

Quinacrine But Not Chloroquine Inhibits PMA Induced Upregulation of Matrix Metalloproteinases in Leukocytes: Quinacrine Acts at the Transcriptional Level Through a PLA₂-Independent Mechanism

KARL M. STUHLMEIER and CHRISTINE POLLASCHEK

ABSTRACT. *Objective.* Macrophages play an important role in rheumatoid arthritis (RA). RA is a disease characterized by the successive accumulation of leukocytes resulting in subsequent destruction of affected joints. Activation of matrix metalloproteinases (MMP) is essential for many physiological as well as many pathological events owing to the essential role of MMP in cell migration. We analyzed the effectiveness of quinacrine as an inhibitor of MMP activation in leukocytes and investigated the mode of action.

Methods. Leukocytes were isolated and treated with quinacrine with or without phorbol myristate acetate (PMA). ELISA and RT-PCR were used to monitor production of MMP-1, MMP-2, MMP-3, and MMP-8 at the mRNA and protein level.

Results. Quinacrine suppressed PMA induced MMP-1 release in mononuclear cells (MNC) in a dose- and time-dependent manner. RT-PCR showed that quinacrine downregulated induced as well as noninduced steady-state mRNA levels of MMP-1, MMP-2, and MMP-8, but had no effect on MMP-3. The observed inhibition was not due to effects of quinacrine on phospholipase A₂ (PLA₂) activity. Adding exogenous arachidonic acid to reconstitute the blocked PLA₂ signaling pathways did not result in restoration of PMA induced mRNA transcription.

Conclusion. Inhibition of MMP by quinacrine might, in part, account for its reported immunosuppressive action. Synthesizing more potent derivatives of quinacrine may be a means of suppressing undesired MMP activation. (J Rheumatol 2006;33:472–80)

Key Indexing Terms:

QUINACRINE

MATRIX METALLOPROTEINASES

INFLAMMATION

Quinacrine (mepacrine) has been administered to millions of individuals and has been used for decades to successfully treat a series of ailments ranging from malaria to certain forms of rheumatic diseases¹. Besides being used to treat rheumatoid arthritis (RA), quinacrine and other antimalarials have a successful record in the management of patients with mild and moderate systemic lupus erythematosus (SLE)¹⁻⁶. Despite its well documented antirheumatic properties, quinacrine has never been carefully evaluated. For a variety of reasons and despite the lack of negative RA studies, interest in this drug faded and its potential was not optimally exploited¹. As a result of reports on its apparent effectiveness for the treatment of Creutzfeldt-Jakob disease, interest has again focused on this drug and its exceptional safety record⁷⁻¹⁰.

Rheumatic disorders are characterized by the migration and activation of leukocytes into affected sites. The pathogenesis of RA involves the migration of T cells, B cells, plasma cells, macrophages, dendritic cells, neutrophils, granulocytes, and mast cells¹¹. It has been demonstrated that quinacrine can act as a potent inhibitor of such cell migration¹²⁻¹⁵. In addition to these effects, quinacrine has also been shown to reduce infarct size after transient cerebral artery occlusion¹⁶, to attenuate cyclosporine induced nephrotoxicity¹⁷, to inhibit lipopolysaccharide induced tissue factor expression, to decrease lung leaks in rats given interleukin 1¹⁸, and to prevent myocardial reperfusion injury¹⁹. In general it is thought that most of the beneficial effects of quinacrine as an anti-inflammatory drug are due to inhibition of phospholipase A₂ (PLA₂)^{20,21}. Nonetheless, the mechanisms of the effects of quinacrine are not well defined.

Accumulation of cells at the site of inflammation depends strongly on the ability of cells to penetrate cell barriers and to enter into tissue. Matrix metalloproteinases (MMP) make up a family of extracellular matrix-degrading enzymes that are believed to act in many biological as well as pathological processes including cell migration²²⁻²⁶. We demonstrated that quinacrine has the potential to block the upregulation of certain metalloproteinases expressed on fibroblast-like synovio-

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

Supported in part by grants from the City of Vienna, the "Medizinisch Wissenschaftlicher Fonds des Bürgermeisters der Bundeshauptstadt Wien," the Austrian Ministry of Social Security and Generations, and the Austrian Ministry of Education, Science and Culture.

Address reprint requests to Dr. K.M. Stuhlmeier, Ludwig Boltzmann Institute for Rheumatology and Balneology, Kurbadstrasse 10, 1100 Vienna, Austria. E-mail: karlms@excite.com

Accepted for publication October 14, 2005.

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2006. All rights reserved.

cytes²⁷. Here we investigated whether quinacrine has any effect on the expression of metalloproteinases that are important for the migration of leukocytes. It is the migration of leukocytes with which the cycle leading to joint and tissue destruction commences. As noted, a number of studies describe effective inhibition of leukocyte migration following quinacrine treatment, yet the mechanisms behind these observations are unknown. We studied the mode of action of quinacrine by testing and comparing the effectiveness of this drug as an MMP inhibitor with the more commonly used drug chloroquine. We also tested whether the observed effects on phorbol myristic acetate (PMA) induced MMP-1 expression were due to the inhibition of PLA₂ activity by quinacrine.

MATERIALS AND METHODS

Reagents. Quinacrine (mepacrine; 6-chloro-9-(4-(diethylamino-1-methylbutylamino)-2-methoxyacridine dihydrochloride); chloroquine [N⁴-(7-chloro-4-quinolinyl)-N1,N1-dimethyl-1,4-pentanediamine diphosphate salt]; Dulbecco's modified Eagle's medium (DMEM); fetal calf serum (FCS); trypsin; dimethyl sulfoxide; Histopaque-1077; 12-O-tetradecanoylphorbol 13-acetate (PMA); TRIzol; and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were from Sigma Chemical Co., Vienna, Austria. MMP-1 ELISA kit (sensitivity 0.023 ng/ml) was from Calbiochem-Novobiochem, San Diego, CA, USA. In some experiments, the MMP-1 human Biotrak ELISA system (Amersham Biosciences, Freiburg, Germany) was used for comparison. Antibodies for Western blots were from Cell Signaling Technology, Beverly, MA, USA.

Cells and cell cultures. Blood from healthy laboratory volunteers was collected in EDTA tubes and diluted with an equal volume of phosphate buffered saline (PBS). Mononuclear cells (MNC) were isolated using Histopaque-1077 according to standard protocols. Cells were cultured and incubated (37°C, 5% CO₂) in DMEM containing 10% FCS, penicillin (100 U/ml), streptomycin (50 U/ml), and L-glutamine.

