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# Triptolide suppresses proinflammatory cytokine-induced matrix metalloproteinase and aggrecanase-1 gene expression in chondrocytes

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**Abstract**

A hallmark of rheumatoid- and osteoarthritis (OA) is proinflammatory cytokine-induced degeneration of cartilage collagen and aggrecan by matrix metalloproteinases (MMPs) and aggrecanases (ADAMTS). Effects of the Chinese herb, *Tripterygium wilfordii* Hook F (TWHF), on cartilage and its anti-arthritic mechanisms are poorly understood. This study investigated the impact of a purified derivative of TWHF, PG490 (triptolide), on cytokine-stimulated expression of the major cartilage damaging proteases, MMP-3, MMP-13, and ADAMTS4. PG490 inhibited cytokine-induced MMP-3, MMP-13 gene expression in primary human OA chondrocytes, bovine chondrocytes, SW1353 cells, and human synovial fibroblasts. Triptolide was effective at low doses and blocked the induction of MMP-13 by IL-1 in human and bovine cartilage explants. TWHF extract and PG490 also suppressed IL-1-, IL-17-, and TNF-α-induced expression of ADAMTS-4 in bovine chondrocytes. Thus, PG490 could protect cartilage from MMP- and aggrecanase-driven breakdown. The immunosuppressive, cartilage protective, and anti-inflammatory properties could make PG490 potentially a new therapeutic agent for arthritis.


*Keywords:* Cytokines; Chondrocytes; Matrix metalloproteinases; Aggrecanase; Triptolide

Patients with rheumatoid arthritis (RA) and osteoarthritis (OA) experience variable degrees of inflammation and degeneration of articular cartilage ultimately resulting in exposure of underlying bone, pain, and disability [1,2]. Elevated proinflammatory cytokines, interleukin-1 (IL-1), IL-17, and tumor necrosis factor-α (TNF-α) in the joints of arthritis patients are prominent stimuli for inducing cartilage catabolism [3–5]. The cartilage extracellular matrix is cleaved by cytokine-stimulated proteases, matrix metalloproteinases (MMPs), and aggrecanases or ADAMTS (a disintegrin and metalloproteinase with thrombospondin motif) [6–8]. MMP-3 and MMP-13 cleave aggrecan and collagen II, and cartilage-specific overexpression of human MMP-13 in mice mimics human osteoarthritis [9,10]. These enzymes and their cleavage products are increased in arthritic joint tissues and synovial fluid [11–14]. Similarly, ADAMTS-4 and -5 are major proteases degrading aggrecan [6,8,15]. MMP-13 and aggrecanases are main targets for developing cartilage protective therapies either by direct inhibition of enzyme activities or by interception in proinflammatory signal transduction [16,17].

Chinese herb, *Tripterygium wilfordii* Hook F (TWHF), has therapeutic activity against arthritis and other autoimmune diseases due to its immunosuppressive and anti-inflammatory properties including inhibition of cytokine gene expression in T cells [18]. These actions are attributed to inhibition of cyclooxygenase-2 (COX-2) and prostaglandin $E_2$ ($PGE_2$) in rheumatoid fibroblasts and other cell-types [19]. In our quest for

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novel agents for blocking the cartilage damaging actions of proinflammatory cyokines, we demonstrated that the crude extract of TWHF containing multiple ingredients suppressed cytokine-stimulated MMP-3 and MMP-13 gene expression in chondrocytes and synovial fibroblasts [20]. However, the precise identity of the effective agent is not known. A purified active ingredient of TWHF, triptolide or PG490 (a diterpene triepoxide) also has anti-inflammatory and immunosuppressive activities [21]. PG490 suppresses MMP-1 and MMP-3 induction by IL-1 in synovial fibroblasts [22], however its effect on MMP-13 and aggrecanase expression is unknown. Besides inflamed RA synovium (pannus) that invades articular cartilage and contributes to its depletion, cartilage is the main tissue damaged by arthritis. Aside from our study on TWHF extract [20], the impact of purified triptolide on MMP and aggrecanase-1 gene expression has not been studied in chondrocytes, an important cell-type in the context of arthritis. Here, we investigated whether PG490 was the active ingredient responsible for inhibition of MMP gene induction in chondrocytes and if it could down-regulate induction of principal cartilage degrading enzymes, MMP-13 and ADAMTS-4.

