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Regulation of metalloproteinases and NF-κB activation in rabbit synovial fibroblasts *via* E prostaglandins and Erk: contrasting effects of nabumetone and 6MNA

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1 Nabumetone is a prodrug that is converted *in vivo* into 6-methoxy-2-naphthylacetic acid (6MNA), a cyclooxygenase inhibitor with anti-inflammatory properties. We tested the effects of nabumetone and 6MNA on the inflammatory responses of synovial fibroblasts (SFs).

2 Brief exposures to 6MNA (50–150 μM) had no effect on IL-1β/TNF-α (each 20 ng ml^{-1})-stimulated Erk activation. Longer exposures depleted prostaglandin E_1 (PGE_1) as much as 70%, and stimulated Erk as much as 300%. Nabumetone (150 μM) inhibited Erk activation by 60–80%.

3 6MNA (50–150 μM) stimulated (≈200%) and nabumetone (150 μM) inhibited (≈50%) matrix metalloproteinase (MMP)-1, but not MMP-13 secretion from SFs. 6MNA stimulation of MMP-1 secretion was inhibited ≈30% by PGE_1 (1 μM) and ≈80% by the Erk pathway inhibitor UO126 (10 μM), confirming that PGE depletion and Erk activation mediate MMP-1 secretion by 6MNA.

4 Consistent with its role as an Erk inhibitor, nabumetone (150 μM) abrogated 6MNA enhancement of MMP-1 secretion.

5 UO126 (10 μM) and nabumetone (150 μM) inhibited (≈70 and 40%, respectively), but 6MNA (150 μM) enhanced (≈40%), NF-κB activation.

6 Our data indicate that 6MNA shares with other COX inhibitors several proinflammatory effects on synovial fibroblasts. In contrast, nabumetone demonstrates anti-inflammatory and potentially arthroprotective effects that have not been previously appreciated.
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Keywords: 6MNA; nabumetone; Erk; synoviocyte; matrix metalloproteinase; prostaglandin; cyclooxygenase; MAP kinase; NF-κB

Abbreviations: COX, cyclooxygenase; LDH, lactate dehydrogenase; MMP, matrix metalloproteinase; 6MNA, 6-methoxy-2-naphthylacetic acid; NF-κB, nuclear factor-κB; NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin; SF, synovial fibroblast

Introduction

Synovial fibroblasts (SF) are the stromal cells of the joint capsule and regulate both lubricative and structural properties of the joint as well as immune functions. In rheumatoid arthritis (RA), SF express a hyperplastic, inflammatory and chondrodestructive phenotype, including secretion of cytokines and matrix metalloproteinases (MMPs) (Firestein, 1998; Yamanishi & Firestein, 2001). Cyclooxygenase (COX)-2-selective and nonselective COX inhibitors have direct, potentially adverse effects on the phenotypic responses of SF (Dayer *et al*., 1976; He *et al*., 2002; Pillinger *et al*., 2003). Exposure of SF to various selective and nonselective COX inhibitors results in the activation of the mitogen-activated protein kinase (MAPK) Erk as well as Erk-dependent secretion of MMP-1 (collagenase 1), an important mediator of cartilage degradation. Conversely, E prostaglandins (PGs) inhibit SF Erk activation as well as MMP-1 message expression and protein secretion (Salvatori *et al*., 1992; DiBattista *et al*., 1994; Suzuki *et al*., 1997; Pillinger *et al*., 2003). Thus, the effects of COX inhibitors on Erk and MMP-1 are linked with their ability to deplete PG. Whether other components of the inflammatory SF response, for instance, NF-κB activation and NO production, are similarly affected is not known.

Nabumetone is a nonsteroidal anti-inflammatory drug (NSAID) used to treat osteoarthritis and RA (Helfgott, 1994). In contrast to most other NSAIDs, nabumetone is a prodrug that undergoes hepatic conversion to 6-methoxy-2-naphthylacetic acid (6MNA) (Haddock *et al*., 1984; Torre *et al*., 1987), an active inhibitor of COX with some selectivity for COX-2 (Davies, 1997). Based on a limited number of pharmacokinetic studies, it has been assumed that nabumetone

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itself is either irrelevant or biologically inert. However, the effects of nabumetone on cells involved in arthritis have not been rigorously evaluated.

