Mepacrine Inhibits Matrix Metalloproteinases-1 (MMP-1) and MMP-9 Activation in Human Fibroblast-like Synoviocytes

KARL M. STUHLMEIER

ABSTRACT. ***Objective.*** Matrix metalloproteinases (MMP) are enzymes known to be involved in normal physiological and in many pathological conditions. Rheumatic diseases are among the ailments where MMP have been shown to exert detrimental effects. Mepacrine is used alone or in combination with other drugs to treat lupus and other rheumatic diseases. We investigated whether mepacrine's beneficial effects might be due to its influence on MMP.

Methods. Human fibroblast-like synoviocytes were used to study the effect of mepacrine on phorbol myristic acetate (PMA) induced activation of MMP. Western blot, reverse transcription-polymerase chain reaction, and electrophoretic mobility shift assay (EMSA) experiments were used to investigate the effect of mepacrine.

Results. Mepacrine selectively inhibited MMP in human fibroblast-like synoviocytes. Mepacrine inhibited MMP-1 as well as MMP-9, but had no effect on MMP-3 at the mRNA level. Possible mechanisms to explain these findings were investigated, and it was found that mepacrine had a strikingly different effect on c-Jun, as opposed to c-Fos activation. While mepacrine treatment alone led to increased concentrations of c-Jun within the nuclear compartment, c-Fos translocation into the nucleus was blocked in synoviocytes treated with mepacrine and stimulated with PMA. Accordingly, EMSA showed reduced AP-1 binding in mepacrine treated synoviocytes. These results imply that the observed effects of mepacrine on immediate early genes resulted in reduced AP-1 binding. That ultimately led to a selective suppression of genes that rely on unhindered assembling of these transcription factors for their activation.

Conclusion. Selective inhibition of MMP by mepacrine might in part explain the beneficial effects of this drug in the treatment of certain diseases. (J Rheumatol 2003;30:2330–7)

Key Indexing Terms:
MATRIX METALLOPROTEINASE MEPACRINE QUINACRINE GENE REGULATION
HUMAN FIBROBLAST-LIKE SYNOVIOCYTES TRANSCRIPTION FACTOR

Matrix metalloproteinases (MMP) are a family of over 20 enzymes that play an important role in normal physiological as well as in many pathological conditions. In healthy individuals, a finely tuned balance between tissue/matrix degradation and synthesis must be maintained. An imbalance in tissue remodeling will lead to a variety of pathological manifestations. Accordingly, MMP are involved in nearly all aspects of life — normal fetal development, wound healing, normal as well as pathological cell migration, angiogenesis, the activation of cytokines, intercellular communication, and apoptosis[1,2]. MMP play a key role in pathological cell growth and in the development of many forms of cancer[3-9].

Rheumatic diseases are another cluster of ailments in which MMP are involved in disease progression[10-13]. Among the many forms of MMP, collagenase (MMP-1) has been among the first molecules shown to be able to induce arthritis[14]. Since then, many reports have described the presence of active MMP-1 in synovial fluid as well as in cartilage samples of patients with rheumatoid arthritis (RA) or osteoarthritis (OA). Moreover, MMP-1 has also been implicated in the pathologic loss of articular cartilage[15-20].

Mepacrine (quinacrine) alone or in combination with other drugs has been used for many years to treat certain forms of rheumatic diseases. Interestingly, mepacrine, originally employed as an antimalarial drug, has recently been proposed as a possible remedy to treat prion caused diseases, including Creutzfeld-Jakob disease[21].

High doses of mepacrine are known to inhibit phospholipase A_2 (PLA_2)[22]. For a long time it has been assumed that most of the beneficial effects of mepacrine are due solely to this effect. Only recently is it becoming evident that mepacrine exerts many effects independently of its inhibition of PLA_2. Sargent, *et al*[23], for example, concluded that the cardioprotective effect of 1–50 µM mepacrine in an

From the Ludwig Boltzmann Institute for Rheumatology and Balneology, Vienna, Austria.

Supported by the Austrian Ministry of Social Security and Generations (GZ. 236.065/9-VIII/A/6/00 and 236.065/7/VIII/A/6/00) and the City of Vienna, Austria.

K. M. Stuhlmeier, PhD.

Address reprint requests to Dr. K.M. Stuhlmeier, Ludwig Boltzmann Institute for Rheumatology and Balneology, Kurbadstrasse 10, PO Box 78, A-1107 Vienna-Oberlaa, Austria. E-mail: karlms@excite.com

Submitted December 2, 2002; revision accepted April 24, 2003.

isolated rat heart model is independent of possible effects of mepacrine on PLA_2. Bugge, *et al* demonstrated that mepacrine did not inhibit PLA_2 while protecting the heart during hypoxia and reoxygenation[24]. In other reports, the modes of action of the well documented antiinflammatory properties of mepacrine also seem to be less clear but likely independent of the inhibition of PLA_2. This drug has also been shown to affect polymorphonuclear leukocyte activation and migration[25,26] and prevent cyclosporine-induced nephrotoxicity[27], and has even been shown to stimulate PLA_2 activity in polymorphonuclear cells[28].

Mepacrine has been available for more than 60 years and has been administered to millions of individuals with no significant side effects, while its antirheumatic properties have been well documented[29,30]. In the management of patients with lupus, use of mepacrine, alone or in combination with other drugs, has had some success[31-34]. Due to the reported beneficial effects of mepacrine and because MMP are important in the progression of rheumatic disorders, we investigated to what degree MMP might be influenced by this substance. For comparative reasons, we included several other MMP; however, the focus of this work was on MMP-1.

MATERIALS AND METHODS

Reagents. Mepacrine (quinacrine) (6-chloro-9-[4-diethylamino)-1-methylbutyl]amino-2-methoxy-acridine dihydrochloride; lipopolysaccharide (LPS) (*Escherichia coli* serotype 055:B5); protease inhibitor cocktail; phorbol myristic acetate (PMA); TRIzol; and MTT were from Sigma (Sigma Chemical Co., Vienna, Austria). Anti-MMP antibodies were from NeoMarkers, Fremont, CA, USA. The mouse monoclonal antibodies anti-MMP-1 [AB-5 (clone III12b)], recognizing doublets of 52 kDa/57 kDa and 42 kDa/47 kDa, which are identified as glycosylated and unglycosylated species of latent and active forms of MMP-1; anti-MMP3 (clone SL-1 IIIC4), recognizing latent and active forms of MMP-3; and the anti-MMP-9 (clone IIA5), recognizing proteins of 92 kDa and 86 kDa (the latent and activated forms MMP-9), were used. The antitubulin antibody [tubulin AB-4 (clone DM1A + DM1B)], recognizing alpha-tubulin at 57 kDa and beta-tubulin at 54 kDa, was from NeoMarkers.