MMP measurements and data analysis. For ELISA measurements of MMP-1, leukocytes (5 × 10⁶ per well) were kept in 24-well tissue culture plates for indicated times in a total volume of 400 µl medium per well. At the end of experiments, supernatants from individual wells were transferred into 1.5 ml Eppendorf tubes and centrifuged for 5 min at 5000 g to remove any cells. ELISA were carried out in duplicates as described in the accompanying manuals. Signal intensity (optical density) was measured at appropriate wavelengths using a microplate reader (Bio-Rad Laboratories, Vienna, Austria). Effects of quinacrine on PMA induced MMP-1 release were measured in 3 independent experiments. Data analysis was done using a curve-fitting program (AssayZap, Cambridge, UK).

Reverse transcription-polymerase chain reaction (RT-PCR). RNA was isolated using TRIzol according to instructions provided by the manufacturer. For block-cycler experiments RNA was resuspended in Tris-EDTA buffer, quantitated on a spectrophotometer, reverse transcribed (RT-PCR kit, Amersham), and stored at -60°C. An Eppendorf cycler (Eppendorf, Hamburg, Germany) was used for conventional block-cycler 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. mRNA for GAPDH or actin were coamplified and used as controls. 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', respectively; MMP-3: 5'-CTC ACA GAC CTG ACT CGG TT-3' and 5'-CAC GCC TGA AGG AAG AGA TG-3'; MMP-8: 5'-ATG GAC CAA CAC CTC CGC AA-3' and 5'-GTC AAT TGC TTG GAC GCT GC-3'²⁸. GAPDH: 5'-TCA AAG GCA TCC TGG GCT ACA-3' and 5'-GAG GGG AGA TTC AGT GTG GTG-3'.

Actin: 5'-CAC CTT CTA CAA TGA GCT GC-3' and 5'-AGG CAG CTC GTA GCT CTT CT-3'. Block-cycler PCR was carried out in 0.2 ml tubes containing 50 µl of the following reagents: 5 µl 10x buffer, 1 µl dNTP, 2 µl each (forward and reverse) primer, 1 µl TAQ polymerase (1.5 U final), cDNA, and ddH₂O. As described²⁹, different numbers of cycles were tested regularly in order to ensure that PCR reactions were stopped in the linear-phase of cDNA amplification. Fragments for GAPDH and actin were amplified using 20 to 24 cycles, while 29 to 34 cycles were used for amplification of MMP. The lengths of amplified fragments were as follows: MMP-1, 428 bp; MMP-2, 390 bp; MMP-3, 294 bp; and MMP-8, 282 bp. Aliquots of PCR were separated on agarose gels and scanned and analyzed on a Fluorimager 595 (Amersham). All RT-PCR experiments were repeated at least 3 times; one representative experiment is illustrated.

Real-time RT-PCR and PCR data analysis was as follows: RNA was resuspended in water, quantitated on a spectrophotometer, and set to equal concentrations. Reverse transcription was performed using the StrataScript First Strand synthesis system (Stratagene, Amsterdam, The Netherlands). Total RNA (500 ng) was transcribed, using random hexamer primers, and diluted with water to a final concentration of 250 ng in order to improve amplification efficiency. For real-time RT-PCR, SYBR Green RT-PCR amplification was conducted on a Mx3000P real-time PCR system (Stratagene), using Brilliant SYBR Green QPCR Master Mix (Stratagene). After optimization of the primer pairs, RT-PCR was performed using the following standard conditions: initial denaturation, 10 min at 95°C; denaturation 10 s; annealing, 15 s 57°C; extension 15 s 72°C. Each RT-PCR experiment included a dissociation curve to verify the specificity of the amplicon, as well as no-template controls. mRNA for hypoxanthine-guanine phosphoribosyl transferase 1 (HPRT) was coamplified and used as a control for quantification. Primers (MWG Biotech AG) used in real-time PCR experiments were selected based on published data³⁰⁻³² and dissolved at a concentration of 100 pmol/ml in Tris-EDTA. The correct length of the PCR product was confirmed by agarose gel electrophoresis. Standards and samples were assayed in a 25 µl reaction mixture containing 2x Brilliant SYBR Green QPCR Master Mix, 30 nM reference dye (ROX), 1.5 ml of forward and reverse primer, cDNA (2.5 ml), and ddH₂O. The following equations were used to calculate real-time PCR data:

$$\begin{aligned} \text{MMP-1; } y &= -3.507 \times \log(x) + 33.63, \text{ Eff.} = 98 \\ \text{MMP-2; } y &= -3.336 \times \log(x) + 23.11, \text{ Eff.} = 99.4 \\ \text{MMP-8; } y &= -3.306 \times \log(x) + 30.48, \text{ Eff.} = 100.7 \end{aligned}$$

Western blot experiments. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting: cells were washed twice in ice-cold PBS and then dissolved in SDS sample buffer [62.5 mM Tris/HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue]. For 3 cm and 10 cm culture dishes, 100 µl and 500 µl sample buffer, respectively, were used. Aliquots of whole-cell protein extract (10–25 µl/well) were separated on 10% mini-gel. Proteins were blotted onto PVDF membranes (Amersham) using a semidry apparatus (Bio-Rad, Hercules, CA, USA). Blots were flushed with ddH₂O, dipped into MeOH, and dried for 20 min. Then blots were transferred to a blocking buffer solution (1x PBS, 0.1% Tween-20, 5% w/v nonfat dry milk) and incubated 1 h. Membranes were then incubated with the appropriate diluted primary antibody in 5% BSA, 1x PBS, and 0.1% Tween-20, at 4°C overnight in a roller bottle. After 3 washing steps in wash buffer (1x PBS, 0.1% Tween-20), blots were incubated with appropriate secondary antibodies diluted in PBS. After 45 min gentle agitation, blots were washed 5 times for 5 min in wash buffer, and proteins were made visible using either LumiGLO (New England Biolabs, Beverly, MA, USA) or Renaissance Plus (Perkin-Elmer Life Science, Boston, MA, USA) or Kodak BioMax MR films.

Viability assay. Viability of cells was confirmed by the trypan blue exclusion assay at the end of experiments as well as by visual inspection of cells under a phase-contrast microscope. In some cases, MNC were incubated up to 2 weeks in quinacrine (10 µM) and tested for viability daily. At the conditions and concentrations used, viability of MNC was not affected by treatment with quinacrine.

