department of Obstetrics and Gynecology. (Control: n=19; I. std: n=22; II. std: n=8; III. std: n=6; IV. std: n=3)

## Results

1. In the peritoneal fluids elevated MIF levels was detected.
2. No correlation could have been obtained between enol-keto conversion rates, on the other hand, compelling correlation was found between phenylpyruvate keto-enol conversion rates and MIF immunoreactivities.
3. Both of these parameters show significantly higher values in PFs of women with stage I and II endometriosis compared to patients without endometriosis (Garai et al., 2003).
4. MIF inhibitors had almost the same effect on PF's enolase activity than in MIF tautomerase.
5. MIF Western blots showed positive bands at 170, 130, 45 kDa also. At 37 kDa a faint band was also in some cases appreciable.

## Conclusions

Both in synovial fluids and in PFs only the enolase activity was detectable. Its rate was correlated with MIF immunoreactivities assessed by Duo-Set MIF ELISA. It is interesting, because ketonase (exergon) and not enolase reaction is spontaneous. We could assume that something (eg.protein) inhibit the free (ketonase) way in the human samples. The finding of detectable ketonase rection after sample dilution might confirm our thesis.

In both human studies 130 and 45 kDa bands were detected with Western blot. These results suggest that MIF forms a complex with several different proteins. Almost the same result was written by Vera and coworkers (2005). They detected MIF at 170, 130 and 12 kDa from intraluminal fluid during bladder inflammation in the rat; they identified the associates' protein as a member of the  $\alpha$ 2-makroglobulin proteinase inhibitor family.

### II.3. Changes in serum MIF level after laparotomy

Within 6 hours of bacterial inoculation the MIF production increases in a lot of different cells (in hepatic cells also) (Meinhardt et al., 1997). Gando reported the highest MIF levels following hepatectomy and this was explained by the surgical trauma induced hypothalamo-hypophyseal activation and also by the liberation of the endotoxin (Gando et al. 2001) and associated with an increased level of ACTH, cortisol and MIF.

In our prospective, descriptive study the induction of MIF levels was assessed in cancer patients undergoing bowel resection (elective bowel opening) in comparison with cancer patients undergoing liver resection (no surgical bowel opening), and its correlation with early postoperative morbidity and mortality was evaluated. Our study was carried out in cooperation with the Department of Anesthesia and Intensive Therapy and the Department of Clinical Chemistry.

This study was carried out with 28 volunteers. Group A included cancer patients who had liver resection without surgical bowel opening (10 patients), in group B patients underwent bowel resection and surgical bowel opening (18 patients). The levels of MIF, TNF $\alpha$ , IL-1 $\beta$ , IL-8, prealbumin, albumin, fibrinogen and CRP were monitored prior to the procedures ( $t_0$ ), immediately after the surgery ( $t_1$ ) and on three consecutive postoperative days ( $t_2$ ,  $t_3$ ,  $t_4$ ). Our laboratory tested only the serum MIF level.

## **Results**

1. In both study groups the preoperative MIF values (MIF<sub>0</sub>) were found to be within the normal range and there was no significant difference between the two sets of patients.
2. Immediately after the operations (MIF<sub>1</sub>) the values measured in those patients undergoing hepatic resection were significantly higher.
3. On the first postoperative day normal values of MIF were measured in both study groups, and these values did not increase either on the second, or the third postoperative day.

## **Conclusions**

Since both study groups comprised cancer patients and the preoperative MIF values did not differ, we assume that highly elevated immediate postoperative MIF levels of patients undergoing liver resection are explained by tissue damage related to the release of MIF from hepatocytes rather than by inflammatory stimulus driven secretion (Márton et al., 2005). Further studies are warranted to delineate the postoperative kinetics of MIF in different surgical procedures.

### **III. *IN VIVO* TESTING OF CAFFEIC ACID, AS AN EFFECTIVE MIF INHIBITOR IN MOUSE ARTHRITIS MODEL**

#### **Introductions**

Leech et al (1998) reported a role for MIF in the evolution of a more severe model of RA, rat adjuvant-induced arthritis. MIF was increased in the synovial tissues and serum of rats with arthritis. Our best tautomerase inhibitor, caffeic acid was tested in CFA arthritis. This compound is used by conventional medicine to treat arthritis. Experiments were performed on male Balb/c mice in the Department of Pharmacology and Pharmacotherapy.