Anti-c-Jun [c-Jun/AP-1(D): sc-44], and anti-c-Fos [c-FOS(6-2H-2F): sc-447] antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA, USA, or from Sigma (rabbit anti-AP-1/c-Jun). Horseradish peroxidase-conjugated secondary antibodies (ImmunoPure goat antimouse IgG, #31430; goat antirabbit IgG, #31461; and rabbit antigoat IgG, #31403) and the bicinchoninic acid protein assay (BCA) kit were from Pierce Co. (Rockford, IL, USA).

Cell culture. Human fibroblast-like synoviocytes isolated from patients with RA were a gift from Dr. G. Partsch, Vienna. Synoviocytes were cultured as described[35]. In brief, synoviocytes were cultured in T75 tissue culture flasks or culture dishes (10 cm diameter) in RPMI-1640, supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), L-glutamine, and 50 U/ml penicillin/streptomycin. Medium was changed every 3 days. Cells were split at a ratio of 1:3 and used at passage numbers from 3 to 9. For experiments, cells were transferred to 6-well plates (for polymerase chain reaction, PCR) or 15 cm diameter tissue culture dishes (for Western blotting). Complete medium [10% fetal calf serum (FCS), L-glutamine, penicillin/streptomycin] was replaced with medium without FCS or with medium containing 1% FCS 16 h before experiments. The inhibitory effects of mepacrine were independent of the amount of FCS used.

Viability assay. Viability of cells was confirmed after harvesting by staining aliquots of mepacrine-treated cells with a trypan blue solution. Further, in some cases, cells were incubated up to 6 h with mepacrine (50 µM), after which the medium containing mepacrine was replaced with complete medium (without mepacrine). Viability of these cells was monitored in intervals ranging from 4 to 48 h. At the conditions and concentrations used, viability of synoviocytes was not affected by mepacrine.

Western blot experiments. Proteins in culture supernatant were recovered by trichloroacetic acid precipitation[36]. Cytoplasm and nuclear extract were prepared as described[37]. Equal amounts of proteins were combined with reducing Laemmli sample buffer and separated on 10% polyacrylamide gels. Gels were blotted onto Immobilon-P transfer membranes (Millipore, Vienna, Austria); nonspecific binding was blocked by incubating the blots in phosphate buffered saline (PBS)-Tween-20 solution containing 5% nonfat milk for 45 min at 25°C (or overnight at 4°C). Each lot of primary antibody was tested and diluted accordingly (1:500 to 1:2000). Secondary antibodies were used at a dilution of 1:5000. Antibodies were diluted in PBS-Tween-20, 5% nonfat milk. Blots were incubated 1 h in each antibody solution, then extensively washed in PBS-Tween-20 and exposed to chemiluminescence substrate for 1 min. Bands were visualized by exposure of blots to x-ray films for times ranging from 10 s to 45 min.

Electrophoretic mobility shift assay (EMSA). Nuclear extract was prepared as described[37], with the exception that 0.7% Igepal was used. The double-stranded, blunt-ended oligonucleotides used in all experiments were end-labeled using T4 polynucleotide kinase and (^{32}P)ATP. Oligonucleotides for AP-1 (5'-CGC TTG ATG AGT CAG CCG GAA-3') were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After labeling, 5 µg of nuclear extract were incubated with 50,000 cpm of labeled probe in the presence of 1.5 µg poly(dI-dC) at 4°C for 30 min, followed by separation of this mixture on a 6% polyacrylamide gel in Tris-glycine-EDTA buffer at pH 8.5. For specific competition, 5 pmol unlabeled AP-1 oligonucleotides were included in the reaction. Gels were transferred to 3MM filter paper using a vacuum device and exposed to x-ray films.

PCR. RNA was isolated using TRIzol according to manufacturer's instructions. RNA was resuspended in Tris-EDTA buffer, quantitated on a spectrophotometer, reverse transcribed (RT-PCR kit, Amersham Biosciences, Freiburg, Germany), and stored at –60°C. A Techne cycler (Techgene, Cambridge, UK) was used for PCR under the following standard conditions: initial denaturation, 5 min at 94°C; annealing, 1 min at indicated temperatures; amplification, 1 min at 72°C; denaturation, 1 min 94°C; followed by final extension at 72°C for 5 min. GAPDH was used as control. Primers were from MWG Biotech AG (Ebersberg, Germany), and were dissolved at a concentration of 100 pmol/µl in Tris-EDTA. Primer sequences: MMP-1: 5'-CTG AAG GTG ATG AAG CAG CC-3' and 5'-AGT CCA AGA GAA TGG CCG AG-3'; MMP-3: 5'-CTC ACA GAC CTG ACT CGG TT-3', and 5'-CAC GCC TGA AGG AAG AGA TG-3'; MMP-9: 5'-CGC AGA CAT CGT CAT CCA GT-3' and 5'-GGA TTG GCC TTG GAA GAT GA-3'. GAPDH: 5'-TCA AAG GCA TCC TGG GCT ACA-3' and 5'-GAG GGG AGA TTC AGT GTG GTG-3'. PCR was carried out in 0.5 ml tubes containing a total of 50 µl of the following reagents: 5 µl (10× buffer), 1 µl dNTP, 2 µl each (forward and reverse) primer, 1 µl TAQ polymerase (5 U final) cDNA, and double distilled H_2O. Different numbers of cycles were tested regularly to ensure linear-phase amplification of the cDNA. Fragments for GAPDH were amplified using 20 to 24 cyles, and for amplification of MMP 29 to 34 cycles were used. The lengths of amplified fragments were: MMP-1, 428 bp; MMP-3, 294 bp; and MMP-9, 406 bp. Aliquots of PCR were separated on agarose gels and scanned and analyzed on a Fluorimager 595 (Amersham Biosciences).