RESULTS

Pretreating leukocytes with quinacrine inhibited MMP-1 secretion. Mononuclear cells were isolated by density gradient centrifugation as described above. Cells were transferred into 10 cm tissue culture dishes and were left untreated for 16 h (Figure 1A). Figure 1B shows the results when all cells were exposed to 10 μ M quinacrine for 16 h. After this period, MNC were removed, washed twice with prewarmed medium (DMEM plus 10% FCS), and allotted into wells of 24-well tissue culture plates. Where indicated (“Quin,” Figure 1), MNC were exposed to quinacrine (10 μ M) for 30 min. Subsequently, cells were either left untreated (“Medium,” Figure 1) or were treated with PMA (2 ng/ml). Cell culture media were harvested after additional incubation of 10 h. Aliquots of supernatants were used to measure differences in MMP-1 secretion.

A representative experiment showing that MMP-1 is readily detectable in supernatants of unstimulated MNC is illustrated in Figure 1A. Stimulation of these cells with PMA led to a 2.3-fold increase of MMP-1 levels released into the supernatant. In experiments (Figure 1A) in which cells were preincubated with quinacrine for only 30 min and then stimulated with PMA, MMP-1 protein levels were consistently higher than in cells treated with PMA only. In this experiment, levels of MMP-1 in unstimulated cells were 2.4 ± 0.66 ng/ml; in PMA treated cells MMP-1 values rose to 5.8 ± 0.14 ng/ml, and increased to 6.7 ± 0.1 ng/ml in cells treated with quinacrine and PMA.

However, in experiments where cells were first incubated overnight with quinacrine (Figure 1B) and then washed to remove quinacrine, results were reproducibly different. Figure

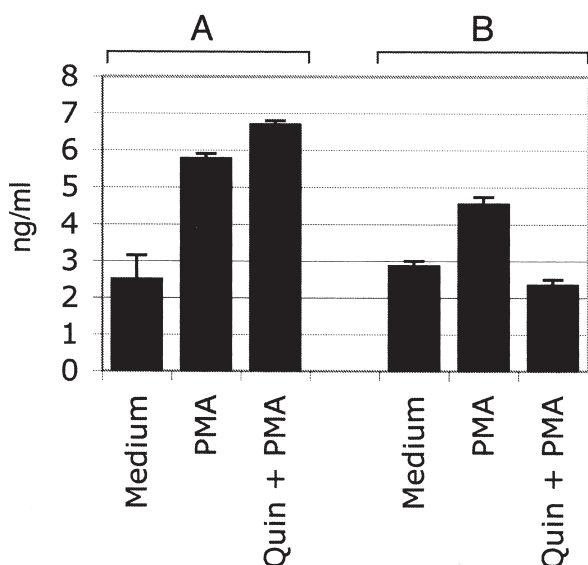


Figure 1. Cells were transferred into 10 cm tissue culture dishes and as illustrated in panel A were left untreated for the next 16 h. In panel B, all cells were exposed to 10 μ M quinacrine for 16 h. After this period, MNC were removed, washed twice with prewarmed medium (DMEM plus 10% FCS), and allotted into wells of 24-well tissue culture plates.

1B (“Medium”) shows that cells precultured in quinacrine for 16 h and then kept in complete medium without quinacrine or any other stimulus released similar amounts of MMP-1 over the course of the experiments (10 h) as cells that were never treated with quinacrine (Figure 1A, “Medium”). Cells incubated for 16 h with quinacrine, then washed to remove quinacrine and subsequently stimulated with PMA, released significantly lower amounts of MMP-1 over the course of the experiment (10 h) than did cells that were not pretreated with quinacrine (5.8 ± 0.14 vs 4.5 ± 0.2 ng/ml). Comparing “PMA” in Figure 1A with “Quin + PMA” in Figure 1B, pretreatment of cells for 16 h and then stimulating these cells with PMA in the continuous presence of quinacrine resulted in complete inhibition of PMA induced MMP-1 release (5.8 ± 0.14 vs 2.3 ± 0.16 ng/ml). Similar results were observed in experiments where cells were stimulated with PMA for up to 24 h (data not shown).

Effect of quinacrine on steady-state mRNA levels of MMP in MNC. To determine whether the observed effect of quinacrine on MMP-1 release in leukocytes was due to interference at the transcriptional or the translational level, a series of RT-PCR experiments was performed using freshly isolated MNC from healthy donors. MNC were pretreated with quinacrine and stimulated with PMA. Figure 2A illustrates the results of a representative experiment where MNC were treated with 0, 5, and 25 μ M quinacrine for 30 min. Then PMA 2.5 ng/ml was added and cells were incubated an additional 6 h. As shown in Figure 2B, MMP-1 mRNA levels in untreated MNC are very low or undetectable, while stimulating cells with PMA resulted in a substantial increase of MMP-1 mRNA. Further, a short treatment with quinacrine resulted in dose-dependent suppression of MMP-1 mRNA. While in this particular experiment 5 μ M quinacrine was not sufficient to exert any significant inhibitory effects, increasing quinacrine levels to 25 μ M resulted in > 70% inhibition of PMA induced MMP-1 mRNA.

We also tested the effect of quinacrine on the gene encoding MMP-8. As shown in Figure 2A, PMA stimulation led to the activation of MMP-8 in MNC. As with MMP-1, quinacrine treatment of MNC suppressed PMA induced MMP-8 mRNA transcription in a dose-dependent manner. Again, 25 μ M quinacrine completely abolished the PMA effect and 5 μ M quinacrine treatment for 30 min was not sufficient to significantly affect MMP-8.

As shown in Figure 2A, MMP-2 mRNA is readily detectable in unstimulated cells. More importantly, treatment of MNC with quinacrine resulted in suppression of MMP-2 mRNA levels. Interestingly, while transcription of MMP-1 and MMP-8 was induced by PMA, MMP-2 mRNA levels were reduced to nearly undetectable levels when MNC were exposed to PMA. Further, and as a demonstration of the gene specificity of the effects of quinacrine, MMP-3 mRNA levels in MNC were not affected by quinacrine. Figure 2A also shows mRNA levels of actin following amplification by RT-PCR. mRNA for this gene was coamplified in each experiment and served as a control for the equal use of RNA.