## Materials and methods

*Human and bovine chondrocytes, SW1353 cells, synovial fibroblasts and treatments.* Normal bovine articular cartilage from the knee and femoral head of adult animals was from a local abattoir. Human chondrocytes were obtained from the femoral heads of patients undergoing hip replacement surgery due to OA. Chondrocytes were released by enzymatic digestion and high-density monolayers were cultured as before [20]. Cells were kept in serum-free DMEM for 24 h before treating with PG490 (Calbiochem, San Diego, CA) or equivalent amount of its vehicle (DMSO, control). The agent was added 30 min before treatment with human recombinant IL-1 (10 ng/ml), IL-17 (20 ng/ml) or TNF-α (10 ng/ml) (R&D Systems, Minneapolis, MN) for 24 h. Human chondrosarcoma cell line, SW1353, from American Type Culture Collection (Manassas, VA) was treated as described for primary chondrocytes. Human knee synovial fibroblasts at passage 4 were maintained in medium with 0.5% FCS and were treated similarly.

*Northern hybridizations.* RNA was extracted by the guanidinium isothiocyanate procedure [23] and 5 μg aliquots were analyzed by Northern hybridization with the human MMP-3, MMP-13, and 28S rRNA probes as described previously [20]. Aggrecanase-1 probe was generated by RT-PCR and characterized as recently described [24]. This probe yields a band of 4.2 kb in Northern blots.

*Western immunoblot analysis.* The conditioned media were concentrated by 10% (final) trichloroacetic acid (TCA) precipitations, the protein was quantified by Bio-Rad assay, and 10–20 μg aliquots were fractionated by a 4% stacking and 10% SDS–PAGE gel (Bio-Rad, Mississauga, Ont.), transferred to nitrocellulose membranes, and reacted overnight at 4 °C with 1–2 μg/ml of the rabbit anti-human MMP-13 antibodies (Sigma–Aldrich, Oakville, ON). Subsequently, membranes were washed 5 times with PBS–0.1% Tween, incubated with the anti-rabbit secondary peroxidase-conjugated IgG and, washed 7 times, and MMP-13 bands were revealed by chemiluminescence system of Roche Biochemicals (Laval, Que.).

*Cartilage explants.* Human or bovine femoral head cartilage explants were maintained for one week for acclimatization in DMEM with 10% FCS, medium was then changed with 0.01% serum-containing DMEM for 3 days until treatments. Cells were treated with PG490 and IL-1 vehicles as control (DMSO and PBS–0.1% BSA) or exposed to 80 nM PG490 and IL-1 (33 ng/ml) for 15 days (to ensure delivery) with replacement of the fresh reagents every two days, the secreted medium was concentrated by precipitation with 10% TCA, and equal amounts of protein (16 μg/lane for human and 20 μg/lane for bovine explants) were subjected to Western immunoblotting as described above.

All experiments were performed at least three times and the reported results were reproducible.

## Results

### *Triptolide inhibits proinflammatory cytokine-induced MMP-3 and MMP-13 gene expression in primary bovine and human chondrocytes*

To investigate the impact of purified triptolide, PG490 on MMP gene expression, primary bovine chondrocytes were exposed to different doses of PG490 and then stimulated with proinflammatory cytokines, TNF-α, IL-17, and IL-1β. PG490 at 125–250 nM concentrations, dose-dependently down-regulated MMP-3 and MMP-13 mRNA induction without affecting the control 28S ribosomal RNA levels (Fig. 1A). Similar down-regulation of MMP-13 protein (the prominent MMP involved in type II collagen degradation) induction by PG490 was observed in the conditioned media from these cells (Fig. 1B). Pretreatment of human OA femoral head chondrocytes with different doses of PG490 (from low 100 to 600 nM) and induction with cytokines either completely suppressed or down-regulated MMP-3 and MMP-13 RNA and MMP-13 protein expression. DMSO, the vehicle for PG490, had no impact on the induction by cytokines (Fig. 2).