RESULTS

MMP-1 release and protein synthesis is inhibited by mepacrine. Stimulation of human fibroblast-like synoviocytes with PMA or tumor necrosis factor-α (TNF-α) leads to the release of MMP. First, we tested whether preincubation

of synoviocytes has any effect on PMA or TNF-α induced release of MMP-1. Synoviocytes were kept in serum-free medium for 16 h, then mepacrine (75 µM) was added. PMA (33 ng/ml) or TNF-α (10 ng/ml) was added 45 min later. Cells thus treated were incubated an additional 16 h, then the supernatant was taken off, centrifuged at 12,000 g for 10 min, aliquoted, and stored for further analyses at –60°C. Proteins in the supernatant were precipitated using TCA, resuspended, and quantitated using the BCA kit. Equal amounts of proteins were mixed with reducing Laemmli sample buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described in Materials and Methods. Figure 1 shows representative data illustrating that stimulation of synoviocytes with PMA or TNF-α for 16 h leads to enhanced release of MMP-1 into the cell culture medium. The figure also shows that preincubation of synoviocytes for 45 min with mepacrine reduces PMA (MEP+PMA lane) as well as TNF-α (MEP+TNF) induced MMP-1 secretion to background levels detected in unstimulated cells (MED). These effects are dose dependent, as lower doses of mepacrine led to lesser reduction of PMA or TNF-α-induced MMP-1 release (data not shown).

Next, we investigated whether mepacrine affects the release of MMP-1 or interferes with protein synthesis mechanisms of this proteinase. Western blot experiments were performed using cytoplasm extract of synoviocytes. Cells cultured overnight in serum-free medium were preincubated with mepacrine (10 or 50 µM) for 30 min. PMA (33 ng/ml) was added for 3 h. Cells were washed with ice-cold PBS and removed from culture plates using a rubber policeman. After collecting cells by centrifugation (500 g for 5 min) at 4°C, cytoplasm proteins were collected as described. The data shown in Figure 2 demonstrate that mepacrine interferes with MMP-1 protein synthesis. In cells treated only with PMA for 3 h, increased levels of MMP-1 could be detected

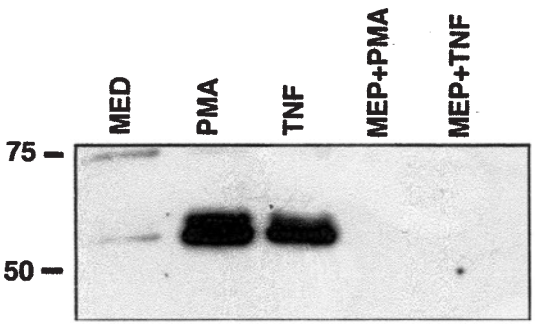

Figure 1. Mepacrine inhibits MMP-1 release. Fibroblast-like synoviocytes were left untreated (MED) or were treated with PMA, TNF, or mepacrine 30 min prior to stimulation with PMA or TNF for 16 h. Supernatant proteins were concentrated and separated by SDS-PAGE. These Western blot data show that mepacrine blocks PMA (MEP+PMA) as well as TNF induced MMP-1 release (MEP+TNF). The antibody used recognized both the pro (latent) and activated forms of MMP-1. Position of size markers is indicated on the left.

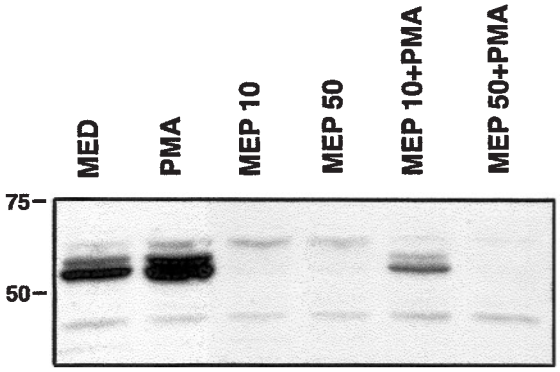

Figure 2. Levels of MMP-1 protein found in cytoplasm of fibroblast-like synoviocytes are suppressed by mepacrine. After preincubation with mepacrine, synoviocytes were stimulated with PMA, and levels of MMP-1 found in cytoplasm were compared to cells left untreated (MED), treated with 10 µM mepacrine (MEP 10), 50 µM mepacrine (MEP 50), pretreated with mepacrine (10 µM) followed by PMA (MEP 10+PMA), or pretreated with mepacrine (50 µM) followed by PMA (MEP 50+PMA). Treatment of synoviocytes with mepacrine reduced basal levels of MMP-1 and suppressed PMA induced MMP-1 dose dependently, as 10 µM was not sufficient to completely block PMA induced MMP-1 production. The MMP-1 antibody used recognizes pro and activated forms of MMP-1. Position of size markers is indicated on the left.

in the cytoplasm (PMA lane). Preincubation of synoviocytes blocked MMP-1 synthesis in a dose dependent manner, as incubating cells with 50 µM mepacrine resulted in complete inhibition of MMP-1 release (MEP50+PMA). Adding smaller amounts of mepacrine led to a less pronounced inhibitory effect. In cells pretreated with 10 µM mepacrine for 30 min, PMA (33 ng/ml, 3 h) was able to induce the synthesis of small amounts of MMP-1 (MEP10+PMA). These low levels of MMP-1 induced by PMA in the presence of mepacrine are still lower than the basal levels of MMP-1 found in untreated cells (MED). That mepacrine could also suppress the basal level of MMP-1 synthesis (which might be elevated due to cell handling, medium changes, etc.) is shown by a comparison of MMP-1 levels in cells cultured in medium (MED) without stimuli with cells treated with mepacrine only (MEP 10 and MEP 50). All Western blot membranes were stained for tubulin, confirming equal loading and protein transfer (data not shown).