We compared the effects of 6MNA and nabumetone on the responses of SF and chondrocytes. Our data indicate that, whereas 6MNA exerts proinflammatory effects on SF comparable to other COX inhibitors, nabumetone reduces inflammatory responses, and reverses potentially adverse effects of COX-2 inhibition.

Methods

SF culture and preparation for experiments

Rabbit SF (HIG-82 cell line; American Type Culture Collection catalog #CRL1832 (Georgescu *et al*., 1988)) were grown in Ham's F-12 medium containing 10% fetal bovine serum (FBS) and 1% Pen/Strep. Human RA SF were obtained from rheumatoid synovium obtained at the time of elective total knee replacement for RA. RA SF were prepared and cultured as described (Pillinger *et al*., 2003). Unless otherwise stated, all experiments were performed using rabbit SF.

PG assays

SF, grown to near confluence, were serum starved for 24 h, followed by incubation for 30 min ± nabumetone or 6MNA, as indicated. Cells were treated with IL-1β (20 ng ml^{-1}) and TNF-α (20 ng ml^{-1}) for 18 h in the continued presence of nabumetone or 6MNA. Supernatants were harvested and analyzed for PGE_1 or $PGF_{2\alpha}$ using PG ELISA assay kits (R & D Systems, Minneapolis, MN, U.S.A.), according to the manufacturer's instructions.

Cell viability

Cell death after 18 h treatment with 6MNA or nabumetone was measured as release of lactate dehydrogenase (LDH) into the supernatant as described (Pillinger *et al*., 1994), and expressed as % of maximal possible cell death (LDH activity in the supernatant of SF treated with 1% Triton X-100 for 30 min at 4°C). Cell viability was defined (100%-cell death) for each condition.

Measurement of Erk activation

Erk 1 and 2 activation was measured as described (Pillinger *et al*., 2003). Briefly, SFs were serum starved (24–48 h, 0% FBS), then incubated with inhibitors for 30 min and stimuli for an additional 30 min. SFs were lysed with ice-cold lysis buffer (20 mM Tris, pH 7.4, 1 mM EGTA, 2 mM sodium vanadate, 25 mM sodium fluoride, 0.5% (v v^{-1}) Triton X-100, 2 mM PMSF, 5 kallikrein inhibitory units ml^{-1} aprotinin and 10 μg ml^{-1} each of chymostatin, antipain and pepstatin) for 20 min at 4°C. Adherent remnants were liberated with a rubber spatula, and lysates collected and supplemented with a sample buffer (Laemmli, 1970) containing 5% β-mercaptoethanol. Samples were heated (100°C for 5–10 min), analyzed by SDS–PAGE on 10% Tris-glycine gels, and electrophoretically transferred to nitrocellulose. Nitrocellulose papers were imaged with Ponceau S solution to confirm even loading of the gel lanes, washed 3 × with Tris-buffered saline (pH 7.4) containing 0.05% Tween-20 (TBS-T), blocked with 3% BSA in TBS-T, washed 3 × and immunoblotted with anti-phosphoErk antibody in TBS-T (1 : 200). Blots were again washed 3 × with TBS-T, incubated with [^{125}I]protein A, again washed, and dried. Blots were imaged and quantitated by phosphorimaging (Storm imaging system, Molecular Dynamics™, Sunnyvale, CA, U.S.A.), and subsequently stripped and reblotted using antibodies recognizing total Erk 1 and 2 (each at 1 : 200). Erk activation was calculated as the ratio of phosphoErk/total Erk and expressed as percentage of either the control or stimulated condition.

MMP secretion

SF cultures grown to near confluence were serum starved for 24–48 h. Supernatants were discarded and replaced with Ham's F-12 and, after 30 min equilibration, SFs were treated with inhibitors and/or stimuli for 18 h. This duration of incubation was selected based on prior studies by our laboratory (Pillinger *et al*., 2003). Supernatants were concentrated by centrifugation in Centricon centrifugal filter devices (MW cutoff 30,000) for 35 min at 5000 r.p.m. at 4°C. Aliquots of concentrated supernants were supplemented with sample buffer, and analyzed for MMP-1 or -13 (10% tris glycine SDS–PAGE, immunoblotting with anti-MMP antibody (1 : 200 dilution), and visualization/quantitation as described for Erk.