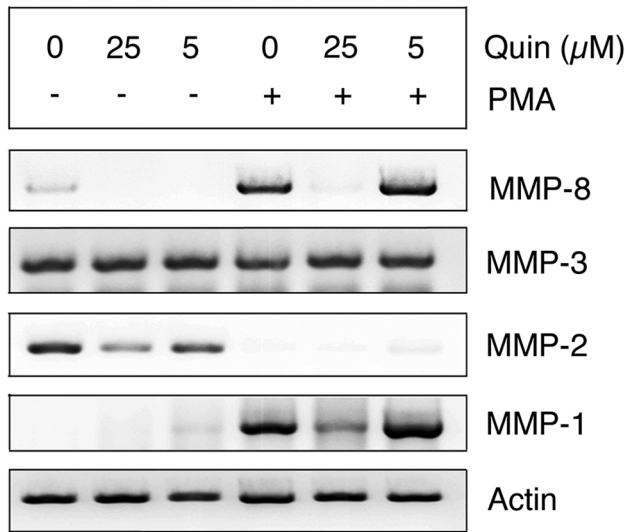


Figure 2A. Quinacrine selectively inhibited MMP-1, MMP-2, and MMP-8 transcription in MNC pretreated (30 min) with quinacrine 5 and 25 μ M and stimulated with PMA 2.5 ng/ml for 6 h. Conventional block-cycler RT-PCR experiments showed that 30 min pretreatment with 25 μ M quinacrine completely blocked PMA induced MMP-8 transcription, suppressed MMP-1 mRNA levels, and downregulated steady-state levels of MMP-2 mRNA. The inhibition was specific, as mRNA levels for genes encoding MMP-3 and actin were not affected.

Subsequently, real-time PCR was used to confirm and extend data obtained by PCR on a conventional block-cycler. The data shown in Figure 2B resulted from real-time PCR experiments where MNC were left untreated (“MED”) or were treated with quinacrine (6, 12, 25, and 50 μ M) for 30 min, and then stimulated with PMA 2.5 ng/ml as indicated. After 8 h, total RNA was isolated and amplified as described in Materials and Methods. Real-time PCR data were used to calculate fold-induction of MMP-1 mRNA. In the experiment shown in Figure 2B, MMP-1 mRNA levels in PMA treated MNC are ≥ 50 times higher than in untreated cells. As well, addition of quinacrine to MNC prior to treatment with PMA resulted in a dose-dependent inhibition of PMA induced MMP-1 upregulation. Adding 50 μ M quinacrine completely blocked PMA induced MMP-1 transcription. Lowering quinacrine concentrations reduced the inhibitory effect. As shown in the upper panel of Figure 2B, treating MNC with 25 and 12 μ M quinacrine, respectively, resulted in dose-dependent reduction of PMA induced MMP-1 mRNA levels. However, concentrations of quinacrine lower than 10 μ M resulted in no significant effect.

We also tested quinacrine’s effects on MMP-8 by real-time PCR. Representative data are shown in the lower panel of Figure 2B: real-time PCR data confirmed the effect of quinacrine on MMP-8 in block-cycler experiments. mRNA levels of this gene were constitutively elevated in unstimulated MNC (“MED”). Stimulating these cells with PMA induced MMP-8 transcription (“PMA 2.5 ng/ml”). As shown in the lower panel of Figure 2B, incubating MNC with increasing

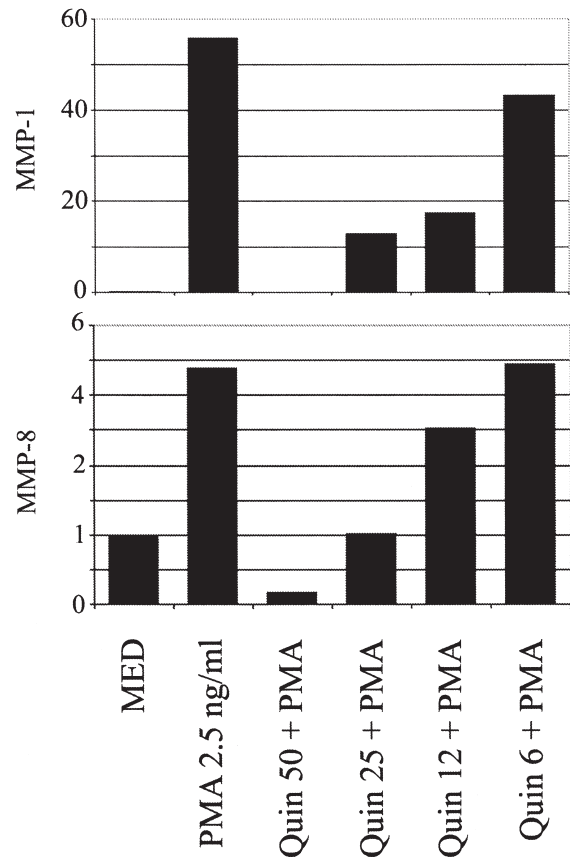


Figure 2B. Quinacrine inhibited MMP-1 and MMP-8 transcription in a dose-dependent manner, shown by real-time PCR data. MNC were left untreated (MED) or pretreated with concentrations of quinacrine (50, 25, 12, 6 μ M) for 30 min, then exposed to PMA 2.5 ng/ml for 8 h. Induction and inhibition were calculated using standard curve equations generated on the real-time cycler using serial dilutions. Quinacrine 50 μ M completely blocked PMA induced MMP-1 transcription, and significantly reduced constitutively elevated levels of MMP-8. While quinacrine 25 and 12 μ M was sufficient to reduce mRNA levels of these genes significantly, < 10 μ M quinacrine had no significant effect. Given on the y-axis are levels of mRNA (n-fold induction/inhibition); indicated on the x-axis are culture conditions.

concentrations of quinacrine resulted in dose-dependent suppression of PMA induced MMP-8 transcription. At the highest concentration of quinacrine (50 μ M), MMP-8 mRNA levels were lower than in untreated cells. Lower quinacrine concentrations were less effective. Similar to PMA induced transcription of MMP-1, levels of MMP-8 were not significantly affected when MNC were incubated with less than 10 μ M quinacrine. While coamplifications of the housekeeping gene actin served as a control in RT-PCR block-cycler experiments, HPRT was used in real-time RT-PCR experiments. Figure 2B shows culture conditions (x axis) and n-fold levels of mRNA of indicated genes (y axis). For calculating fold-induction or inhibition, basal MMP-1 and MMP-8 mRNA levels in unstimulated cells were considered to equal 1. In real-time experiments, HPRT was coamplified and used to recalculate mRNA expression.

Chloroquine did not affect PMA induced MMP-1 activation. Chloroquine, like quinacrine, belongs to a group of disease-modifying drugs that are very similar in structure. We therefore tested the effectiveness of chloroquine as an inhibitor of MMP-1 and MMP-2. Figure 3A shows a representative experiment comparing the effects of chloroquine and quinacrine on these genes on a block-cycler. MNC were freshly isolated and left untreated or were treated with 5 or 25 μ M chloroquine and quinacrine, respectively; after 30 min, PMA 2.5 ng/ml was added to indicated wells, and cells were incubated an additional 6 h, when the experiment was terminated. The upper panel of Figure 3A shows results of experiments obtained treating cells with 0, 5, and 25 μ M quinacrine with and without PMA 2.5 ng/ml. The lower panel shows data obtained after pretreating cells with 0, 5, and 25 μ M chloroquine and with or without PMA 2.5 ng/ml. As shown in the upper panel, data for MMP-1 are consistent with data shown in Figure 2. Preincubating MNC with equimolar amounts of chloroquine did not result in a reduction in PMA induced MMP-1 mRNA levels. Further, while quinacrine reproducibly suppressed the constitutively elevated MMP-2 mRNA levels in MNC, chloroquine had no effect on this gene, even when used at the high concentration of 25 μ M.