PCR reveals selective effects of mepacrine on MMP. To further investigate the mechanisms by which mepacrine interferes with MMP-1 upregulation, PCR experiments were performed. Mepacrine was added to synoviocytes for 30 min, then non-incorporated mepacrine was removed by washing cells once with medium. PMA was added to indicated culture dishes for times ranging from 30 to 120 min, and then total RNA was extracted. RNA (3 µg) was used for reverse transcription performed in a total volume of 33 µl using the Amersham reverse transcriptase kit. From the resulting cDNA, 2.5 µl was used to amplify the housekeeping gene GAPDH and 5 µl each was used to amplify

cDNA using primer pairs for MMP-1, MMP-3, and MMP-9. Figure 3 illustrates findings from one representative experiment, showing that mepacrine suppresses MMP-1 and MMP-9, but does not influence transcription of MMP-3. PMA (33 ng/ml) was added for 2 h to cells pretreated with mepacrine (50 µM) for 30 min. The data reveal that in such experiments, PMA induced upregulation of MMP-1 is suppressed by more than 90% and MMP-9 expression is reduced to levels observed in unstimulated cells. While in these cells basal levels of MMP-3 mRNA are high, as reported[38], there is still a further induction upon PMA stimulation. As illustrated in Figure 3, mepacrine neither reduces basal levels of MMP-3 nor does it affect PMA induced MMP-3 mRNA levels. These data testing the effect of mepacrine on MMP-3 correspond to findings in Western blot experiments (data not shown), which showed that mepacrine did not suppress basal MMP-3 or PMA induced MMP-3 release into the culture medium.

EMSA to test the effect of mepacrine on transcription factor AP-1. AP-1 seems to play an important role in the activation of MMP-1[39]. We tested whether mepacrine had any effect on the AP-1 protein complex using EMSA and nuclear extract isolated from human synoviocytes. Cells were pretreated for 30 min with increasing amounts of mepacrine (20 to 75 µM), washed once with medium, and stimulated with PMA (33 ng/ml) for 2 h. Nuclear proteins were isolated as described[37]. EMSA experiments using consensus AP-1 oligonucleotides and control experiments were performed as described[40]. The data shown in Figure 4 indicate that in mepacrine-treated synoviocytes, AP-1 binding to its consensus element is diminished in a dose dependent manner. Out of several control experiments conducted to confirm specificity of the AP-1-DNA interactions, 2 are illustrated in Figure 4. "Free probe" indicates the lane where no nuclear protein was added, and in the lane marked

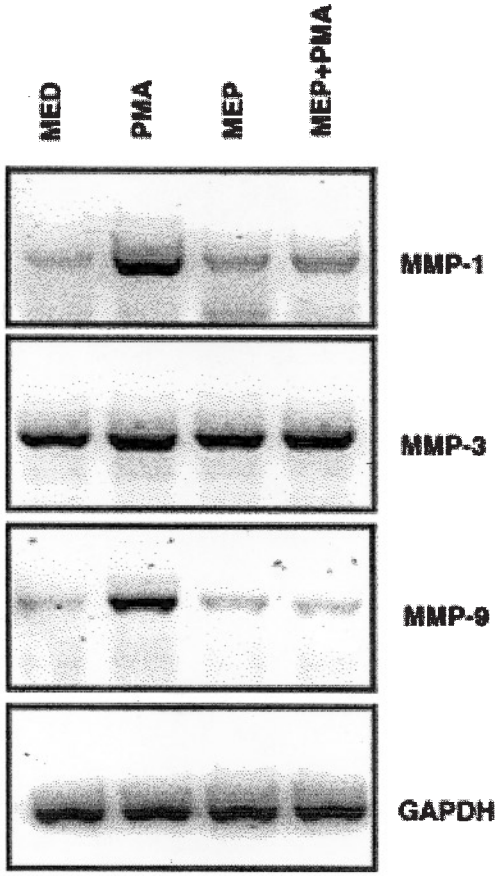

Figure 3. Suppression of mRNA production for MMP-1 and MMP-9 but not MMP-3 by mepacrine. Equal amounts of mRNA were amplified with indicated primer pairs (right side of panels). In unstimulated fibroblast-like synoviocytes (MED), MMP-3 is constitutively expressed, while mRNA levels for MMP-1 and MMP-9 are low. Stimulation with PMA led to increased levels of mRNA for the tested MMP. Mepacrine treatment only (MEP) did not lead to changes of mRNA levels, but prevented induction of MMP-1 and MMP-9 by PMA (MEP+PMA). GAPDH was included as a control.

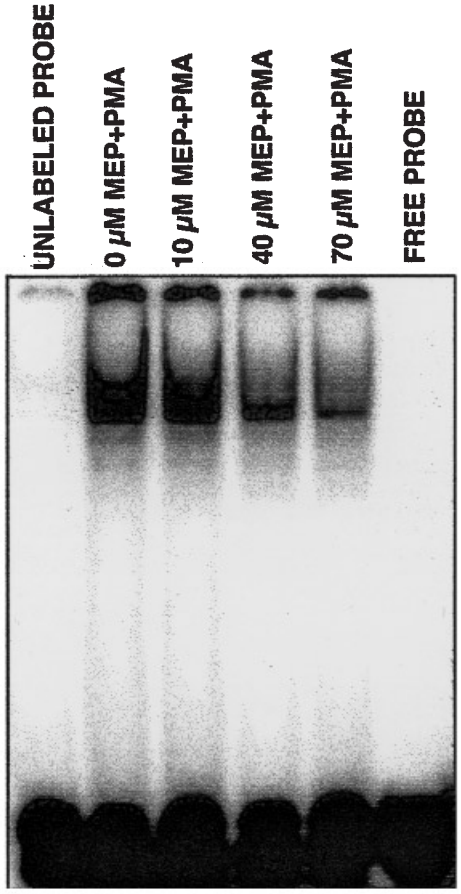

Figure 4. EMSA revealed reduced binding of AP-1 proteins in fibroblast-like synoviocytes treated with mepacrine. Synoviocytes were treated with the indicated amounts of mepacrine prior to stimulation with PMA. After 2 h, nuclear proteins were isolated. Equal amounts of protein were incubated with labeled AP-1 binding elements and separated on a native gel. For "Unlabeled probe," nuclear proteins of PMA-only treated cells were used and incubated with excess of unlabeled AP-1 oligonucleotides. No protein was included in the reaction mixture loaded onto the lane labeled "Free probe."

"Unlabeled probe" a competition experiment with unlabeled AP-1 oligonucleotides was performed.