NF-κB activation

NF-κB activation was assayed as translocation of the NF-κB subunit p65 from cytosol to the nucleus. SFs, grown to 70% confluence on glass coverslips at 37°C and 5% CO_2, were serum starved for 24 h. SF were incubated for 1 h ± 6MNA, nabumetone, U0126 or SB203580, stimulated for 14 h with IL-1β and TNF-α (each 20 ng ml^{-1}) fixed and permeabilized (15 min with ice-cold methanol), and blocked (30 min with 1% goat serum in phosphate-buffered saline containing 0.1% calcium chloride (PBS-C)). SF were incubated for 1 h at room temperature with anti-p65 antibody (1 : 100) in PBS-C with 1% goat serum, washed (PBS-C x3), and incubated with FITC-conjugated goat anti-rabbit antibody at 1 : 100 dilution (together with Hoechst 1 : 2000 to visualize the nuclei) in PBS-C/1% goat serum for 45 min. After washing again with PBS-C x3, coverslips were mounted onto glass slides and imaged in a Zeiss fluorescence microscope (FITC at 490 nm for 20,000 ms and Hoechst at 405 nm for 2000 ms); FITC and Hoechst images were overlaid and stored as Tiff files. For each condition, the percentage of cells demonstrating p65 translocation to the nucleus was determined. Results were expressed relative to the stimulated condition.

NF-κB activation was also assayed as p65 binding to the NF-κB promoter on DNA, determined using an NF-κB Family Transcription Factor Assay Kit (Active Motif, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. In all, 3 μg of nuclear extract was hybridized with the NF-κB-specific oligo bound into the ELISA plates. The bound NF-κB protein was analyzed by an ELISA system supplied with the kit.

NO production

NO production was measured as nitrate + nitrite in the supernatant using a Greiss assay kit according to the instructions of the manufacturer, The Nitrate Elimination Co., Inc. (Lake Linden, MI, U.S.A.).

Drugs

Unless otherwise noted, all materials were from Sigma-Aldrich (St Louis, MO, U.S.A.). Anti-phosphoErk, anti-Erk 1 and 2 and anti-NF-κB p65 antisera were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-MMP-1 antiserum was from Chemicon International (Temecula, CA, U.S.A.). Anti-MMP-13 antiserum was from R & D Systems (Minneapolis, MN, U.S.A.). UO126 was from Upstate Biotechnology (Lake Placid, NY, U.S.A.), and SB203580 was from Promega (Madison, WI, U.S.A.); the concentrations used (10 μM each) were selected based on previous reports (Cuenda *et al*., 1995; Favata *et al*., 1998) indicating that these drugs are specific for their designated substrates at these concentration ranges. Similarly, the concentrations of PGE_1 employed were based on prior studies (Pillinger *et al*., 1995). [^{125}I]protein A was from Amersham (Arlington Heights, IL, U.S.A.). Ham's F-12 medium and FBS were from BioWhittaker (Walkersvile, MD, U.S.A.). Penicillin G sodium (10,000 U ml^{-1})/streptomycin sulfate (10,000 μg ml^{-1}) in 0.85% NaCl was from Life Technologies (Rockville, MD, U.S.A.). Hoechst 33258 (bisbenzimide) was from Molecular Probes, Inc. (Eugene, OR, U.S.A.).

Statistical analysis

Except for NF-κB assays (described above), results of all experiments were normalized either to the percentage of a control condition or the percentage of the maximal stimulated signal, as appropriate. The mean and s.e.m. for multiple experiments of the same category were then calculated. Statistical significance for each experiment was determined using the one-way ANOVA, one-way repeated-measures ANOVA, or Kruskal–Wallis one-way ANOVA on ranks, as appropriate for the data. Subsequent pairwise comparisons were calculated using the Dunnett's method or the Holm–Sidak method, as appropriate. All statistical analyses were performed using SigmaStat.