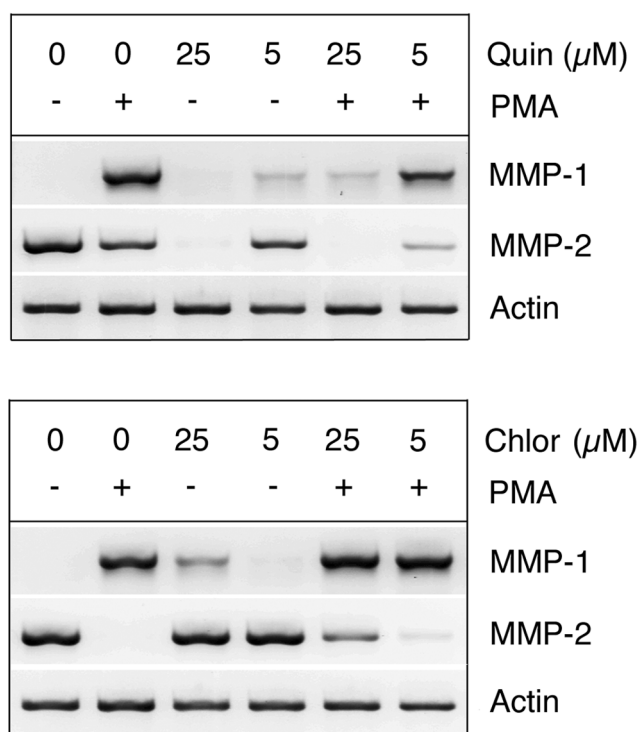


Figure 3A. Comparison of the effectiveness of quinacrine and chloroquine on induced and noninduced activation of MMP-1 and MMP-2. MNC were exposed to equimolar amounts of quinacrine and chloroquine for 30 min, then stimulated with PMA 2.5 ng/ml for 6 h. While quinacrine 25 μ M completely blocked PMA induced MMP-1 activation, it also exerted weak inhibitory effects on this gene at 5 μ M. Chloroquine had no effect on MMP-1 mRNA levels at either 5 μ M or 25 μ M. Similarly, no chloroquine mediated effects were observed on constitutive levels of MMP-2 mRNA.

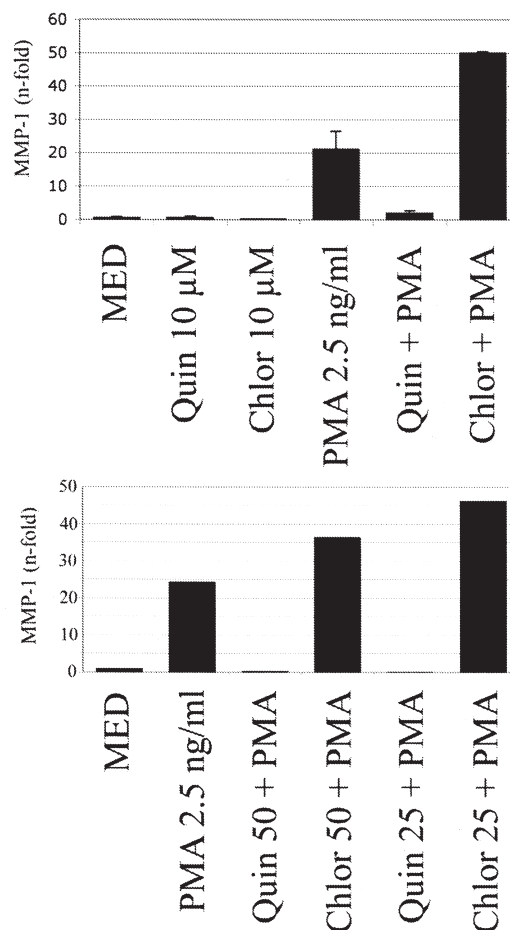


Figure 3B. Real-time PCR data show that, unlike quinacrine, chloroquine does not suppress PMA induced MMP-1 transcription. Upper panel: data generated on a real-time cycler extend block-cycler data shown in Figure 3A. MMP-1 mRNA levels were calculated in 3 experiments. While quinacrine 10 μ M significantly ($p = 0.009$) reduced mRNA levels of this gene, chloroquine did not. mRNA levels of MMP-1 were significantly higher in cells exposed to chloroquine and PMA ($p = 0.033$). Lower panel: increasing the concentration of chloroquine to 50 μ M had no effect on PMA induced MMP-1 mRNA levels. While quinacrine was highly effective in blocking PMA induced MMP-1 transcription, chloroquine failed to show suppressive effects even at a concentration of 50 μ M.

Again, real-time PCR was used to confirm these findings and to allow more accurate estimation of effects of quinacrine and chloroquine on MMP-1. Figure 3B illustrates 2 such experiments. The upper panel of Figure 3B shows a comparison of the effect of equimolar concentrations of quinacrine and chloroquine on PMA induced MMP-1 induction. Collective data from 3 experiments indicated that the low basal levels of MMP-1 were not significantly affected by 10 μ M quinacrine ($p = 0.493$); PMA treatments resulted in significantly ($p = 0.009$) elevated levels of MMP-1 mRNA (21.2 ± 5.42 -fold higher than in untreated cells). While quinacrine reduced PMA induced MMP-1 mRNA levels significantly ($p = 0.009$), chloroquine did not. Real-time PCR data revealed that chloroquine treatment actually led to a significant aug-

mentation of the PMA effect ($p = 0.033$), resulting in 50 ± 0.4 -fold higher MMP-1 mRNA levels than in unstimulated cells.

The lower panel of Figure 3B illustrates a real-time PCR experiment showing that increasing the concentration of chloroquine to 25 or 50 μM did not result in suppression of PMA induced MMP-1 mRNA levels. If anything, mRNA levels of MMP-1 were higher in MNC treated with PMA and high concentrations of chloroquine. Again, quinacrine completely diminished the effect of PMA at these concentrations.