Differential effect of mepacrine on Jun and Fos. c-Jun and c-Fos are members of the AP-1 family. In order to bind to the AP-1 sequence these proteins have to be translocated into the nucleus. We tested whether mepacrine interferes with protein translocation mechanisms and therefore contributes to the reduced binding of AP-1 proteins to the AP-1 binding element observed in the EMSA experiments. Human synoviocytes kept in medium containing 1% FCS for 16 h were pretreated with mepacrine (50 µM) for 30 min. Then, cells were left untreated or were treated with PMA (33 ng/ml) for indicated periods of time. Cytoplasm and nuclear proteins of these cells were isolated and tested for the presence of c-Jun and c-Fos by Western blot. While there was no apparent difference in the levels of either c-Jun or c-Fos detected in the cytoplasm of treated and untreated cells (data not shown), there was a remarkable differential effect of mepacrine on the translocation of these transcription factors. Figure 5 shows results of Western blot experiments where equal amounts of nuclear proteins were blotted onto membranes and located and quantitated using indicated antibodies. Figure 5A illustrates that PMA stimulation (30 min) led to translocation of c-Fos into the nucleus (PMA lane). Interestingly, in cells pretreated with mepacrine and subsequently stimulated with PMA (MEP+PMA), c-Fos could not be detected in the nuclear fraction of synoviocyte proteins. Furthermore, mepacrine itself did not lead to translocation of c-Fos into the nucleus (MEP 50), as levels of c-Fos found in mepacrine treated nuclei equaled those found in the nuclei of untreated cells (MED). When blots containing the nuclear protein fraction of synoviocytes were stained for the presence of c-Jun, the results where remarkably different. As shown in Figure 5B, no c-Jun was detected in unstimulated cells (MED). PMA stimulation for 30 min resulted in a weak band (PMA), and preincubation with mepacrine (30 min) followed by PMA (30 min) yielded a strong band (MEP+PMA). Interestingly, incubating synoviocytes for the same period of time with mepacrine only also led to translocation and detection of c-Jun in the nuclear fraction of these cells (MEP). This effect is dose dependent, because incubation of cells with lower amounts of mepacrine (5 µM) for a similar time resulted in less c-Jun detection in the nuclear fraction.

The above data on the differential effect of mepacrine on PMA induced c-Jun and c-Fos translocation were confirmed by stripping blots of anti-Fos antibodies and restaining the same blots using anti-c-Jun antibodies. Moreover, the method of staining blots with Ponceau red after exposure to x-ray films was used to confirm equal loading and even protein transfer in cases where nuclear proteins had to be detected.

DISCUSSION

MMP are involved in nearly all aspects of life. These enzymes take part in embryonic development and growth, in wound healing, tissue remodeling, cell communication, and cell migration, and in apoptotic as well as in necrotic events[41,42]. In contrast, abnormally regulated MMP are at the center of many pathological conditions ranging from cancer to neurodegenerative and cardiovascular diseases[43]. Involvement of MMP in rheumatic disorders is well characterized. For a long time, inhibiting MMP has been a major goal in the treatment of RA and OA. Mepacrine, which has

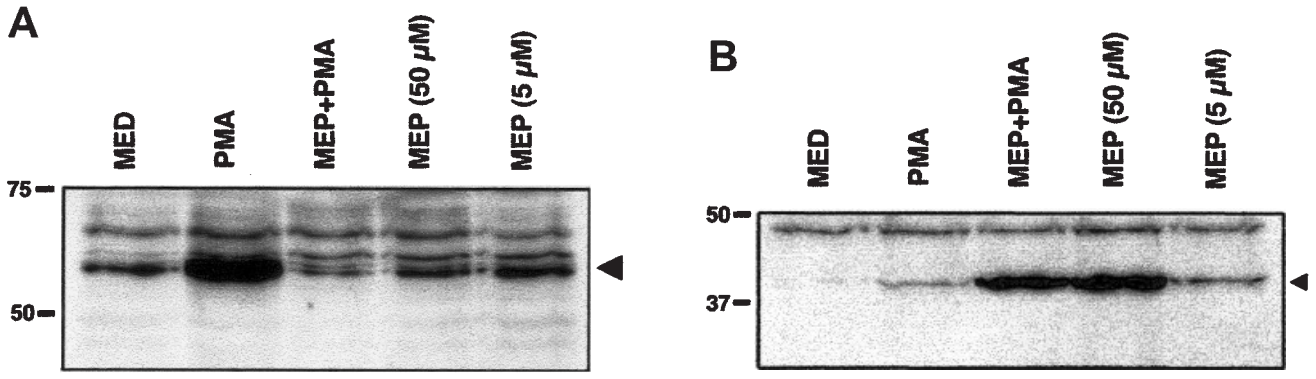

Figure 5. Western blot experiments revealed differential effects of mepacrine on PMA induced c-Jun and c-Fos translocation. Fibroblast-like synoviocytes were treated with mepacrine (50 µM or 5 µM) 30 min prior to stimulation with PMA (33 ng/ml). After an additional 30 min, nuclear proteins were isolated and separated by SDS-PAGE. Blot shown in Figure 5A, stained with anti-c-Fos antibodies, shows that PMA induces nuclear translocation of c-Fos (PMA). More importantly, 50 µM mepacrine prevented PMA induced c-Fos translocation (MEP+PMA). Compared to levels of c-Fos found in untreated cells (MED), mepacrine 50 µM and 5 µM did not lead to significant change in c-Fos detected in the nuclear fraction. The c-Fos-specific antibody recognizes a protein of roughly 62 kDa. Figure 5B shows a representative Western blot where such nuclear extracts were stained to detect the presence of c-Jun. The antibody reacts specifically with AP-1/cJun, a protein of roughly 39 kDa. As shown here, 50 µM mepacrine alone led to detection of high levels of c-Jun in the nuclear fraction [MEP (50 µM)], and 5 µM mepacrine still induced c-Jun translocation [MEP (5 µM)]. In such experiments, PMA was the weakest inducer of c-Jun translocation. Position of size markers is indicated on the left.

been used to treat rheumatic disorders and is currently used as a lupus remedy, has a long history of clinical use. It was first introduced as an antimalarial in 1930 and is known for its few adverse side effects that are generally minor or reversible[30]. Recently, it was suggested that mepacrine may be an immediate candidate for the treatment of Creutzfeldt-Jakob disease, as it prevented formation of the disease-causing form of prions in *in vitro* experiments[21].