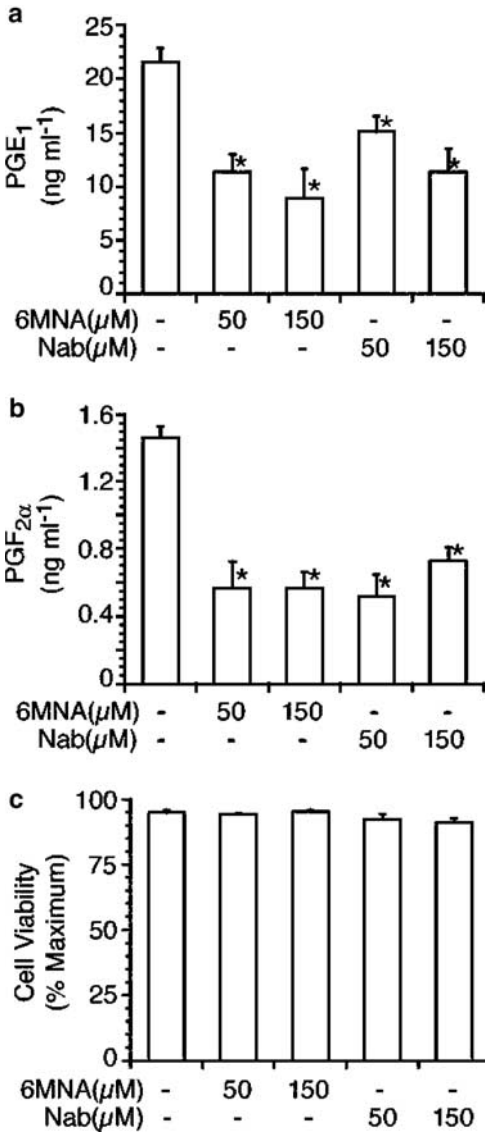

Figure 1 Effects of 6MNA and nabumetone on SF PG secretion and cell viability. Panels a, b: 6MNA and nabumetone on PG secretion. SF were incubated ± 6MNA or nabumetone, stimulated overnight ± IL-1β/TNF-α (each 20 ng ml^{-1}), and supernatants assayed for PGE_1 (panel a) and $PGF_{2\alpha}$ (panel b). Panel c: SF incubated overnight ± 6MNA or nabumetone were assayed for cell viability by LDH release assay. Data shown are the mean ± s.e.m. of three experiments in duplicate for each condition (one-way repeated-measures ANOVA: for panel a, $P = 0.001$; for panel b, $P < 0.001$; for panel c, $P = 0.013$; for pairwise comparisons $^*P \leqslant 0.009$ vs the untreated control condition).

Results

Effects of 6MNA and nabumetone on PG production

Treatment of SF with 6MNA inhibited the secretion of PGE_1 and $PGF_{2\alpha}$ (Figure 1a, b). Nabumetone also inhibited PGE_1 and $PGF_{2\alpha}$ secretion, though somewhat less effectively. Thus, both agents act as COX inhibitors. Inhibition of PG secretion by 6MNA or nabumetone was not due to toxicity, as neither 6MNA nor nabumetone significantly affected SF viability (Figure 1c). SF adherence and morphology were also unaffected by exposure to either nabumetone or 6MNA.

Effects of 6MNA and nabumetone on Erk phosphorylation

COX inhibitors stimulate and E PGs inhibit SF Erk activity (Dayer *et al*., 1976; Salvatori *et al*., 1992; DiBattista *et al*., 1994; Suzuki *et al*., 1997; He *et al*., 2002; Pillinger *et al*., 2003). We therefore tested the effects of 6MNA and nabumetone on Erk activity, measured as Erk phosphorylation (Figure 2). Il-1β/TNF-α (each 20 ng ml^{-1}) stimulated phosphorylation of Erk 1 and 2 (Figure 2a, top). Incubation with 6MNA (50–150 μM) for 30 min did not affect Erk 1 or 2 phosphorylation stimulated by Il-1β/TNF-α. In contrast, 150 μM nabumetone