### *Triptolide suppresses proinflammatory cytokine-induced MMP-3 and MMP-13 RNA expression in human chondrosarcoma cells*

We investigated the impact of cytokines and PG490 on human chondrosarcoma cell line, SW1353, a widely used in vitro chondrocyte model. Both TNF-α and IL-1β potently induced MMP-3 and MMP-13 RNA expression that was not affected by DMSO, the vehicle for PG490. IL-17 was not a strong inducer of MMPs in these cells (results not shown). As in the primary chondrocytes, triptolide was very effective in completely suppressing the MMP-3 and MMP-13 RNA induction by these cytokines without affecting the control 28S rRNA levels (Fig. 3A).

### *PG490 inhibits MMP-3 and MMP-13 RNA induction by IL-1 in human synovial fibroblasts*

Besides chondrocyte-derived MMPs, synovial membranes also contribute to cartilage loss by their ability

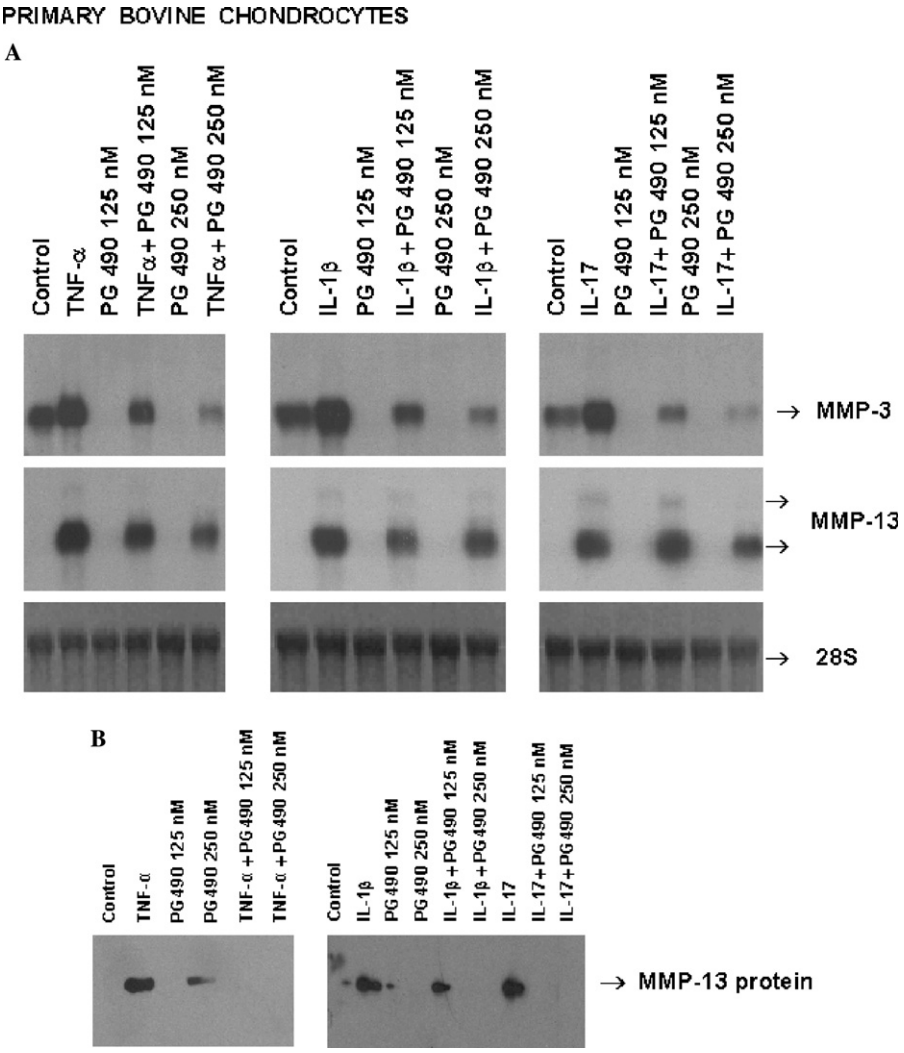


Fig. 1. Down-regulation of proinflammatory cytokine-stimulated MMP-3 and MMP-13 expression by PG490 in bovine chondrocytes. The cells were made quiescent by maintaining for 24 h in serum-free medium, pretreated with the indicated doses of PG490 for 30 min alone or stimulated further with TNF-α, IL-1β, and IL-17 for 24 h. Control cells were treated with cytokine and PG490 vehicles (PBS–0.1% BSA and DMSO, respectively). Northern blots (A) of RNA hybridized with the human MMP-3, MMP-13, and 28S probes are shown. (B) MMP-13 levels measured by Western immunoblotting of the conditioned media are depicted.

to invade cartilage and by digesting its matrix with MMPs. A previous study demonstrated inhibition of IL-1-induced MMP-1 and MMP-3 expression in synoviocytes but effect on MMP-13 was not studied [22]. IL-1, the principal cytokine implicated in cartilage resorption [4], induced MMP-3 and MMP-13 RNA in synovial fibroblasts. Besides MMP-3, PG490 also suppressed the induction of MMP-13 RNA expression (Fig. 3B).