We report that mepacrine dose dependently inhibits the release of MMP-1 in cultured human fibroblast-like synoviocytes stimulated with TNF-α or PMA. We tested the mechanism involved in this phenomenon and found that mepacrine does not inhibit constitutive or PMA-induced MMP-3 mRNA formation, but has a profound inhibitory effect on induced mRNA formation of MMP-1 as well as MMP-9. Since AP-1 seems to be important for the regulation of MMP, EMSA experiments were performed. These studies revealed that mepacrine dose dependently suppresses AP-1 binding to its consensus sequence in human synoviocytes. These findings are in accord with earlier findings, following the unpublished observation that mepacrine improves the survival of transplanted organs, in which treatment of endothelial cells with mepacrine-suppressed AP-1 binding, albeit by yet another mechanism[44].

Several proteins make up the family of AP-1 factors that bind DNA as homo- or heterodimers[45]. Classically, AP-1 dimers bind to AP-1-binding sequences that consist of 2 subunits that are formed either by heterodimerization of a Fos family protein (c-Fos, Fos B, Fra, Fra-2) with a Jun family protein (c-Jun, JunB, JunD) or homodimerization of the Jun family members[1,2]. The effects of mepacrine on the activation/translocation of AP-1 proteins might account for the observed results. We therefore investigated the extent to which mepacrine affects 2 members of this multimeric complex. Of note, we found strikingly different effects of mepacrine on c-Jun activation in contrast to c-Fos activation. In cells pretreated with mepacrine and subsequently stimulated with PMA, no c-Fos could be detected inside the nucleus, while at the same time mepacrine treatment alone led to increased levels of c-Jun within the nucleus. It is tempting to speculate that these events contribute to the observed inhibition of MMP by mepacrine. Since little is known about the functions of specific AP-1 dimers in the regulation of MMP, it will not be possible to judge the consequences of our findings with certainty without further study. However, there are reports supporting the concept that mepacrine-induced blockage of Fos translocation might indeed be one of the mechanisms affecting the activation of AP-1 dependent genes. As an example, it was observed in cotransfection experiments that c-Jun and c-Fos heterodimers bound 25 times more efficiently to the AP-1 DNA site than a c-Jun homodimer[46].

Further complicating the issue is that the AP-1 complexes are not limited to Jun and Fos dimers, since certain Jun and Fos proteins have been shown to dimerize with other factors such as activating transcription factor-2, musculoaponeurotic fibrosarcoma oncogene homolog, and their related proteins[47]. This results in myriad possibilities for these factors to interact with each other. As reported[48], the combinatorial character of the AP-1 transcriptional complex makes even the interpretation of overexpression experiments difficult since introduction of an AP-1 binding protein may affect homodimerization as well as heterodimerization with usually unidentified binding proteins. Further, even subtle changes in relative protein levels can have significant effects[49]. The situation becomes even more complex if one considers that AP-1 DNA binding does not necessarily correlate with transcriptional activation[50]. Shemshedini, *et al*[51], investigating the effect of c-Fos and/or c-Jun coexpression on transcriptional activation of genes, reported that c-Jun could either inhibit or further stimulate induced transcription. All these effects were receptor, promoter, and cell type-specific[51]. Although it seems plausible that the differential effects of mepacrine on MMP could be explained, at least in part, by the observed differential effect on Jun/Fos, only careful promoter analyses in combination with reporter assays will reveal the significance of these changes.

In any case, suppression of MMP could be beneficial in rheumatic diseases, where enzymes like MMP-1 and MMP-9 are overabundant. But there might be indirect beneficial effects of inhibiting MMP as well. MMP-1, for example, has been reported to be involved in cleaving TNF-α into its active form. Thus, blocking MMP-1 might result in lower levels of circulating TNF-α. Indeed, such observations have been made, specifically that the antimalarials chloroquine and mepacrine had inhibitory effects on zymosan-induced expression of interleukin 1α and TNF-α[52]. Although it was noted that mepacrine reduced these cytokines at both the mRNA and the protein level, others found similar suppressive effects of chloroquine and mepacrine on LPS-induced TNF-α expression in peripheral blood mononuclear cells (PBMC)[53]. This group also confirmed our observation[44] that mepacrine does not interfere with the activation of nuclear factor-κB. Another group observed that chloroquine, a closely related compound, did inhibit LPS-stimulated TNF-α release by interfering with TNF-α processing and membrane release[54]. Since no investigators to date have tested the effect of these drugs on the transcription factor AP-1 or the synthesis of MMP-1 in PBMC, it would be worthwhile to investigate whether mepacrine affects the activation of AP-1 in such cells by mechanisms similar to the one reported here on human synoviocytes.

Mepacrine also intercalates into DNA in a sequence-specific manner[55,56]. It has been shown that such specific binding of mepacrine can result in reduced binding of transcription factors[44]. Whether suppressing the binding of tran-

scription factors or the modulatory effect of mepacrine in the translocation of Jun/Fos proteins is of greater relevance for the inhibition of MMP mRNA accumulation is currently under investigation.

Mepacrine has been described and used in many studies as an inhibitor for PLA_2. It might seem that this widely accepted mechanism is solely responsible for the beneficial effects of mepacrine. There are reports that only weak PLA_2 inhibitory activity of mepacrine, e.g., 400 µM mepacrine, is necessary to achieve significant effects on PMA-induced platelet aggregation[57]. As well, several studies have suggested that the protective effects of mepacrine could be independent of such a mechanism. It has been shown, for example, that mepacrine prevented acute lung injury in rats after hypoxia-reoxygenation-induced injury, while another potent PLA_2 inhibitor had almost no effect[58]. A study showed that doses of mepacrine used to protect isolated rat hearts during hypoxia and reoxygenation did so without affecting activity of PLA_2 [24]. Another group reported that mepacrine even stimulated PLA_2 activity in polymorphonuclear leukocytes[59]. Nevertheless, the possibility that the observed effects of mepacrine on MMP activation are ultimately due to inhibition of PLA_2 cannot be excluded until further experiments are performed, especially in light of a recent report that mepacrine, at concentrations used in this study, was able to completely inhibit PLA_2 activity[60].

Findings from this study indicate that the observed differential effects of mepacrine on AP-1 transcription factor family proteins might contribute to many of the antiinflammatory effects of this drug. Although the antimalarial properties of mepacrine might, at least in part, also be attributed to the observed modulation of Jun/Fos, or whether the potential of mepacrine to selectively inhibit enzymes involved in pathological tissue destruction plays any role in prion diseases, such hypotheses have yet to be tested. The observation that mepacrine prevents upregulation of MMP-1 and MMP-9 might explain the favorable effect of this drug in rheumatic diseases, especially in the treatment of lupus, a disease where mepacrine is used successfully[31,32,61] and where elevated levels of circulating MMP-1 can be detected[62,63].

