EXTENDED REPORT

The regulation of human MMP-13 by licofelone, an inhibitor of cyclooxygenases and 5-lipoxygenase, in human osteoarthritic chondrocytes is mediated by the inhibition of the p38 MAP kinase signalling pathway

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Background: MMP-13 is one of the most important metalloproteases (MMP) involved in osteoarthritis. Licofelone, a novel dual inhibitor of cyclooxygenases (COX) and 5-lipoxygenase (5-LOX), can modulate MMP-13 production in human osteoarthritic chondrocytes. 

Objective: To evaluate the impact of licofelone on MMP-13 expression/production, promoter, and major MAP kinase signalling pathways and transcription factors.

Methods: Human osteoarthritic chondrocytes were stimulated by interleukin 1β (IL1β) and treated with or without licofelone (0.3, 1, or 3 μg/ml); NS-398 (10 μM; a specific COX-2 inhibitor); or BayX-1005 (10 μM; a specific 5-LOX inhibitor). MMP-13 synthesis was determined by specific enzyme linked immunosorbert assay, and expression by real time polymerase chain reaction. The effect of licofelone on the MMP-13 promoter was studied through transient transfection; dexamethasone (10^{-7} M) was used as comparison. The effect on IL1β induced MMP-13 signalling pathways was determined using specific ELISA for phosphorylated MAP kinases and transcription factors.

Results: Licofelone dose dependently inhibited the IL1β stimulated production and expression of MMP-13. NS-398 and BayX-1005 had very little effect. Licofelone also inhibited MMP-13 transcription on each of the promoter constructs used. The licofelone inhibition was comparable to that obtained with dexamethasone. Licofelone had no effect on phosphorylated p44/42 or JNK1/2; however, it decreased phosphorylated c-jun and inhibited phosphorylated p38, CREB, and AP-1 activity.

Conclusions: Licofelone inhibited MMP-13 production under proinflammatory conditions on human osteoarthritic chondrocytes, through inhibition of the p38/AP-1 pathway and the transcription factor CREB. This may explain some of the mechanisms whereby licofelone exerts its positive effect on osteoarthritic changes.

In osteoarthritis, the structural changes that take place during the evolution of the disease ultimately result in the degeneration of articular cartilage. The first macroscopic signs of joint destruction include cartilage swelling, fibrillation, and the disruption of the collagen framework. The integrity of the collagen macromolecules plays a primary role in the functional properties of the cartilage. Damage to this network is a critical event in the pathogenesis of osteoarthritis because, when this framework fails, the tissue degenerates irreversibly.

In cartilage, collagen type II is of particular importance as its breakdown results in the irreversible loss of the structural integrity of the tissue. Although not all the mechanisms leading to the development of cartilage erosion in osteoarthritis are completely understood, it is acknowledged that proteolytic degradation of the extracellular matrix is particularly significant. Evidence points to an increased production of matrix metalloproteases (MMP), a family of structurally related zinc dependent endopeptidases that are capable collectively of degrading extracellular matrix components. This family contains approximately 25 members which can be divided into subgroups: collagenases, gelatinases, stromelysins, membrane-type MMPs, and a heterogeneous group. Within this family, collagenases are the enzymes responsible for native collagen degradation.

Recent progress in the identification of the MMP involved in cartilage collagen matrix degradation has shown that, in addition to MMP-1 (collagenase-1), MMP-13 (collagenase-3) is also important in the osteoarthritic pathological process. The latter collagenase cleaves types I, II, and III collagens at a specific site. MMP-13 has 5- to 10-fold greater activity on collagen type II and a gelatinase activity that is over 44 times greater than that of MMP-1. MMP-13 also degrades collagen types IV and X, as well as other matrix molecules including fibronectin and aggrecan core protein. The ability of MMP-13 to degrade a wide range of extracellular matrix components suggests that its physiological expression is involved in situations where the rapid and effective remodelling of collagenous extracellular matrices is required—for example, during fetal bone development. The demonstration of a functional role of MMP-13 in osteoarthritis has raised the possibility of using therapeutic interventions directed against its production.