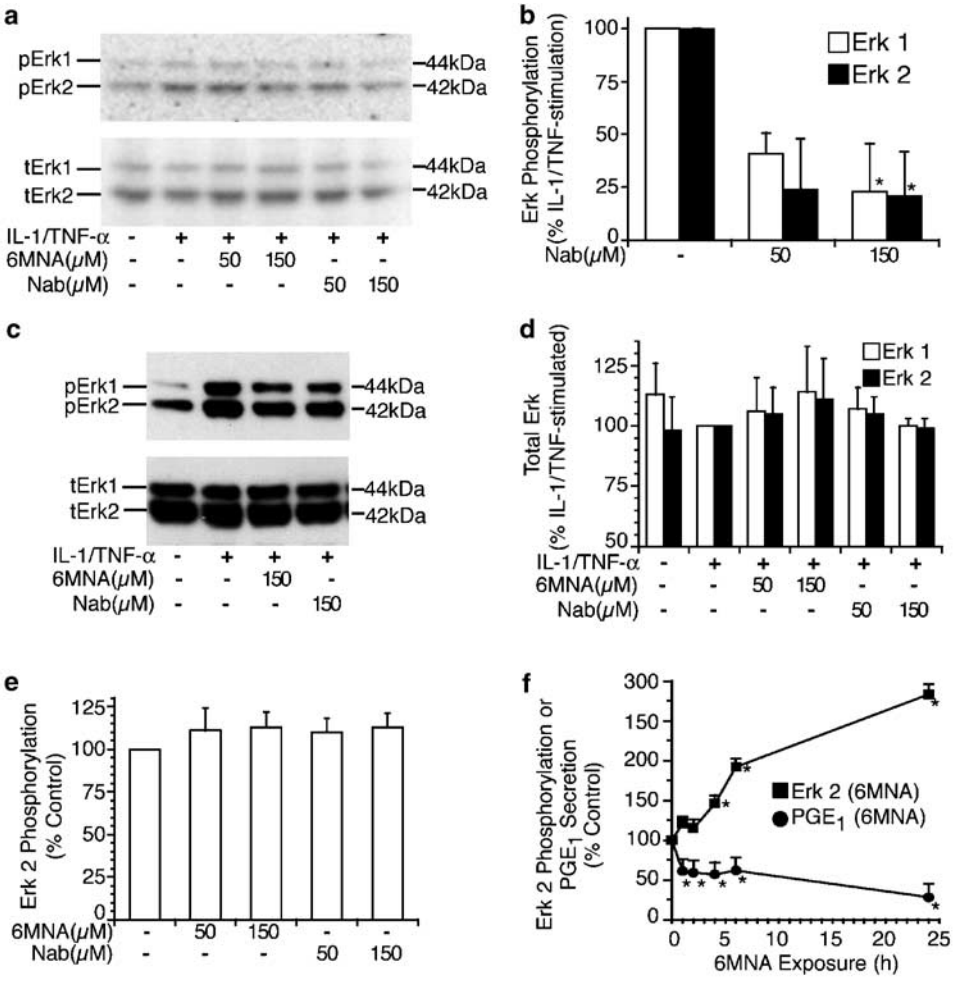

Figure 2 Effects of 6MNA and nabumetone on Erk activation in SF. Panel a: SF incubated for 30 min ± 6MNA or nabumetone were stimulated with IL-1β/TNF-α (each 20 ng ml^{-1}) for 30 min and assayed for Erk phosphorylation (top) or total Erk, bottom). Panel b: SF from multiple experiments were treated as in panel a and assayed for Erk 1/2 phosphorylation, expressed as % of IL-1/TNF-α stimulation (Kruskal–Wallis one-way ANOVA on ranks: $P=0.05$ for Erk 1, $P=0.025$ for Erk 2). Panel c: Human RA SFs were treated ± 6MNA or nabumetone, stimulated with IL-1β/TNF-α and assayed for phospho- (top) and total (bottom) Erk. Panel d: Lack of effect of 6MNA, nabumetone and IL-1β/TNF-α on total Erk levels (one-way ANOVA: for Erk 1, $P=0.91$; for Erk 2, $P=0.95$). Panel e: brief exposures to 6MNA and nabumetone do not affect constitutive Erk 2 activity. SF were incubated for 30 min ± 6MNA or nabumetone in the absence of other stimuli, and assayed for Erk phosphorylation normalized to total Erk (one-way ANOVA, $P=0.82$). Panel f: Longer exposures to 6MNA inhibit PGE production and induce Erk 2 activation. SF were incubated with 150 μM 6MNA or nabumetone for indicated times; SF lysates were assayed for Erk phosphorylation and SF supernatants for PGE_1 levels (one-way repeated-measures ANOVA: for Erk 2, $P<0.001$; for PGE_1, $P<0.001$). Data shown are representative or the mean ± s.e.m. of three (panels a–e) or four (panel f) experiments for each condition. (For pairwise comparisons, $^*P\leqslant 0.05$ vs the appropriately matched control condition without 6MNA or nabumetone.)

significantly inhibited Il-1β/TNF-α-stimulated phosphorylation of both Erk isoforms (Figure 2a, top, 2b). Similar results were observed in human RA SF stimulated with Il-1β/TNF-α (Figure 2c). Nabumetone, but not 6MNA, also inhibited Erk phosphorylation in SF stimulated with epidermal growth factor (EGF), demonstrating that Erk regulation by nabumetone is not limited to SF stimulated with inflammatory cytokines (data not shown). Neither 6MNA nor nabumetone significantly affected total Erk levels (Figure 2a, bottom; 2c, bottom; and 2d).