Quinacrine mediated inhibition of PMA induced MMP-1 mRNA transcription was not due to its effects on the activity of PLA₂. Quinacrine has the ability to block activation of PLA₂, thereby blocking the release of arachidonic acid and its many bioactive metabolites. Reconstituting the arachidonic acid pathway by adding high concentrations of exogenous arachidonic acid was used to test whether suppression of arachidonic acid release by quinacrine is the underlying mechanism of quinacrine mediated inhibition of MMP-1. Figure 4A illustrates an experiment in which freshly isolated MNC were pretreated with quinacrine 20 μM . After 30 min, PMA 2.5 ng/ml was added simultaneously with arachidonic acid 60 μM . After 6 h, total RNA was isolated and semiquantitative RT-PCR experiments were performed on a block-cycler. As shown again in this representative experiment, PMA treatment led to detection of high levels of MMP-1 mRNA, and pretreatment with 20 μM quinacrine completely abolished the PMA effect. Adding arachidonic acid to cells treated with quinacrine and PMA did not restore the effects of PMA.

As shown in Figure 4A, exogenous arachidonic acid did not restore PMA induced MMP-1 activation in cells treated with quinacrine. That the exogenous arachidonic acid used in these experiments is both bioactive and sufficient to highly activate cells was tested next. Since the mitogen activated protein kinases (MAPK) p38, JNK, and ERK are located at the

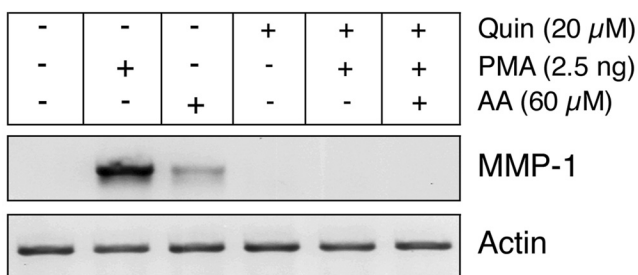


Figure 4A. Data show that exogenous arachidonic acid does not abolish the effect of quinacrine on PMA induced MMP-1 mRNA levels. MNC were left untreated or were preincubated with quinacrine 20 μM for 30 min and subsequently stimulated with PMA. Arachidonic acid 60 μM was added simultaneously with PMA. Total RNA was isolated after 6 h incubation. As shown, PMA treatment resulted in high levels of MMP-1 mRNA; quinacrine prevented PMA induced MMP-1 activation, and adding exogenous arachidonic acid to cells treated with quinacrine and PMA did not restore the PMA effect. Use of equal amounts of RNA in this and other experiments is shown by coamplification of the housekeeping gene actin.

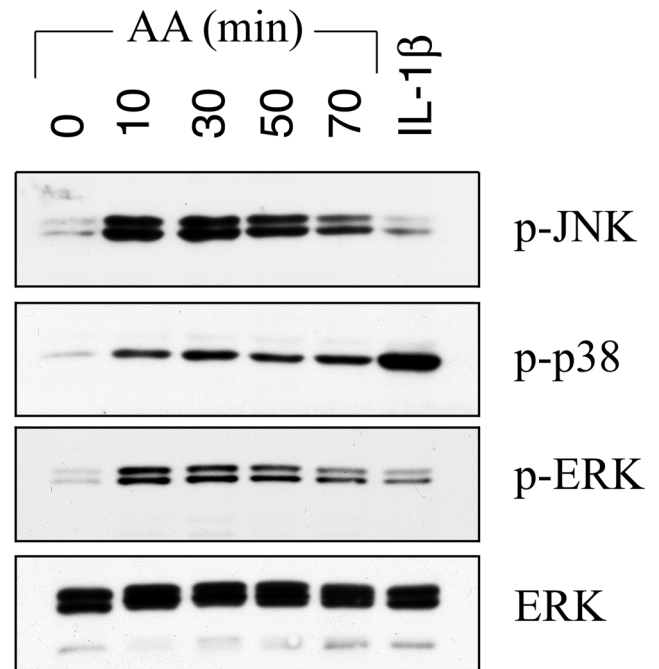


Figure 4B. Arachidonic acid (AA) induces phosphorylation of MAPK. Blots show the bioactivity of the exogenous arachidonic acid used to restore the PLA₂ mediated signaling pathway. Cells were exposed to arachidonic acid 60 μM for times ranging from 0 to 70 min. Interleukin 1 β (1 ng/ml) was included as control. Blots were stained with antibodies that specifically recognize the phosphorylated MAPK JNK (p-JNK), p38 (p-p38), and ERK (p-ERK). One blot stained with non-phosphospecific anti-ERK antibodies is included to confirm equal use of protein. Exogenous arachidonic acid activated all MAPK in a time-dependent fashion.

crossroads of many intracellular signaling pathways, monitoring the activation of MAPK is well suited to determining the biological activities of substances. Data presented in Figure 4B show that adding exogenous arachidonic acid activates MAPK. Cells were treated with 60 μM arachidonic acid for times ranging from 0 to 70 min, and with interleukin 1 β 5 ng/ml for 50 min. Whole-cell protein extracts were separated by SDS-PAGE and stained with antibodies specific for phosphorylated JNK (p-JNK), p38 (p-p38), and ERK (p-ERK), as well as for nonphosphorylated ERK. As shown, the exogenous arachidonic acid used to restore PLA₂ mediated signaling pathways was highly active, in that it induced phosphorylation of all 3 MAPK in a time-dependent manner. Equal loading of proteins was monitored by staining blots with Ponceau red (data not shown) and is also confirmed by the identical signals obtained in Western blots stained with nonphosphorylation state-specific anti-MAPK antibodies.

DISCUSSION

Unrestrained expression of MMP plays a key role in many pathological conditions ranging from cancer to rheumatic disorders^{23,26,33}. Considerable effort has been put into finding efficient MMP inhibitors that might prevent undesirable cell

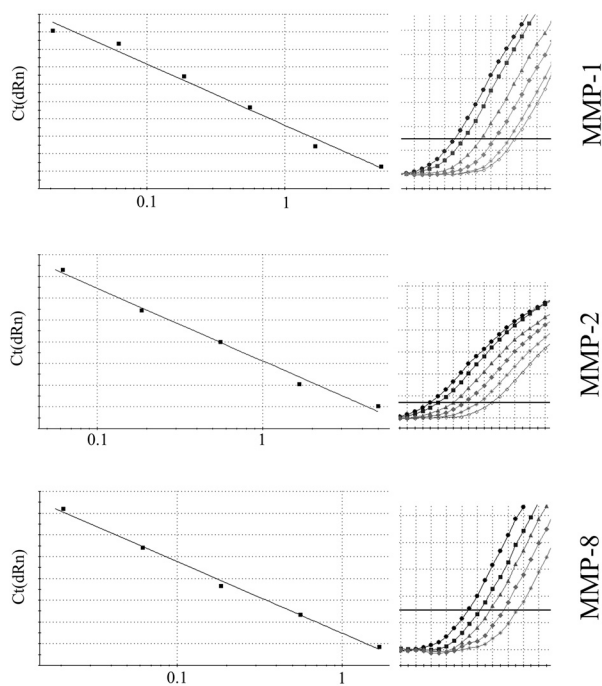


Figure 5. A series of dilutions of cDNA was amplified on a real-time cyclor; the resulting equation was used for quantification of real-time RT-PCR experiments. Left panels show amplification curves for MMP-1, MMP-2, and MMP-8; right panels depict standard curves used for quantification.