Licofelone, a novel dual inhibitor of cyclooxygenases (COX) and 5-lipoxygenase (5-LOX), has been shown in vivo in animal models to have a positive effect on the...
structural changes that articular tissue undergoes during arthritis. In the in vivo adjuvant arthritis model, this compound induced a significant reduction in secondary lesions, paw swelling, synovial cell proliferation, histological damage, and joint erosion. On an induced canine osteoarthritis model, licofelone reduced the severity of cartilage erosion, subchondral bone remodelling, and the severity of the histological damage, and had potent anti-inflammatory and anti-apoptotic properties.

In this study, we evaluated the effects of licofelone on IL-1β induced MMP-13 production in human osteoarthritic chondrocytes by analysing the impact of licofelone on MMP-13 expression and on its promoter, as well as on the major signalling pathways involved in MMP-13 enhanced transcription. Our data show that licofelone has a potent inhibitory effect on IL-1β induced MMP-13 production in human osteoarthritic chondrocytes, which results in inhibition at the transcriptional level and is related to the inhibition of the activation of MAP kinase p38, AP-1, and CREB.

**METHODS**

**Specimen selection**

Human osteoarthritis cartilage (femoral condyles and tibial plateaus) was obtained from patients undergoing total knee arthroplasty (mean (SD) age, 67 (9) years). All patients were evaluated by a certified rheumatologist and the diagnosis was evaluated using the American College of Rheumatology criteria. These specimens represented moderate to severe osteoarthritis, as defined by macroscopic criteria. The University of Montreal Hospital Centre Clinical research ethics committee at the Notre-Dame Hospital approved the use of human articular tissue.

**Chondrocyte cultures and treatments**

Chondrocytes were released from full thickness strips of articular cartilage through sequential enzymatic digestion at 37°C, as previously described. The cells were seeded at high density (10⁵ cells/cm²) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, Burlington, Canada) supplemented with 10% heat inactivated fetal calf serum (FCS; Gibco-BRL) and an antibiotic mixture (100 units/ml penicillin base and 100 μg/ml streptomycin base, Gibco-BRL) at 37°C in a humidified atmosphere of 5% CO₂/95% air. Only first passage cultured chondrocytes were used. Human osteoarthritic chondrocytes were incubated in DMEM/0.5% FCS with 1–100 pg/ml rhIL1β (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) at 37°C, after which the temporal and dose dependent effects of licofelone on MMP-13 synthesis were studied. Three therapeutic concentrations of the drug were used (0.3, 1.0, and 3.0 μg/ml), with incubation periods of 24 to 96 hours. The licofelone concentrations used are within the therapeutic concentrations of the drug were used (0.3, 1.0, and 3.0 μg/ml) for 24 to 96 hours. The licofelone concentrations used are within the therapeutic range, based on clinical studies (Merckle GmbH, internal files). Two comparators were used: NS-398, a specific COX-2 inhibitor (10 μM; Calbiochem, La Jolla, California, USA); and BayX-10005, a specific 5-LOX inhibitor (10 μM, generously provided by Merckle, GmbH, Germany). At these concentrations, studies showed that each comparator had maximal effects on its target molecule. The conditioned medium was used for the MMP-13 synthesis determination, employing a specific enzyme linked immunosorbent assay (ELISA) (Amersham Pharmacia Biotech, Baie D’Urfé, Quebec, Canada). The ELISA allowed the measurement of both pro and active collagenase-3 with a sensitivity of 32 pg/ml.

**RNA extraction, reverse transcription, and real time polymerase chain reaction**

Total cellular RNA was extracted from osteoarthritic chondrocytes with TRIzol™ reagent (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer’s specifications after 24 hours of stimulation, in the presence or absence of 1–100 pg/ml rhIL1β, with or without 3 μg/ml of licofelone. RNA was treated with a DNA-free™ DNase treatment and removal kit (Ambion, Austin, Texas, USA) to ensure the complete removal of chromosomal DNA. The RNA was quantitated using a RiboGreen RNA quantitation kit (Molecular Probes, Eugene, Oregon, USA). Reverse transcription reactions were primed with random hexamers, with 2 μg of total RNA in a 100 μl final reaction volume.