### *PG490 down-regulates MMP RNA induction by IL-1 and TNF-α in SW1353 cells at very low doses*

We explored if PG490 affected MMP gene induction at doses even lower than 100 nM. SW1353 cells were pretreated with PG490 at concentrations ranging from 2.5 to 40 nM and then stimulated with IL-1β and TNF-α. Triptolide dose-dependently reduced the induction of MMP-3 and MMP-13 RNA expression. Thus, triptolide down-regulates MMP genes at very low doses (Fig. 4).

### *PG490 diminishes IL-1-induced MMP-13 in human and bovine cartilage*

To examine the effect of PG490 on chondrocyte MMP-13 in native cartilage matrix, human and bovine cartilage explants were maintained in low, 0.1%-serum containing medium and exposed to PG490 (80 nM) and IL-1β for 15 days with changes of reagents every two days. PG490 drastically reduced the secreted MMP-13 protein levels in human (Fig. 5A) and bovine cartilage (Fig. 5B) as measured by Western blotting of the conditioned media. Thus, PG490 diminishes IL-1-stimulated MMP-13 production in cartilage explants.

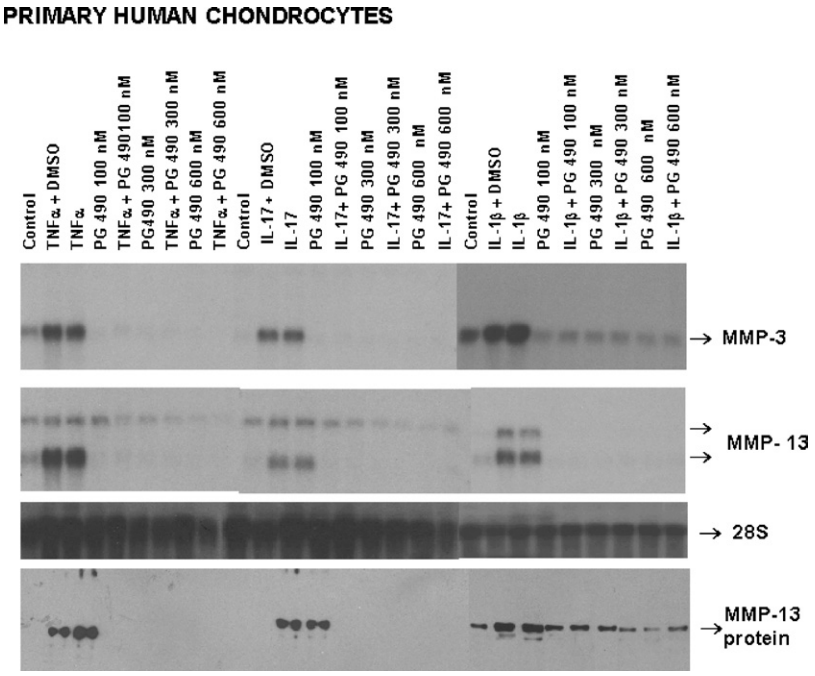


Fig. 2. Inhibition of TNF-α-, IL-17-, and IL-1β-induced MMP-3 and MMP-13 gene expression by PG490 in human femoral head chondrocytes. Cells from different patients were grown to confluence, rendered quiescent by serum starvation for 24 h, pretreated with the indicated doses of PG490 for 30 min alone or treated additionally with cytokines for 24 h. Results of Northern analysis with MMP-3, MMP-13, and 28S RNA are shown in upper 3 panels. Total protein (10 μg/lane) from the conditioned media of treated chondrocytes was subjected to SDS–PAGE, transferred to nitrocellulose, and reacted with MMP-13 antibodies, and the bands were revealed by chemiluminescence.