migration. We investigated the effects of quinacrine and chloroquine on PMA induced MMP activation in MNC. We also investigated whether the observed inhibition of MMP-1 was due to the inhibition of PLA₂ activity by quinacrine. Our results show that quinacrine at relevant doses acts as a potent inhibitor of MMP-1, while the related compound chloroquine does not. MMP-1-specific ELISA revealed a dose-dependent inhibition of MMP-1 release. In addition, our RT-PCR experiments showed that quinacrine not only inhibited induction of MMP-1 in a dose-dependent fashion, but also downregulated MMP-2 mRNA. In addition, quinacrine suppressed the induced as well as uninduced expression of MMP-8 in MNC, but did not influence the transcription of MMP-3.

With regard to the mechanism of these observations, quinacrine mediated inhibition of PMA induced MMP-1 mRNA accumulation was independent of the observed suppression of PLA₂ activity by quinacrine. Restoring arachidonic acid mediated signaling pathways through the addition of exogenous arachidonic acid did not result in restoration of PMA induced MMP-1 activation — suggesting a PLA₂-independent mechanism.

Drug effects are often not universal, but rather cell type-specific, a consequence of the utilization of distinct signaling pathways in different cell types. Previously we reported the effects of quinacrine on MMP activation patterns in fibroblast-like synoviocytes²⁷. The data presented in this report are in accord with our earlier observations using different types of

cells, and extend our previous findings significantly by demonstrating the PLA₂ independence of the observations. Further, determining that quinacrine is able to block MMP-1, MMP-2, and MMP-8 upregulation in MNC underlines the potential of this substance as a regulator of MMP.

Quinacrine, often referred to as mepacrine or atabrine, has been used for more than 60 years and was administered to millions of individuals during World War II as an antimalarial agent¹. Although quinacrine has been replaced by other antimalarials, it is approved for the treatment of giardiasis, with a cure rate of more than 90%, and is similarly recommended for the treatment of certain rheumatic diseases. Dosage of quinacrine 100 mg orally for a few days is recommended for treatment of giardiasis, while the drug has been administered orally for treatment of malaria in daily doses from 100 mg to 1000 mg for many months¹⁰. Quinacrine distributes slowly and achieves steady-state levels only 4 weeks after the start of therapy, corroborating observations that it takes several weeks for it to be maximally effective, for example, in the treatment of SLE¹.

As an antimalarial remedy, quinacrine was eventually replaced by the more potent chloroquine. The better efficacy of chloroquine in treating malaria seems to have resulted in the perception that chloroquine will therefore be a more efficient drug for the treatment of other ailments. It seems that to this day physicians are more prone to use chloroquine than quinacrine. Nevertheless, there is ample evidence that quinacrine is underused and underestimated, and might hold great potential for treatment of certain diseases^{1,2}.

Although they are related in structure, it has been noted that quinacrine and chloroquine can be vastly different in their effectiveness. Quinacrine has an EC₅₀ of 0.3 μM when used to obliterate the formation of disease-causing prion isoforms, whereas more than 10-fold concentrations had to be used to achieve similar effects with chloroquine¹⁰. The significant improvement of patients after combination therapy with both quinacrine and chloroquine also points at differences in their mode of action^{4,5,34,35}. In other cases very high concentrations of chloroquine had to be used to achieve significant effects; for example, inhibition of tumor necrosis factor-α synthesis in lipopolysaccharide treated macrophages was achieved by the use of 150 μM chloroquine³⁶. Our data also emphasize differences between these 2 drugs, for while quinacrine is a potent inhibitor of PMA induced MMP-1 activation, chloroquine is not.

Our observation that quinacrine has the potential to inhibit upregulation of MMP might also explain numerous observations made in *in vitro* and *in vivo* experiments. Mikes, *et al* reported, for example, that among several PLA₂ inhibitors tested, only quinacrine was able to significantly inhibit leukocytosis in a rat model¹³. In another *in vivo* model, quinacrine decreased lung neutrophil accumulation¹⁸.

As noted, quinacrine is best known for its ability to prevent PLA₂ activation, a property first reported in 1977³⁷.

Consequently, quinacrine is used in numerous studies as a “specific” PLA₂ inhibitor. However, as reported, quinacrine can affect cells at many levels, and as shown here, inhibition of MMP activation by quinacrine is independent of the effects of quinacrine on PLA₂. Figure 4 shows that supplementing cell culture medium with high doses of arachidonic acid did not lead to restoration of PMA induced MMP-1 mRNA accumulation. That PLA₂ inhibition is not solely responsible for the effects of quinacrine has been noted before²¹. Interestingly, investigators have even reported activation of PLA₂ activity by quinacrine³⁸. Such differences might again be consequences of cell type-specific effects.

In light of the observed inhibition of MMP-1 at the transcriptional level, it is of interest (as shown in Figure 1) that quinacrine reproducibly leads to somewhat higher levels of MMP-1 protein in culture supernatants of cells pretreated with quinacrine for only a short time prior to stimulation with PMA. This might be due to quinacrine induced changes in the rate of degradation and/or synthesis of MMP-1 proteins, that is, inhibitory effects of quinacrine on other MMP-degrading proteins. We resorted to replenishing cells with fresh medium shortly before stimulating cells with PMA in experiments where quinacrine’s effects on MMP-1 protein levels were monitored. Such measures enabled us to quantify the inhibitory effect of quinacrine on MMP-1 by ELISA.

More importantly, as illustrated in the representative experiment shown in Figures 2, 3, and 4, treatment of MNC with quinacrine for 30 min was sufficient to block PMA induced mRNA synthesis in a dose-dependent manner. In addition, treatment of MNC with equimolar amounts of chloroquine did not suppress MMP-1 or MMP-2 mRNA levels.

Taken together, our data indicate that quinacrine but not chloroquine has the potential to be a potent inhibitor of MMP. Major resources have been devoted to finding ways to control unwanted MMP activation and, although there are compounds that reportedly can achieve this, quinacrine seems to stand out due to its notable safety record. Thus it may be worth investigating the chemical/structural determinants of MMP inhibition, with the objective of designing more potent derivatives of quinacrine.