ACKNOWLEDGMENT

The author thanks Dr. B. Rifkin and Dr. J.J. Kao (Maimonides Medical Center, Brooklyn, NY, USA) for helpful discussion and critical reading of the manuscript; C. Linnert for technical assistance; and E. Karner for help in preparing the manuscript.

REFERENCES

1. Angel P, Karin M. Specific members of the Jun protein family regulate collagenase expression in response to various extracellular stimuli. Matrix 1992;1 Suppl:156-64.
2. Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1991;1072:129-57.
3. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. Chem Biol 1996;3:895-904.
4. Coussens LM, Werb Z. Inflammatory cells and cancer: think different! J Exp Med 2001;193:F23-6.
5. Lochter A, Bissell MJ. An odyssey from breast to bone: multi-step control of mammary metastases and osteolysis by matrix metalloproteinases. APMIS 1999;107:128-36.
6. McCawley LJ, Matrisian LM. Matrix metalloproteinases: multifunctional contributors to tumor progression. Mol Med Today 2000;6:149-56.
7. Stamenkovic I. Matrix metalloproteinases in tumor invasion and metastasis. Semin Cancer Biol 2000;10:415-33.
8. Furcht LT, Skubitz AP, Fields GB. Tumor cell invasion, matrix metalloproteinases, and the dogma. Lab Invest 1994;70:781-3.
9. Blavier L, Henriet P, Imren S, Declerck YA. Tissue inhibitors of matrix metalloproteinases in cancer. Ann NY Acad Sci 1999;878:108-19.
10. Elliott S, Cawston T. The clinical potential of matrix metalloproteinase inhibitors in the rheumatic disorders. Drugs Aging 2001;18:87-99.
11. Jackson C, Nguyen M, Arkell J, Sambrook P. Selective matrix metalloproteinase (MMP) inhibition in rheumatoid arthritis — targetting gelatinase A activation. Inflamm Res 2001;50:183-6.
12. Katrib A, Tak PP, Bertouch JV, et al. Expression of chemokines and matrix metalloproteinases in early rheumatoid arthritis. Rheumatology Oxford 2001;40:988-94.
13. Gordon JL, Drummond AH, Galloway WA. Metalloproteinase inhibitors as therapeutics. Clin Exp Rheumatol 1993;11:S91-4.
14. Steffen C, Menzel J, Zeitlhofer J, Smolen J, Lanzer G. Experimental arthritis induced by granulocyte collagenase. Scand J Rheumatol 1980;9:179-86.
15. Nguyen Q, Mort JS, Roughley PJ. Preferential mRNA expression of prostromelysin relative to procollagenase and in situ localization in human articular cartilage. J Clin Invest 1992;89:1189-97.
16. Lohmander LS, Lark MW, Dahlberg L, Walakovits LA, Roos H. Cartilage matrix metabolism in osteoarthritis: markers in synovial fluid, serum, and urine. Clin Biochem 1992;25:167-74.
17. Zafarullah M, Pelletier JP, Cloutier JM, Martel-Pelletier J. Elevated metalloproteinase and tissue inhibitor of metalloproteinase mRNA in human osteoarthritic synovia. J Rheumatol 1993;20:693-7.
18. Wolfe GC, MacNaul KL, Buechel FF, et al. Differential in vivo expression of collagenase messenger RNA in synovium and cartilage. Quantitative comparison with stromelysin messenger RNA levels in human rheumatoid arthritis and osteoarthritis patients and in two animal models of acute inflammatory arthritis. Arthritis Rheum 1993;36:1540-7.
19. Huebner JL, Otterness IG, Freund EM, Caterson B, Kraus VB. Collagenase 1 and collagenase 3 expression in a guinea pig model of osteoarthritis. Arthritis Rheum 1998;41:877-90.
20. Bluteau G, Conrozier T, Mathieu P, Vignon E, Herbage D, Mallein-Gerin F. Matrix metalloproteinase-1, -3, -13 and aggrecanase-1 and -2 are differentially expressed in experimental osteoarthritis. Biochim Biophys Acta 2001;1526:147-58.
21. Korth C, May BC, Cohen FE, Prusiner SB. Acridine and phenothiazine derivatives as pharmacotherapeutics for prion disease. Proc Natl Acad Sci USA 2001;98:9836-41.
22. Vigo C, Lewis GP, Piper PJ. Mechanisms of inhibition of phospholipase A_2. Biochem Pharmacol 1980;29:623-7.
23. Sargent CA, Vesterqvist O, McCullough JR, Ogletree ML, Grover GJ. Effect of the phospholipase A_2 inhibitors quinacrine and 7, 7-dimethyleicosadienoic acid in isolated globally ischemic rat hearts. J Pharmacol Exp Ther 1992;262:1161-7.
24. Bugge E, Gamst TM, Hegstad AC, Andreasen T, Ytrehus K. Mepacrine protects the isolated rat heart during hypoxia and reoxygenation — but not by inhibition of phospholipase A_2. Basic