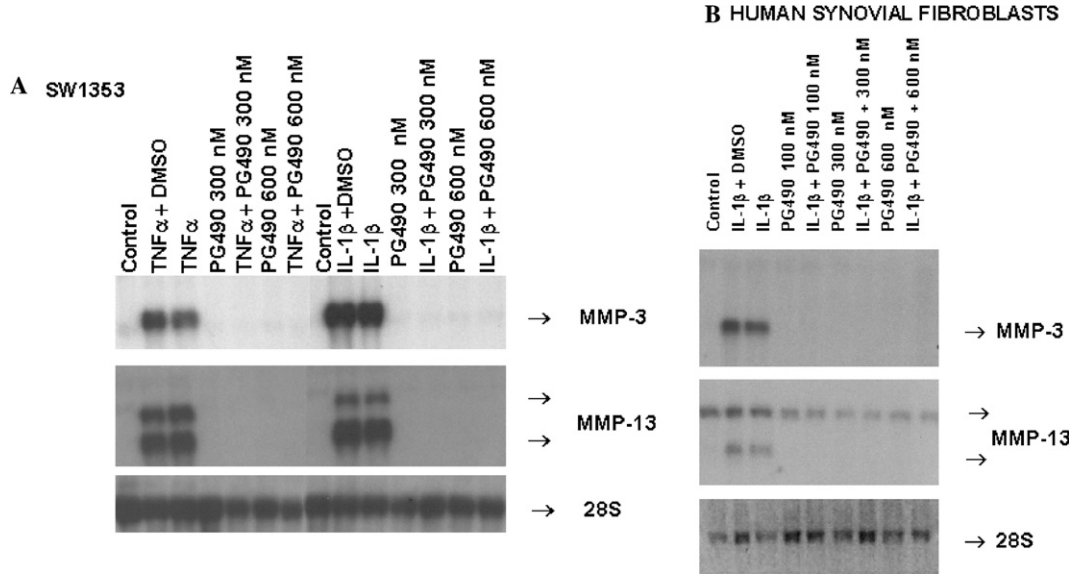


Fig. 3. Inhibition of cytokine-induced MMP-3 and MMP-13 RNA expression by PG490 in human chondrosarcoma (SW1353) and synovial fibroblast cells. (A) The quiescent SW1353 cells were treated as in previous figures. (B) The confluent synovial fibroblasts were made quiescent for 24 h in serum-deficient (0.5% serum) medium, pretreated with PG490 for 30 min alone or further stimulated with IL-1β for 24 h. Results of Northern hybridization with the positions of MMP-3, MMP-13, and 28S RNA bands are shown.

### *Tripterygium wilfordii Hook F extract and PG490 down-regulate aggrecanase-1 gene expression in primary bovine chondrocytes*

Aggrecanase-1 or ADAMTS-4 is a major enzyme cleaving cartilage aggrecan [6,8]. To examine the effect of cytokines, TWHF, and triptolide on ADAMTS-4 gene expression, primary bovine chondrocytes were pretreated with TWHF extract (2.5–5 ng/ml) or PG490 (100–600 nM) followed by stimulation with IL-1β, IL-17, and TNF-α. All three cytokines induced ADAMTS-4 RNA induction in bovine chondrocytes. TWHF extract