REFERENCES

- Wallace DJ. The use of quinacrine (atabrine) in rheumatic diseases: a reexamination. *Semin Arthritis Rheum* 1989;18:282-96.
- Wallace DJ. Is there a role for quinacrine (atabrine) in the new millennium? *Lupus* 2000;9:81-2.
- 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.
- 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.
- Chung HS, Hann SK. Lupus panniculitis treated by a combination therapy of hydroxychloroquine and quinacrine. *J Dermatol* 1997;24:569-72.
- D’Cruz D. Antimalarial therapy: a panacea for mild lupus? *Lupus* 2001;10:148-51.
- Nakajima M, Yamada T, Kusuvara T, et al. Results of quinacrine administration to patients with Creutzfeldt-Jakob disease. *Dement Geriatr Cogn Disord* 2004;17:158-63.
- Dohgu S, Yamauchi A, Takata F, et al. Uptake and efflux of quinacrine, a candidate for the treatment of prion diseases, at the blood-brain barrier. *Cell Mol Neurobiol* 2004;24:205-17.
- Vogtherr M, Grimme S, Elshorst B, et al. Antimalarial drug quinacrine binds to C-terminal helix of cellular prion protein. *J Med Chem* 2003;46:3563-4.
- 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.
- Pierer M, Wagner U, Häntzschel H, Gay R, Gay S. Pathogenesis of inflammatory rheumatic diseases of the joints — current research findings. In: Laufer S, Gay S, Brune K, editors. *Inflammation and rheumatic diseases*. Stuttgart: Georg Thieme Verlag; 2003:1-14.
- Panus PC, Jones HP. Inhibition of neutrophil response by mepacrine. *Biochem Pharmacol* 1987;36:1281-4.
- 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.
- Mikes PS, Polomski LL, De Musis MR, Gee JB. The neutrophil, friend or foe: pharmacologic manipulation. *Respiration* 1988;54:9-15.
- Foldes-Filep E, Filep JG. Mepacrine inhibits fMLP-induced activation of human neutrophil granulocytes, leukotriene B₄ formation, and fMLP binding. *J Leukoc Biol* 1992;52:545-50.
- Estevez AY, Phillis JW. The phospholipase A₂ inhibitor, quinacrine, reduces infarct size in rats after transient middle cerebral artery occlusion. *Brain Res* 1997;752:203-8.
- al Khader A, al Sulaiman M, Kishore PN, Morais C, Tariq M. Quinacrine attenuates cyclosporine-induced nephrotoxicity in rats. *Transplantation* 1996;62:427-35.
- Lee YM, Hybertson BM, Terada LS, Repine AJ, Cho HG, Repine JE. Mepacrine decreases lung leak in rats given interleukin-1 intratracheally. *Am J Respir Crit Care Med* 1997;155:1624-8.
- Otani H, Engelman RM, Breyer RH, Rousou JA, Lemeshow S, Das DK. Mepacrine, a phospholipase inhibitor. A potential tool for modifying myocardial reperfusion injury. *J Thorac Cardiovasc Surg* 1986;92:247-54.
- Sargent CA, Vesterqvist O, McCullough JR, Ogletree ML, Grover GJ. Effect of the phospholipase A₂ inhibitors quinacrine and 7, 7-dimethyleicosadienoic acid in isolated globally ischemic rat hearts. *J Pharmacol Exp Ther* 1992;262:1161-7.
- Bugge E, Gamst TM, Hegstad AC, Andreassen T, Ytrehus K. Mepacrine protects the isolated rat heart during hypoxia and reoxygenation — but not by inhibition of phospholipase A₂. *Basic Res Cardiol* 1997;92:17-24.
- McCawley LJ, Matrisian LM. Matrix metalloproteinases: they’re not just for matrix anymore! *Curr Opin Cell Biol* 2001;13:534-40.
- Katirji A, Tak PP, Bertouch JV, et al. Expression of chemokines and matrix metalloproteinases in early rheumatoid arthritis. *Rheumatology Oxford* 2001;40:988-94.
- Stamenkovic I. Matrix metalloproteinases in tumor invasion and metastasis. *Semin Cancer Biol* 2000;10:415-33.
- Johnson LL, Dyer R, Hupe DJ. Matrix metalloproteinases. *Curr Opin Chem Biol* 1998;2:466-71.
- McCawley LJ, Matrisian LM. Matrix metalloproteinases: multifunctional contributors to tumor progression. *Mol Med Today* 2000;6:149-56.
- Stuhlmeier KM. Mepacrine inhibits matrix metalloproteinases-1 (MMP-1) and MMP-9 activation in human fibroblast-like synoviocytes. *J Rheumatol* 2003;30:2330-7.
- Kontinen 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.
29. Stuhlmeier KM, Pollaschek C. Differential effect of transforming growth factor-beta on the genes encoding hyaluronan synthases and utilization of the p38 MAPK pathway in TGF-beta-induced hyaluronan synthase 1 activation. *J Biol Chem* 2004;279:8753-60.
30. Du H, Olivo M, Mahendran R, Bay BH. Modulation of matrix metalloproteinase-1 in nasopharyngeal cancer cells by photoactivation of hypericin. *Int J Oncol* 2004;24:657-62.
31. Sun HB, Liu Y, Qian L, Yokota H. Model-based analysis of matrix metalloproteinase expression under mechanical shear. *Ann Biomed Eng* 2003;31:171-80.
32. Goffin F, Munaut C, Frankenne F, et al. Expression pattern of metalloproteinases and tissue inhibitors of matrix-metalloproteinases in cycling human endometrium. *Biol Reprod* 2003;69:976-84.
33. Chang C, Werb Z. The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis. *Trends Cell Biol* 2001;11:S37-43.
34. Toubi E, Kessel A, Rosner I, et al. Quinacrine added to ongoing therapeutic regimens attenuates anticardiolipin antibody production in SLE. *Lupus* 2003;12:297-301.
35. Lipsker D, Piette JC, Cacoub P, Godeau P, Frances C. Chloroquine-quinacrine association in resistant cutaneous lupus. *Dermatology* 1995;190:257-8.
36. Jeong JY, Jue DM. Chloroquine inhibits processing of tumor necrosis factor in lipopolysaccharide-stimulated RAW 264.7 macrophages. *J Immunol* 1997;158:4901-7.
37. Horrobin DF, Manku MS, Karmazyn M, Ally AI, Morgan RO, Karmali RA. Quinacrine is a prostaglandin antagonist. *Biochem Biophys Res Comm* 1977;76:1188-96.
38. Authi KS, Traynor JR. Stimulation of polymorphonuclear leucocyte phospholipase A2 activity by chloroquine and mepacrine. *J Pharm Pharmacol* 1982;34:736-8.