The ability of nabumetone to inhibit Erk phosphorylation suggested that it might either act directly on Erk, or target an element of the Erk activation pathway. We therefore tested the effects of nabumetone, as well as 6MNA, on constitutive Erk 2 phosphorylation in unstimulated SF (Figure 2e). SF demonstrated measurable baseline levels of constitutive Erk 2 phosphorylation, which were not inhibited by 30 min incubation with nabumetone. Thus, nabumetone appears to inhibit the activation, rather than the activity, of Erk. 6MNA also had no effect on Erk activity under these conditions.

We have previously reported that longer exposures to COX inhibitors deplete PGEs and enhance Erk activation (Pillinger *et al*., 2003). We therefore tested the effects of longer 6MNA exposures on SF PGE secretion and Erk phosphorylation (Figure 2f). In the absence of other stimuli, treatment with 6MNA (150 μM) for $\geqslant$1 h depleted PGE_1 and subsequently induced persistent Erk 2 phosphorylation. Additionally, PGE_1 inhibited Erk activation in SF stimulated with IL-1/TNF-α (data not shown). These data indicate that Erk activation is downregulated by PGEs. While nabumetone inhibits Erk phosphorylation, 6MNA resembles other COX inhibitors in stimulating Erk, concordant with PGE depletion.

Effects of 6MNA and nabumetone on MMP secretion

MMP-1 but not MMP-13 secretion from SF is positively regulated by Erk (Pillinger *et al*., 2003), and COX inhibitors enhance MMP-1 secretion *via* PG depletion and Erk activation (Dayer *et al*., 1976; He *et al*., 2002; Pillinger *et al*., 2003). We therefore tested the effects of 6MNA and nabumetone on MMP secretion (Figure 3). IL-1β/TNF-α stimulated SF MMP-1 secretion (Figure 3a). 6MNA enhanced IL-1/TNF-α-stimulated secretion of MMP-1 but not MMP-13 (Figure 3a, c), confirming that these MMPs are differentially regulated (Mengshol *et al*., 2000; Vincenti & Brinckerhoff, 2002). 6MNA also enhanced EGF-stimulated SF MMP-1 secretion (data not shown), as well as IL-1β/TNF-α-stimulated MMP-1 secretion by RA SF (Figure 3b). In the absence of other agents, 6MNA stimulated MMP-1 secretion (Figure 3d), and 6MNA stimulation of MMP-1 secretion was inhibited by 10 μM PGE_1 (Figure 3e). UO126 (50 μM) also inhibited 6MNA-stimulated MMP-1 secretion (Figure 3e), suggesting that, as in the case of other COX inhibitors (Pillinger *et al*., 2003), 6MNA regulates MMP-1 *via* effects on Erk. In contrast, 10 μM SB203580 enhanced 6MNA-stimulated MMP-1 secretion, suggesting that p38 activity inhibits MMP-1 secretion (Figure 3e).

In contrast to 6MNA, 150 μM nabumetone inhibited IL-1β/TNF-α-stimulated MMP-1 secretion (Figure 3a, c).