Res Cardiol 1997;92:17-24.
25. Ferrante A, Rowan-Kelly B, Seow WK, Thong YH. Depression of human polymorphonuclear leucocyte function by anti-malarial drugs. Immunology 1986;58:125-30.
26. Mikes PS, Polomski LL, Gee JB. Mepacrine impairs neutrophil response after acute lung injury in rats. Effects on neutrophil migration. Am Rev Respir Dis 1988;138:1464-70.
27. Al Khader A, Al Sulaiman M, Kishore PN, Morais C, Tariq M. Quinacrine attenuates cyclosporine-induced nephrotoxicity in rats. Transplantation 1996;62:427-35.
28. Authi KS, Traynor JR. Stimulation of polymorphonuclear leucocyte phospholipase A_2 activity by chloroquine and mepacrine. J Pharm Pharmacol 1982;34:736-8.
29. Wallace DJ. Is there a role for quinacrine (atabrine) in the new millennium? Lupus 2000;9:81-2.
30. Wallace DJ. The use of quinacrine (atabrine) in rheumatic diseases: a reexamination. Semin Arthritis Rheum 1989;18:282-96.
31. D'Cruz D. Antimalarial therapy: a panacea for mild lupus? Lupus 2001;10:148-51.
32. Chung HS, Hann SK. Lupus panniculitis treated by a combination therapy of hydroxychloroquine and quinacrine. J Dermatol 1997;24:569-72.
33. Lipsker D, Piette JC, Cacoub P, Godeau P, Frances C. Chloroquine-quinacrine association in resistant cutaneous lupus. Dermatology 1995;190:257-8.
34. Toubi E, Rosner I, Rozenbaum M, Kessel A, Golan TD. The benefit of combining hydroxychloroquine with quinacrine in the treatment of SLE patients. Lupus 2000;9:92-5.
35. Matucci-Cerinic M, Marabini S, Jantsch S, Cagnoni M, Partsch G. Effects of capsaicin on the metabolism of rheumatoid arthritis synoviocytes in vitro. Ann Rheum Dis 1990;49:598-602.
36. Ozols J. Amino acid analysis. Methods Enzymol 1990;182:587-601.
37. Dyer RB, Herzog NK. Isolation of intact nuclei for nuclear extract preparation from a fragile B-lymphocyte cell line. Biotechniques 1995;19:192-5.
38. Konttinen YT, Ainola M, Valleala H, et al. Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis. Ann Rheum Dis 1999;58:691-7.
39. Vincenti MP, White LA, Schroen DJ, Benbow U, Brinckerhoff CE. Regulating expression of the gene for matrix metalloproteinase-1 (collagenase): mechanisms that control enzyme activity, transcription, and mRNA stability. Crit Rev Eukaryot Gene Expr 1996;6:391-411.
40. Stuhlmeier KM, Csizmadia V, Cheng Q, Winkler H, Bach FH. Selective inhibition of E-selectin, ICAM-1 and VCAM in endothelial cells. Eur J Immunol 1994;24:2186-90.
41. McCawley LJ, Matrisian LM. Matrix metalloproteinases: they're not just for matrix anymore! Curr Opin Cell Biol 2001;13:534-40.
42. Chang C, Werb Z. The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis. Trends Cell Biol 2001;11:S37-43.
43. Johnson LL, Dyer R, Hupe DJ. Matrix metalloproteinases. Curr Opin Chem Biol 1998;2:466-71.
44. Stuhlmeier KM. Effects of quinacrine on endothelial cell morphology and transcription factor–DNA interactions. Biochim Biophys Acta 2001;1524:57-65.
45. Shaulian E, Karin M. AP-1 in cell proliferation and survival. Oncogene 2001;20:2390-400.
46. Halazonetis TD, Georgopoulos K, Greenberg ME, Leder P. c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. Cell 1988;55:917-24.
47. Bakiri L, Lallemand D, Bossy-Wetzel E, Yaniv M. Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression. EMBO J 2000;19:2056-68.
48. Bakiri L, Matsuo K, Wisniewska M, Wagner EF, Yaniv M. Promoter specificity and biological activity of tethered AP-1 dimers. Mol Cell Biol 2002;22:4952-64.
49. Wisdom R, Johnson RS, Moore C. c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. EMBO J 1999;18:188-97.
50. Rutberg SE, Saez E, Lo S, et al. Opposing activities of c-Fos and Fra-2 on AP-1 regulated transcriptional activity in mouse keratinocytes induced to differentiate by calcium and phorbol esters. Oncogene 1997;15:1337-46.
51. Shemshedini L, Knauthe R, Sassone-Corsi P, Pornon A, Gronemeyer H. Cell-specific inhibitory and stimulatory effects of Fos and Jun on transcription activation by nuclear receptors. EMBO J 1991;10:3839-49.
52. Bondeson J, Sundler R. Antimalarial drugs inhibit phospholipase A_2 activation and induction of interleukin 1 beta and tumor necrosis factor alpha in macrophages: implications for their mode of action in rheumatoid arthritis. Gen Pharmacol 1998;30:357-66.
53. Weber SM, Levitz SM. Chloroquine interferes with lipopolysaccharide-induced TNF-alpha gene expression by a nonlysosomotropic mechanism. J Immunol 2000;165:1534-40.
54. Jeong JY, Jue DM. Chloroquine inhibits processing of tumor necrosis factor in lipopolysaccharide-stimulated RAW 264.7 macrophages. J Immunol 1997;158:4901-7.
55. Scaria PV, Craig JC, Shafer RH. Differential binding of the enantiomers of chloroquine and quinacrine to polynucleotides: implications for stereoselective metabolism. Biopolymers 1993;33:887-95.
56. Rivas L, Murza A, Sanchez-Cortes S, Garcia-Ramos JV. Interaction of antimalarial drug quinacrine with nucleic acids of variable sequence studied by spectroscopic methods. J Biomol Struct Dyn 2000;18:371-83.
57. Jerushalmy Z, Englender T, Shaklai M. Phorbol-myristate-acetate-induced platelet aggregation in the presence of inhibitors. Acta Haematol 1988;80:210-5.
58. Shen CY, Wang D, Chang ML, Hsu K. Protective effect of mepacrine on hypoxia-reoxygenation-induced acute lung injury in rats. J Appl Physiol 1995;78:225-31.
59. Authi KS, Traynor JR. Stimulation of polymorphonuclear leucocyte phospholipase A_2 activity by chloroquine and mepacrine. J Pharm Pharmacol 1982;34:736-8.
60. Cussac D, Schaak S, Denis C, Paris H. Alpha 2B-adrenergic receptor activates MAPK via a pathway involving arachidonic acid metabolism, matrix metalloproteinases, and epidermal growth factor receptor transactivation. J Biol Chem 2002;277:19882-8.
61. von Schmiedeberg S, Ronnau AC, Schuppe HC, Specker C, Ruzicka T, Lehmann P. Combination of antimalarial drugs mepacrine and chloroquine in therapy refractory cutaneous lupus erythematosus. Hautarzt 2000;51:82-5.
62. Keyszer G, Lambiri I, Nagel R, et al. Circulating levels of matrix metalloproteinases MMP-3 and MMP-1, tissue inhibitor of metalloproteinases 1 (TIMP-1), and MMP-1/TIMP-1 complex in rheumatic disease. Correlation with clinical activity of rheumatoid arthritis versus other surrogate markers. J Rheumatol 1999;26:251-8.
63. Nakamura T, Ebihara I, Tomino Y, Koide H. Effect of a specific endothelin A receptor antagonist on murine lupus nephritis. Kidney Int 1995;47:481-9.