The real time quantitation of MMP-13 and GAPDH mRNA was carried out in the GeneAmp 5700 sequence detection system (Applied Biosystems, Foster City, California, USA) with 2X Quantitect SYBR Green polymerase chain reaction (PCR) master mix (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer’s specifications, and as previously described by our laboratory. The primer sequences were 5’-CTTAGAGTGACTGGCAAC-3’ (sense) and 5’-GCCATCAATGGTTAGAG-3’ (anti-sense) for MMP-13, and 5’-CAGAACATCATCCGTCCT-3’ (sense) and 5’-GCTGACAAATGTTCTGAG-3’ (antisense) for GAPDH. Data were given a threshold cycle (Ct), which was converted to number of molecules and calculated as the ratio to GAPDH. Data are expressed as -fold difference with respect to control, which was assigned a value of 1.

**Effect of licofelone on MMP-13 promoter activity**

Transient transfections were done on human osteoarthritic chondrocytes, using the DNA calcium precipitation, with the MMP-13 constructs, as previously described. Twenty-four hours post-transfection, chondrocytes were cultured at 37°C for an additional 24 hours in the presence or absence of phorbol-12-myristate-13-acetate (PMA; 100 ng/ml) (Calbiochem), with or without therapeutic concentrations of licofelone (3 μg/ml, the dose most effective at reducing MMP-13 synthesis) or the comparator dexamethasone (10⁻⁷ M; Sigma-Aldrich, Oakville, Ontario, Canada). After the incubation period, cells were washed with PBS and lysed in the reporter lysis buffer (Promega, Madison, Wisconsin, USA).

Three human collagenase-3 promoter constructs prepared in our laboratory were used. Plasmid –1339 luciferase was constructed by cloning a 1.6 kb fragment of the MMP-13 promoter. The –183 luciferase construct consisted of the first proximal 183 bp of the MMP-13 promoter, and contained OSE-2, AGRE, PEA-3, and AP-1 sites as well as the TATA box. The –133 luciferase corresponds to the –183 luciferase construct without the OSE-2 site. Cells were transfected with the plasmid pCMV β galactosidase to monitor efficiency, and luciferase positive and negative controls were also used. Following incubation, luciferase and β galactosidase levels were measured on cell lysates. The MMP-13 promoter activity level was calculated as RLU/μg of protein and was expressed as a percentage of control, which was assigned a value of 100.

**Effect of licofelone on MMP-13 cell signalling pathways**

Human osteoarthritic chondrocytes were stimulated with rhIL1β (100 pg/ml) and treated with licofelone (3 μg/ml) for 15 to 120 minutes. Cells were lysed and protein levels were quantitated using the bicinchoninic acid (BCA) method. Samples were assayed using ELISA kits for phosphorylated p38, p44/42, IkBα, MAPK-1, c-Jun, and c-fos (Active Motif, MJJ Biolynx, Brockville, Ontario, Canada). AP-1 activity was measured using the electrophoretic mobility shift assay (EMSA) as previously described.

Brieﬂy, osteoarthritic chondrocytes were...
Figure 1  Interleukin (IL) 1β dose and time response study of matrix metalloprotease 13 (MMP-13) production (A) and expression (B) in the presence or absence of licofelone. Human osteoarthritis chondrocytes were incubated for (A) 24 or 72 hours, or (B) 24 hours with or without 1–100 pg/ml IL1β or 3 µg/ml licofelone. MMP-13 production was determined in the conditioned medium using a specific enzyme linked immunosorbent assay (ELISA) (A), and MMP-13 expression was determined by real time polymerase chain reaction (B). (C) Licofelone dose and time response study of MMP-13 production. Human osteoarthritis chondrocytes were incubated for 24 to 96 hours in the absence or presence of 100 pg/ml IL1β and 0.3, 1.0, or 3.0 µg/ml licofelone or NS-398 at 10 µM and Bay-X-1005 at 10 µM. MMP-13 production was determined in the conditioned medium using a specific ELISA. Data are expressed as means of (A) four, (B) six to seven, and (C) four independent experiments. Error bars = SEM; p values indicate significant differences from the respective controls.
incubated in the absence or presence of IL1β (100 pg/ml) and licofelone (3 μg/ml) for 24 hours. Nuclear proteins were extracted as previously described. The double stranded AP-1 oligonucleotides 5′-TAATGATGACTCACCATTGC-3′ were end labelled with [γ-32P]-ATP using T4 polynucleotide kinase (Promega). Binding reactions were conducted with 5 μg of nuclear extracts and 25 fmol of [γ-32P] labelled oligonucleotide probes at 22°C for 20 minutes in a final volume of 10 μl. Binding complexes were resolved on non-denaturing 6% polyacrylamide gels, after which the gels were fixed, dried, and exposed to Kodak X-AR5 films. Data were expressed as -fold difference with respect to control (basal condition), which was assigned a value of 1.