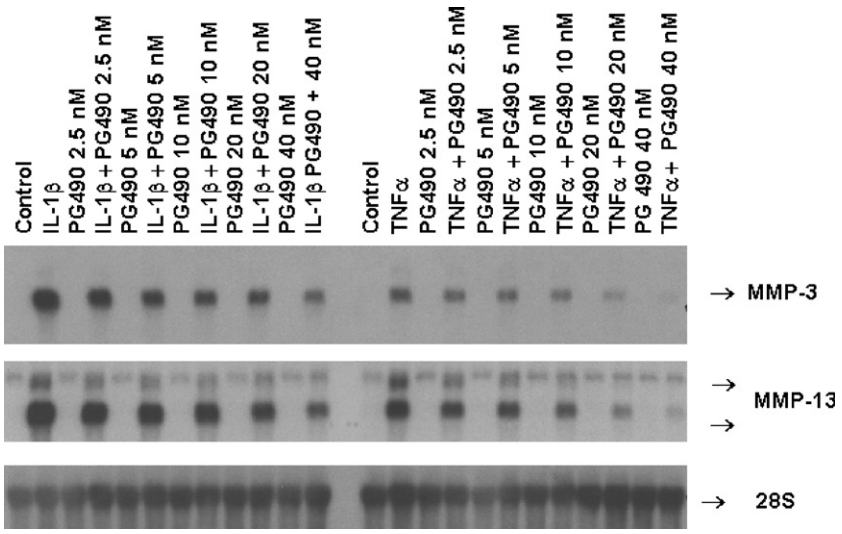


Fig. 4. Dose-dependent inhibitions of IL-1β- and TNF-α-stimulated MMP-3 and MMP-13 RNA expression in human SW1353 cells. Quiescent cells were pretreated with the indicated doses of PG490 for 30 min alone or treated further with IL-1β or TNF-α for 24 h. Northern blots of RNA hybridized with the human MMP-3, MMP-13, and 28S probes are shown.

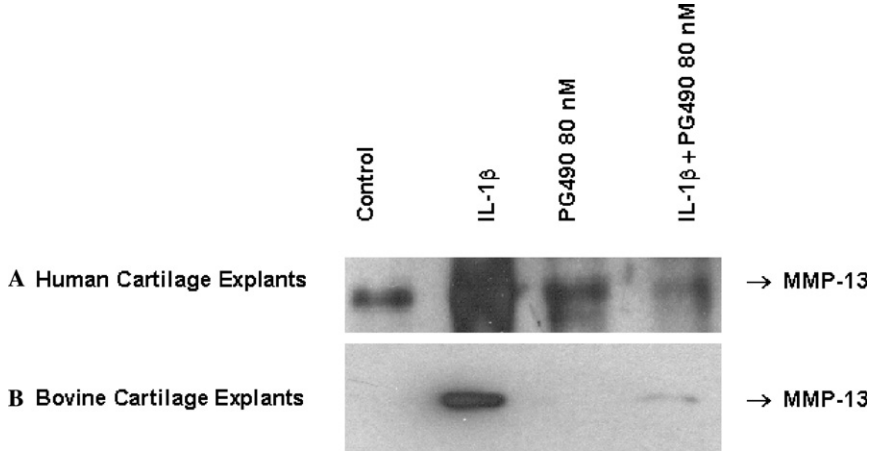


Fig. 5. Effect of PG490 on IL-1β-stimulated MMP-13 protein expression in human (A) and bovine (B) cartilage explants. Tissues maintained in DMEM with 0.01% serum were either treated with PG490 and IL-1β vehicles (DMSO and PBS–0.1% BSA) or exposed to 80 nM PG490 and IL-1β (33 ng/ml) for 15 days with renewal of the reagents every two days. The secreted media were concentrated and equal amounts of protein (human, 16 μg/lane and bovine, 20 μg/lane) were subjected to Western blotting. MMP-13 protein band is shown.

dose-dependently (Fig. 6A) while PG490, at 600 nM concentration (Figs. 6B and C), reduced the induction of ADAMTS-4 mRNA. Thus, both TWHF extract and pure triptolide down-regulate the induction of ADAMTS-4 by cytokines.

## Discussion

Arthritis is a major debilitating disease affecting millions of people all over the world. A painful consequence of arthritis is proinflammatory cytokine-stimulated degradation of cartilage by MMPs (such as MMP-13) and aggrecanases. Purified triptolide from the anti-rheumatic herb, TWHF, can potently down-regulate the induction of these enzymes by principal inflammatory cytokines in chondrocytes from diverse sources. MMP induction in synovial fibroblasts and cartilage explants is also reduced by triptolide. Thus, the observed beneficial anti-arthritic effects of TWHF may be due to its active triptolide component. This study shows that besides its known anti-inflammatory and immunosuppressing activities, triptolide also has the potential to protect cartilage integrity.