Caffeic acid 10mg/kg (n=7) and 100 mg/kg (n=7) was injected intraperitoneally twice a day, and 10% DMSO, 0,9%-os NaCl solvent was given to the control group (n=7). The left tibiotarsal joints were excised after killing the animals on the 21st day after CFA administration, and the IL-1 $\beta$  levels were measured.

#### **Results**

1. The volume of the CFA-injected paw was doses dependent significantly inhibited by caffeic acid (\*: p<0.05, \*\*: p<0.01) in both legs. 100 mg/kg caffeic acid could also inhibit the degree of edema even in the non-treated paw.

2. Mechanical allodynia, developing after CFA induction, was significantly (\*\*\*: $p < 0.001$ ) inhibited on the treated paw by 10 mg/kg, and 100 mg/kg caffeic acid. Dose effect connection was also observed in some days.
3. Caffeic acid treatment could also decrease the IL-1 $\beta$  levels in both treated (left) and untreated (right) tibiotarsal joint homogenisate comparison to the control. This inhibitory effect was potent in both concentrations on left leg. On the untreated leg only 100mg/kg showed significant inhibition.

### **Conclusions**

Caffeic acid, which was a potent MIF tautomerase inhibitor *in vitro*, *in vivo* reduced the paw edema, mechanical allodynia and inflammatory cytokine production in CFA arthritis. In spite of that, our preliminary study could give only indirect proof on the connection between MIF (participate in RA) and its tautomerase inhibiting small molecule. Further histological examinations are in process to confirm the anti-inflammatory effect.

## **IV. STUDIES ON THE CONNECTION BETWEEN MICROTUBULE FUNCTION AND MIF**

### **Introduction**

A lot of indirect data are available on the connection between MIF and microtubule function. Low concentrations of colchicine or vinblastine markedly enhance the migration of guinea pig macrophages causes, and counteract the action of MIF (Pick and Abrahamer, 1973; McCarthy et al., 1979). 16 hours MIF exposition increases (150-200%) significantly the proportion of tubulin present in polymeric form (Pick et al., 1979). Since inhibition of cell motility is accompanied by the elevation of polymerized form of tubule, it may be assumed that intact microtubule is needed for MIF's migration inhibitory effect.

Lots of proteins including MIF can bind to amyloid  $\beta$ -protein (A $\beta$ ) in Alzheimer's disease, which attach pathophysiological importance to plaque formation and/or accumulation (Oyama et al., 2000). Acid denaturing conditions were found to readily induce MIF to undergo amyloid fibril formation (Lashuel et al., 2005). Curcumin, our potent tautomerase inhibitor, prevents fibril and oligomer formation and suppress amyloid accumulation in Alzheimer's disease (Lim et al., 2001).

In rat brain MIF expression was observed in numerous neurons (Bacher et al., 1998). The intracisternal injection of LPS increased MIF mRNA and protein expression in brain (Bacher et al., 1998). So, in the brain not only baseline but also inducible MIF expression is present, but the MIF function in the brain is not known. These observations raise the possibility that MIF participates in a detoxification pathway for catecholamine products (Matsunaga et al., 1999).

We wished to get answer to the following questions:

1. Can we detect MIF immunoreactivity in tubules, isolated from porcine brain?
2. Does the tubule purification performed through more cycles abrogate the immunoreactivity of MIF?
3. Has showable enzyme activity of the tubule after one cycle, include large MAP? /Microtubule associated proteins are stabilizing/destabilizing proteins, bounded to the cytoskeletal polymer./
4. Does the enzyme activity change with the elevation of the purification cycles?
5. How does the MIF amount pro protein changes after purification cycles?
6. How do the best inhibitors of MIF influence the tautomerase activity of tubule with large MAP?
7. Have affect these small molecules on tubule assembly?
8. Does MIF discoverable in porcine brain, and in which cells?
9. Does MIF tubule connection detectable with morphology methods?