Statistical analysis
Data are expressed as mean (SEM). The statistical analysis was assessed by the two tailed paired Student t test, and probability (p) values less than 0.05 were considered significant.

RESULTS
Effect of licofelone on IL1β induced MMP-13 production/expression
To explore the effect of licofelone on MMP-13 synthesis, we first carried out dose and time response experiments with 1–100 pg/ml IL1β. The data showed that, as expected, human osteoarthritis chondrocytes spontaneously produced MMP-13, which increased in a dose dependent manner on IL1β stimulation (fig 1A). Licofelone at 3 μg/ml inhibits IL1β induced MMP-13, and maximum inhibition was reached at 72 hours with 100 pg/ml IL1β. Hence, a concentration of 100 pg/ml IL1β was used for further experiments.

Figure 1B shows that licofelone significantly reduced the IL1β induced MMP-13 mRNA production, suggesting that it acts at the transcriptional level.

A time and dose curve of licofelone on IL1β induced MMP-13 production was also undertaken (fig 1C). Licofelone produced dose dependent inhibition of MMP-13 synthesis. The maximum licofelone effect occurred at 3 μg/ml, at which statistically significant inhibitions of 34%, 41%, 39%, and 50% were found at 24, 48, 72, and 96 hours of treatment, respectively. Even at a lower concentration (0.3 μg/ml), licofelone significantly inhibited IL1β induced MMP-13 production, starting at 48 hours of incubation. Conversely, neither NS-398, a selective COX-2 inhibitor, nor BayX-1005, a specific 5-LOX inhibitor, significantly reduced the IL1β induced MMP-13 production. The maximum inhibitions for NS-398 and BayX-1005 were 22% and 17% and were reached at only 96 hours of incubation.

Effect of licofelone on cell signalling pathways
IL1β significantly increased the level of the phosphorylated forms of p44/42 (p<0.04 at 45–60 minutes) and p38 (p<0.01 at 15–60 minutes), and only very slightly increased the level of phosphorylated JNK1/2 (fig 3A). Licofelone had no effect or only slightly increased the basal level of the phosphorylated forms of p44/42, JNK1/2, and p38. On the IL1β stimulated cells, licofelone had no effect on the level of the phosphorylated forms of p44/42 and JNK1/2; however, it

Figure 2 Functional analysis of the matrix metalloprotease 13 (MMP-13) promoter in human osteoarthritis chondrocytes. Three different constructs of the MMP-13 promoter fused to a luciferase reporter are shown in schematic representation (left). Transfection was carried out in the presence of 100 ng/ml PMA with or without licofelone at 3 μg/ml or dexamethasone at 10–7 M. The cells were also co-transfected with pCMV-β-galactosidase. Data are expressed as the mean percentage of six to seven independent experiments. Error bars = SEM; p values indicate significant difference from the control.
induced a significant decrease in the level of phosphorylated p38 after 15 minutes of incubation (fig 3A). Of note, cell signalling was not carried out before 15 minutes, as preliminary experiments showed no effect before this time (data not shown).