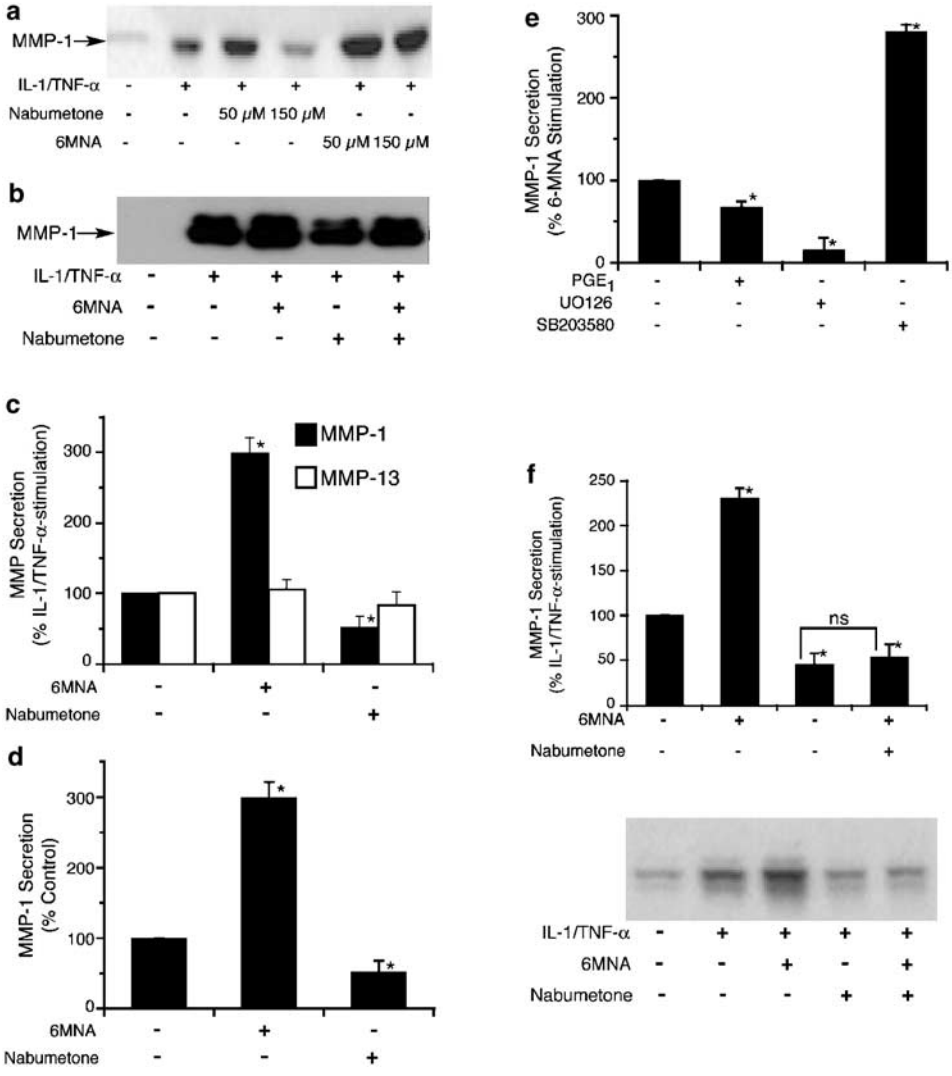

Figure 3 Effects of 6MNA and nabumetone on MMP secretion from SF. Panel a: SF were incubated for 30 min $\pm$ 6MNA or nabumetone, stimulated overnight $\pm$ IL-1β/TNF-α (each 20 ng ml^{-1}), and supernatants assayed for MMP-1 by SDS–PAGE and immunoblot. Panel b: RA SF were incubated for 30 min $\pm$ 6MNA or nambumetone or both, stimulated overnight $\pm$ IL-1β/TNF-α and assayed for MMP-1 secretion. Panel c: SF were incubated for 30 min $\pm$ 6MNA or nabumetone, stimulated overnight $\pm$ IL-1β/TNF-α, and analyzed for MMP-1 and MMP-13 secretion expressed as % IL-1/TNF-α stimulation (one-way repeated-measures ANOVA, $P<0.001$). Panel d: SF were incubated overnight $\pm$ 6MNA or nabumetone, and assayed for MMP-1 secretion (one-way ANOVA, $P<0.001$). Panel e: SF were incubated $\pm$ PGE_1 (1 μM), UO126 (10 μM) or SB203580 (10 μM) for 30 min, followed by overnight incubation with 6MNA (150 μM) and assay for MMP-1 secretion (one-way repeated-measures ANOVA, $P<0.001$). Panel f: top and bottom, SF incubated $\pm$ 150 μM 6MNA, nabumetone, or both together, were stimulated overnight with IL-1β/TNF-α and assayed for MMP-1 secretion (one-way repeated-measures ANOVA, $P<0.001$). Data shown are representative of or the mean $\pm$ s.e.m. for three (panels a–e) or six (Panel F) experiments for each condition. (For pairwise comparisons, $^*P \leqslant 0.05$ vs the appropriately matched stimulated or unstimulated control. ns = not significant.)

Nabumetone also inhibited MMP-1 secretion stimulated by EGF (data not shown), and in RA SF stimulated with IL-1β/TNF-α (Figure 3b). However, nabumetone did not significantly inhibit MMP-13 secretion (Figure 3c). These data are consistent with our observation that Erk regulates the secretion of MMP-1 but not MMP-13 (Pillinger *et al*., 2003), and suggest that nabumetone may inhibit MMP-1 secretion *via* inhibition of Erk.