The suppression by PG490 was reproducible in the important ex vivo cartilage model of bovine chondrocytes as well as in chondrocytes from different patients with OA of the femoral head. The response appeared to be specific as 28S ribosomal RNA controls were not

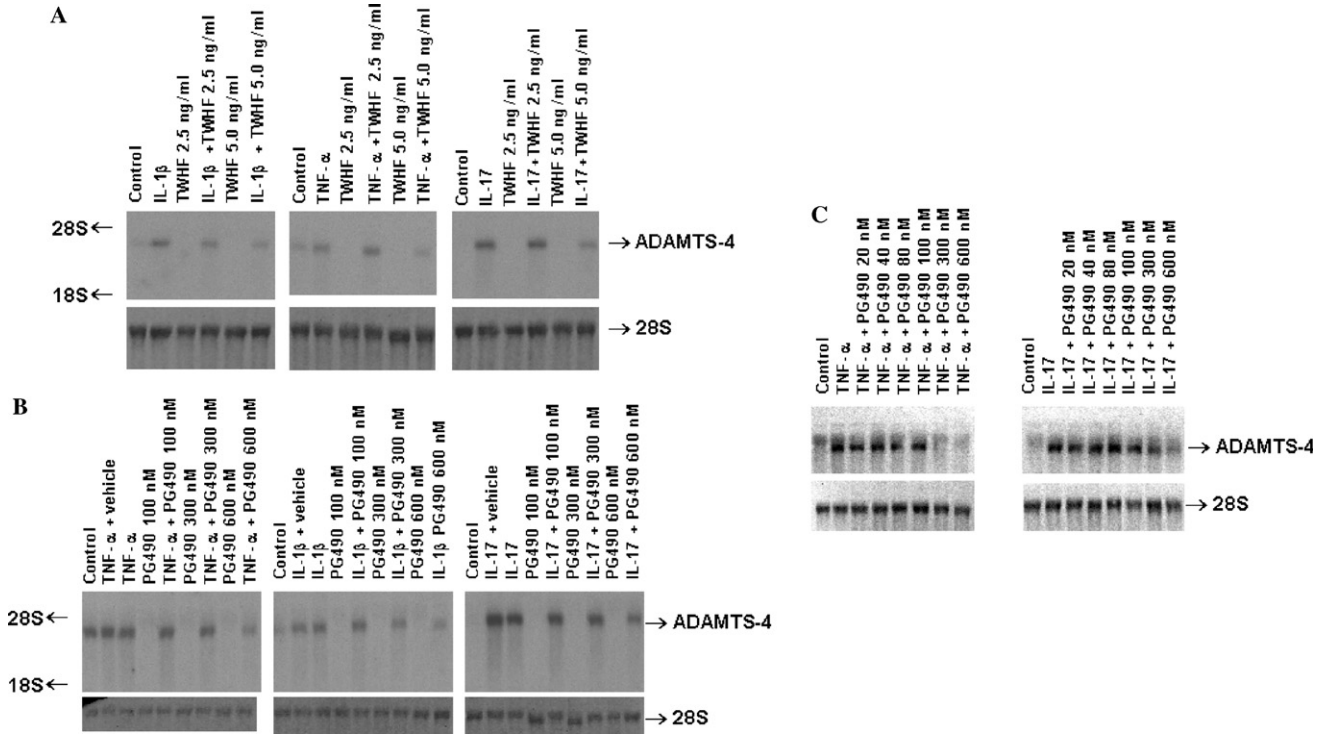


Fig. 6. Down-regulation of cytokine-stimulated aggrecanase-1 (ADAMTS-4) RNA expression by TWHF extract and PG490 in bovine chondrocytes. The cells were made quiescent in serum-free medium, pretreated with the indicated doses of (A) TWHF or (B,C) PG490 for 30 min or exposed further to cytokines for 24 h. Northern blots of RNA hybridized with human aggrecanase-1 and 28S probes are shown.

affected. Similar degree of MMP-13 RNA and protein inhibition suggests a pre-transcriptional mechanism of down-regulation. Specific inhibition of IL-1 with IL-1 receptor antagonist and that of TNF-α by a soluble binding protein have revealed that IL-1 stimulates cartilage destruction and TNF-α stimulates joint inflammation [4]. Triptolide inhibition of all three catabolic cytokines actions in chondrocytes suggests that this active ingredient of TWHF could intercept the process of cartilage degradation during the pathogenesis of arthritis.