On the transcription factor (fig 3B), IL1β markedly increase the level of phosphorylated CREB (p<0.03), reaching a maximum after 45 minutes of incubation. Licofelone reduced the IL1β induced CREB phosphorylation starting at 15 minutes, and achieved its maximum reduction at 60 minutes. Phosphorylated c-jun was only slightly increased by IL1β (15–60 minutes), and licofelone reversed the IL1β induced phospho-c-jun at 30 and 45 minutes (fig 3B); this, however, did not reach statistical significance. The level of phosphorylated junB and c-fos showed no difference in the absence or presence of licofelone (data not shown).

The EMSA experiments (fig 3C) revealed that AP-1 nuclear DNA binding proteins were increased following treatment with IL1β. However, licofelone reversed the IL1β induced AP-1 DNA binding activity (p<0.04). The
specify the binding was confirmed by a 200-fold excess of unlabelled AP-1 oligonucleotides using the nuclear extract of IL1β stimulated chondrocytes.

**DISCUSSION**

Licofelone, a substrate analogue of the arachidonic acid active sites of 5-LOX, COX-1, and COX-2, acts within the same concentration range as arachidonic acid, and inhibits the production of both LTB4 and PGE2. PGE2 and LTB4 are present in articular joint tissues, in which they are able to regulate the synthesis of many catabolic factors, including metalloproteases. Moreover, previous studies showed that licofelone reduced the progressive destruction of cartilage in animal models in vivo, and that this effect occurs, at least in part, through MMP inhibition. Thus we further explored the effect of licofelone on IL1β induced MMP-13 in human osteoarthritis chondrocytes, and the mechanisms whereby this drug modulates the synthesis of this enzyme.

Data showed that licofelone inhibited MMP-13 production under proinflammatory conditions, an effect that occurred at the transcriptional level and implicated the proximal part of the MMP-13 promoter. We also found that the action of licofelone on MMP-13 expression occurs through an inhibition of the activity of p38 MAP kinase, and of the activation of the transcription factors CREB and AP-1.

This study is the first to show that licofelone significantly inhibits MMP-13 synthesis and expression in a time and dose dependent manner. This is in agreement with the data from an animal model showing that this compound inhibits MMP synthesis in cartilage in vivo. Interestingly, neither a selective COX-2 inhibitor (NS-398) nor a selective 5-LOX inhibitor (BayX-1005) was able to affect the IL1β induced MMP-13 expression, thereby stressing the importance of the dual effect of licofelone on COX and 5-LOX for its inhibitory activity against MMP synthesis. These data are also in agreement with the theory that depletion of PGE2 in osteoarthritis chondrocytes induced by COX inhibition leads to a shunt towards LTB4 production, thus inducing the excess production of some catabolic factors. However, it could also be speculated that licofelone affects the MMP-13 production through a COX/5-LOX inhibition independent pathway, as shown for certain NSAIDs on specific cellular mechanisms.

To gain insight into the mechanisms by which licofelone reduces IL1β induced MMP-13 production, we first investigated its effect on the human MMP-13 gene promoter. As we previously described, specific gene sequences play a crucial role in MMP-13 gene induction/repression. Based on these previous findings, we chose three MMP-13 promoter constructs of different lengths that were the most relevant. Data from the transfection experiments, further confirmed by the EMSA, indicated that the AP-1 site is the key factor inhibited by licofelone, as a maximum effect is obtained with the smaller construct containing only AP-1 and two other sites. These data also agree with the findings on several cell types, showing the requirement for AP-1 in the regulation of this MMP. It is possible that the lesser effect obtained with the longer promoters (−1599 and −183Luc) reflects the presence of elements or interactions between upstream sites that may act negatively. Interestingly, the effect of licofelone on the MMP-13 promoter was comparable to that of dexamethasone, one of the most potent anti-inflammatory drugs for inhibiting MMP-13 synthesis. Although the precise mechanisms involved in the downregulation of MMP-13 gene expression by dexamethasone remain to be determined, this process has been shown to occur indirectly through the AP-1 site.