## **Results**

1. MIF could be detected in our tubule preparation by Western blot and by ELISA.
2. Western blot analysis showed that anti-MIF immunoreactivity is decreased in three cycles tubule and supernatant, moreover no band can be detected in the third cycle.
3. We could detect similar, two way enzyme activity parallel to MIF tautomerase in our tubule preparation containing large MAP.
4. The higher the cycle number, the lower the ketonase and enolase activity in tubules and in supernatants.
5. MIF content pro protein is highest after the first and lowest after the third cycle.
6. The small molecules could inhibit phenylpyruvate ketonase and enolase activity of the tubule preparations in a concentration dependent manner. The  $IC_{50}$  values found on MIF and tubule correlated to each other, but  $IC_{50}$  values are higher on tubule. The best inhibitor

of the enolase reaction was caffeic acid. As compared to other substances used, acetaminophen could inhibit tubule tautomerisation only when applied in higher concentrations.

7. Most plant derived compounds inhibited, but acetaminophen, stimulated the tubule assembly.
8. Immunohistochemistry of porcine brain showed that MIF is localized both in inhibitory and in stimulatory neurons of the cortex.
9. Electronmicroscopic studies showed that brain cells having MIF immunoreactivity were *de facto* neurons. MIF immunopositivity was marked in dendrites and in axons containing a lot of parallel microtubule.

### **Conclusions**

Our results might assume that intracellular MIF can behave as a kind of MAP, furthermore in the anti-inflammatory effect of the tested small molecules might play a role their affect on MIF tautomerase and tubule assembly.

Results of light and electronmicroscopy confirm the biochemic achievement to us that MIF and microtubule might compose a functional unit.

MIF immunoreactivity is found in stimulatory and inhibitory neurons alike, a phenomenon difficult to explain. Nevertheless, similar to MIF, other proteins (eg. calbindin) may show a same distribution (Baimbridge et al., 1992).

## **V. POSTMENOPAUSALIS OSTEOPOROSIS PREVENTIONS WITH PHYTOESTROGENS**

### **Introductions**

The role of MIF in osteoporosis was investigated by comparing effects in wild type, ovariectomized (OVX) and MIF knock-out (KO) mice. According to the results MIF is required for the bone loss induced by estrogen depletion after OVX. While MIF has the properties of inducing and regulating the production of cytokines (Baugh and Donnelly, 2003), it may be supposed that there is an up-regulation of bone-resorbing molecules after OVX is, at least in part, triggered by the rapid increase in serum MIF (Oshima et al., 2006).

Nowadays there is praxis to try preventing postmenopausal osteoporosis by hormone replacement therapy, but since it could have many side effects, an alternative method, the use of phytoestrogen compounds has become more and more popular.

In a dietary intervention study, our aim has been to assess the effect of phytoestrogens and of specific exercise program on the progression of osteoporosis in menopausal population. Intervention was carried out with a special seedy cake, containing 32% soy, and 23% linseed. Menopausal volunteers have been asked to consume 100-120 g of this cake. Depending on their choice, the volunteers were assigned into different groups. Control (without intervention) (n=21), seedcake consumers (n=15), exercise group (3x1 hrs weekly, once with a physiotherapist) (n=6), seedcake consumption combined with exercise (n=14). Phytoestrogen serum levels (genistein, daidzein, equol, o-desmethyl-angolensine (O-DMA) and enterolacton) were monitored quarterly by using the TRFIA (DELFI) kit in Helsinki. Bone mineral density (BMD) was monitored by DEXA (Dual-Energy X-Ray Absorptiometry) in the Central Clinical Radioisotope Laboratory. There is concern about the possible goitrogenic effect isoflavonoids (Divi, 1997), therefore, TSH levels were also monitored.