Inhibition of mRNA for the major cartilage degrading enzymes, MMP-3 and MMP-13, in synovial fibroblasts suggests that triptolide can also block cartilage degradation by adjacent synovial membranes, which are severely inflamed and commonly invade the articular cartilage in RA patients. These results are supported by an earlier study where IL-1-stimulated expression of another collagen digesting enzyme MMP-1 as well as MMP-3 was inhibited by triptolide in synovial fibroblasts, though MMP-13 expression was not investigated [22]. Interestingly, triptolide also inhibited IL-1-induced production of inflammatory mediators, $PGE_2$, COX-2 (but not COX-1), in these cells and lipopolysaccharide-stimulated $PGE_2$, IL-1α, IL-β, and IL-6 gene expression in mouse macrophages [22]. Thus, triptolide can potentially reduce arthritis-associated inflammation of synovial membranes and MMP production from this tissue.

Dose–responses revealed that the agent is effective in MMP down-regulation at very low doses (nanomolar range). Chondrocytes from diverse sources tolerated the agent very well at different doses and had no adverse effects. Reduction in the levels of major cartilage degrading enzyme MMP-13 in human and bovine cartilage explants by triptolide suggests that the agent also penetrates and is effective when the chondrocytes are embedded in their native cartilage matrix. Thus, triptolide has the capacity to diminish MMP-mediated degeneration of cartilage in joints.

Aggrecan is degraded predominantly by aggrecanases and to some extent by MMPs such as MMP-3 and MMP-13 [8]. The observed induction of aggrecanase-1 RNA by IL-1 and TNF-α is in agreement with other studies where these cytokines induced ADAMTS-4 gene expression or activity in bovine chondrocytes [25,26]. In agreement with our results, IL-17 also induced aggrecanase activity in porcine and bovine cartilage [27]. Down-regulation of ADAMTS-4 induction by both the crude TWHF extract and pure triptolide suggests that PG490 inhibits the cytokine-induced expression of this major enzyme. Interestingly, a well-studied immunosuppressant, cyclosporin A, also inhibits IL-1α-stimulated MMP-13 and ADAM-TS4 gene expression in bovine cartilage [28]. Thus, triptolide has strong potential to diminish MMP and aggrecanase-driven catabolism of cartilage in joints, making it a very interesting therapeutic agent. Indeed, besides known beneficial

effects in humans, certain extracts of this plant also reduce collagen-induced arthritis in rats and in carrageenan-induced inflammation [29,30].

Since MMP and aggrecanase-1 induction by all the three studied cytokines is inhibited by PG490, a common step leading to expression of these genes may be impaired by triptolide. We previously showed that TWHF may work in part by inhibiting AP-1 and NF-κB activities but did not interfere with the phosphorylation of mitogen-activated protein kinases [20]. Hypoestoxide, a natural diterpene, exerts its anti-inflammatory properties by inhibiting IκB kinase activity [31]. PG490 may work via similar mechanisms. MMP promoters have AP-1 and c-ets-1 transcription factor binding sites [32]. Further NF-κB may also be indirectly implicated in MMP gene regulation [33]. Aggrecanase-1 promoter contains AP-1, AP-2, PEA-3, Sp1, and nuclear factor-1 (NF-1) binding sites [34]. The precise targets of triptolide among these transcription factors remain to be deciphered. A synthetic triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), also inhibits IL-1-induced MMP-1 and MMP-13 induction in SW1353 cells at transcriptional level by unknown mechanisms [35]. Recently, another triptolide was shown to exert its anti-inflammatory activities by inhibiting the transcription of inducible nitric oxide synthase [36]. A triptolide extract induced apoptosis in highly proliferative rheumatoid synovial fibroblasts [37]. These features of triptolide may be useful for blocking pannus formation in the joints of RA patients.

Triptolide has several characteristics of particular interest for rheumatic diseases. It clearly has anti-inflammatory and immunosuppressive effects. It inhibits several proinflammatory cytokines and adhesion molecules, which are all important mediators of rheumatoid arthritis [38]. Triptolide is also effective against allograft rejection after organ transplantation [39] that can be useful in protection of cartilage grafts in arthritis patients. A water-soluble derivative of PG490 blocks bleomycin-induced fibrosis of lungs by reducing TGF-β1 levels [40], a factor implicated in synovial fibrosis and osteophyte formation in patients with arthritis.

To conclude, we demonstrated that triptolide PG490 inhibits the proinflammatory cytokine-stimulated expression of major proteases responsible for cartilage degradation. The cartilage protective ability and previously reported anti-inflammatory and immunosuppressant activities make triptolide a potentially attractive and new therapeutic agent for arthritis.

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