Upstream of the AP-1 transcription factors is the induction of multiple phosphorylation dependent signalling pathways. Studies have shown that in chondrocytes from various origins IL1 mediates its signal mostly through the MAP kinase families ERK (p44/42), JNK1/2, and p38, and also, for some target genes, through NFκB. More precisely, in rabbit chondrocytes and chondrosarcoma cells, the IL1 induction of MMP-13 was shown to occur only through p38 and JNK1/2, although a role for NFκB was suggested. Nevertheless, the exact mechanism of NFκB activity in IL1 induced MMP-13 is as yet unknown and indirect action on other genes, perhaps on the activation of p38, has been suggested.

It is understood that p38 and JNK1/2 are mainly activated by proinflammatory cytokines and stress stimuli, whereas—although it could respond to some cytokines—p44/42 is particularly activated by growth factors. Under the actual experimental conditions, the phosphorylated forms of both p38 and p44/42, but not JNK1/2, were upregulated by IL1β. The absence of an effect of IL1β on the phosphorylation of JNK1/2 may be because this factor is already highly activated in the osteoarthritic chondrocytes through endogenous stimulation. Licofelone significantly and rapidly inhibited p38 activation, but had no effect on the activation of p44/42 and JNK1/2. These findings concur with studies on other cell types showing the critical role of p38 activation in the regulation of MMP-13 expression. Interestingly, licofelone was shown in vivo in the osteoarthritic dog model to inhibit apoptosis, a phenomenon that was found to be mediated, at least in part, by the activation of p38 with the subsequent induction of nitric oxide synthesis.

The nuclear factor AP-1 comprises the products encoded by the members of the jun or fos gene family, or both. Jun proteins homodimerise or heterodimerise with proteins of the Fos family for AP-1 DNA binding activity. Functional specificity among the various members of these gene families determines their differential distribution and transactivating properties. Among members of the jun family, c-jun and junB are of major importance and, because either could make homodimers they could serve as modulators. In contrast to c-jun, which is often required for the full induction of a target gene, junB may act as a negative regulator. More particularly, junB was reported to negatively regulate two collagenases, MMP-1 and MMP-13. Here, we found that licofelone had no effect on junB, but it caused inhibition of IL1 induced c-jun activity. The latter, however, did not reach statistical significance, possibly because of the low level of inducibility of phosphorylated c-jun by IL1β (compared, for example, with phosphorylated CREB), reflecting the high basal level of phosphorylated c-jun in non-stimulated human osteoarthritic chondrocytes.

The effect of the selective inhibition of p38 (using SB203580), leading to a decrease in MMP-13 gene expression, has been shown on different cell types, including animal chondrocytes and transformed keratinocytes, and also on IL1 mediated collagen degradation in cartilage explants. However, to date, as there are no known targets of p38 that directly regulate the MMP promoter; p38 could act indirectly through the phosphorylation of transcription factors. Hence, reduction of p38 activity by licofelone could be responsible for the decreased AP-1 activity, resulting in the inhibition of the activation of c-jun at the AP-1 site. Data also indicated that IL1β increased CREB activation is significantly inhibited by licofelone. Although there is no CREB binding site on the MMP-13 promoter, there is evidence for the binding of CREB on the AP-1 site. Moreover, Masquillier et al showed that antagonists of CREM (proteins with same binding properties as CREB) are capable of negatively modulating the transcriptional activity elicited by Jun/AP-1. In addition, and supporting our data, a link between p38...
activation and the transcription factor CREB has also been documented, in which p38 is shown to be an upstream mediator of CREB activation.53 Thus it could be hypothesised that, in osteoarthritis chondrocytes, MMP-13 is also stimulated by CREB activation through binding to the AP-1 site, and that the inhibition of CREB activity will impair MMP-13 expression. It is therefore logical to assume that the direct inhibition of both p38 and CREB activities by licofelone could lead to a decrease in c-Jun and AP-1 activation, and, as a result, to a decrease in MMP-13 production.

Conclusions

In this study we confirm our previous finding that licofelone inhibits the activity of both p38 and CREB, and that the inhibition of CREB activity will impair MMP-13 and cathepsin K. Further evidence for the possible mode of action. Additionally, we show interactions with anti-inflammatory cytokines. J Rheumatol 2002;29:546–53.


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