## **Results**

1. Those who consumed seedcakes have attained highly elevated serum levels of the phytoestrogens after quarter.
2. The level of the most potent equol was also elevated several fold above control. There was a considerable inter-individual variation especially in the cases of equol and ODMA levels, a phenomenon probably explained by differences in individual gastrointestinal metabolism. According to this, ~80-90% of the population is ODMA producer, (Kelly et al., 1995; Arai et al., 2000), and ~33-50% of them may be so-called equol producer (Rowland et al., 2000; Lampe et al., 2001; Setchell et al., 2002).
3. As for bone density, one year follow-up is probably insufficient to draw definite conclusion. No significant difference has been detected between the groups, but at least there was no obvious fall in BMD levels.
4. In a separate evaluation of the equol producers, phytoestrogen consumption had beneficial effect on BMD levels especially in the hip and lumbar region.
5. In spite of the almost similar basic levels of the BMD in the ODMA producers, seedcake consumption combined with exercise could significantly ( $p < 0.05$ ) inhibit BMD decrease in the lumbar region. In the hip region not only the combined intervention, but even phytoestrogen biscuits alone had a significant effect on BMD.
6. TSH levels remained in the normal (0.5-5.0  $\mu\text{U/ml}$ ) range throughout the study.

## **Conclusions**

Starting levels of phytoestrogen showed that Hungarian diet contains very low levels phytoestrogens. A rise in phytoestrogen intake could achieve notable improvement in the public health indices. Ours was the first study, in which, the serum levels of five phytoestrogens have been followed.

As far as bone density is concerned, a one year follow-up may be insufficient to draw definite conclusion, but it seems to be promising that BMD levels failed to decrease in equol and ODMA producers during the dietary intervention. In subsequent studies combined dietary and exercise intervention programs might prove useful tools as a prevention of bone loss even after menopause.

## SUMMARY OF NEW FINDINGS

- I. Investigation of the phenylpyruvate enzyme activity of MIF
  - I.1. Acetaminophen could inhibit not only MIF's dopachrome (Senter et al., 2002), but also phenylpyruvate tautomerism (Molnár and Garai, 2005). The latter effect could be inhibited by anti-inflammatory small molecules in a concentration dependent manner.
  - I.2. Although some synthesized compounds could achieve almost the same inhibitory concentrations as the plant phenolics, the synthesis of more effective compounds is needed.
  - I.3. Ketones without ring structure seemed to inhibit *in vitro* MIF's ketonase activity effectively (Garai et al., 2005).
  - I.4. LPSs with different structures brought influence to bear on MIF tautomerase. Whether the effect is stimulatory or inhibitory depended on LPS structure.
  - I.5. The most effective compound among those tested to inhibit MIF' phenylpyruvate tautomerase was the phytoestrogen daidzein.
- II. MIF and its enzyme activity could also be detected in human samples, such as synovial fluid and peritoneal fluid, but only the activity of enolase proved to be significant.
  - II.1. Enolase activity and immunoreactivity of PFs were higher stages in I and II of endometriosis as compared to the control samples (Garai et al., 2006).
  - II.2. Extremely high MIF levels were measured in synovial fluids and sera of patients with Seronegativ Spondylarthritis.
  - II.3. Immediately after the operations (MIF<sub>1</sub>) the protein values measured in patients undergoing hepatic resection were significantly higher than in controls. On the first postoperative day MIF reached the normal values in both study groups (Márton et al., 2005).
- III. Edema and paw algesia were decreased *in vivo* after caffeic acid administration in CFA arthritis. This can only be regarded as an indirect proof for the relationship between MIF and its small molecule inhibitor.
- IV. Small molecules and flavonoids inhibited tubule-MIF's ketonase and enolase activity in a concentration dependent manner some of the molecules inhibiting, others stimulating the tubule assembly. According to the results of multiple purification cycles we might assume that MIF can behave as a kind of MAP that can be removed in the course of strong purification procedure. According to our morphologic results MIF is localized both in

inhibitory and in excitatory neurons. In polymerized tubules MIF is located around the tubule cross section as small vesicles.

- V. In our intervention study the effects of elevated the phytoestrogen levels were investigated. In human cases characterized as ODMA or equol producer a positive effect on BMD was expected as found in the lumbar and hip region according in postmenopausal populations.

Although MIF has been discovered in the 1960s, it was intensively investigated only in the past 15 years or so. Still, its receptor has not yet been identified and several of its functional aspects still awaits for clarification. The present thesis is meant as a summary of our modest contribution to learn more of this multifaceted protein.

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