Since administration of nabumetone to patients is likely to result in the simultaneous presence of nabumetone and 6MNA, we assayed the effect of nabumetone/6MNA coadministration on MMP-1 secretion. Simultaneous incubation of SF with 150 μM 6MNA and nabumetone reversed the ability of 6MNA to enhance MMP-1 secretion (Figure 3f, top and bottom). Similar effects were observed in RA SF (Figure 3b).

Effects of 6MNA and nabumetone on NF-κB activation

NF-κB is a transcriptional regulator of inflammatory responses (Ghosh *et al*., 1998), and in some cell types is regulated by Erk (Birkenkamp *et al*., 2000; Garcia-Garcia *et al*., 2001). Since 6MNA and nabumetone regulated Erk in SF, we tested the effects of these agents on NF-κB activation, assayed as translocation of the NF-κB p65 subunit into the nucleus. Stimulation of SF with IL-1β/TNF-α resulted in p65 accumulation in SF nuclei. U0126 (10 μM) abrogated p65 translocation, confirming that Erk regulates SF NF-κB activation (Figure 4). However, EGF did not stimulate p65 translocation despite its ability to stimulate Erk (data not shown), indicating that Erk activation is necessary but not sufficient for p65 translocation. p38 mediates NF-κB activation in a number of cell types. In contrast to the ability of SB203580

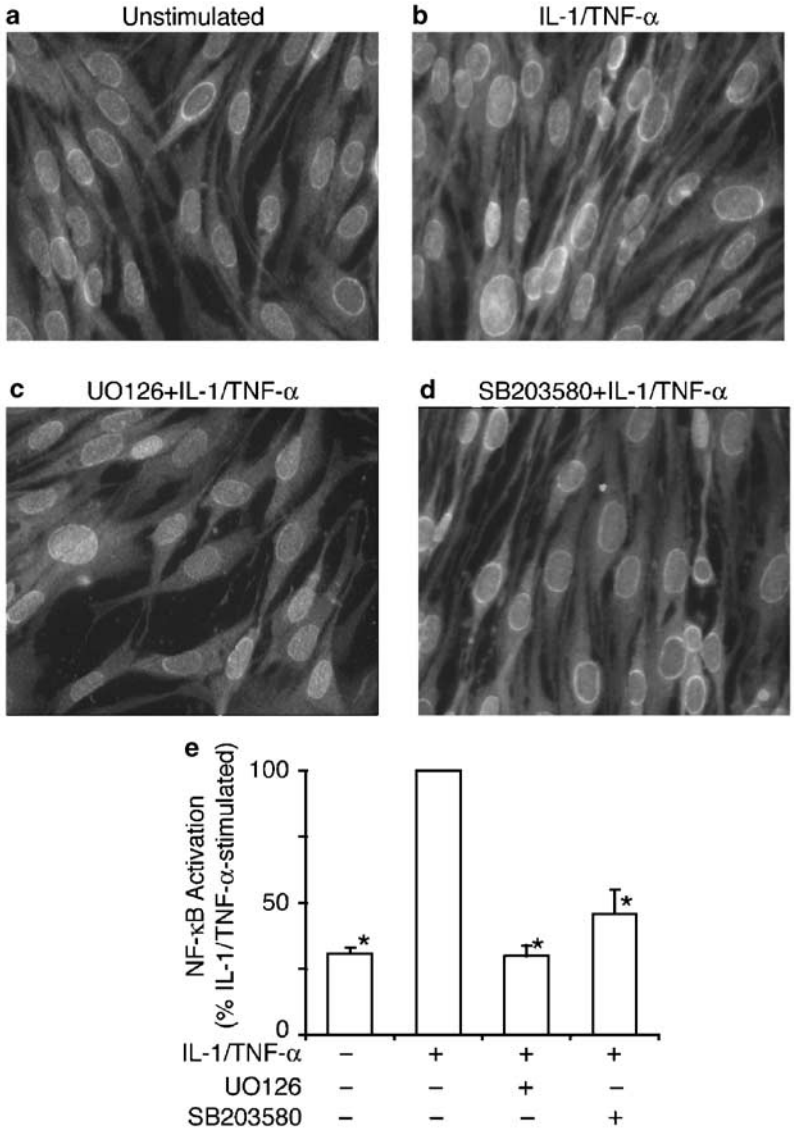

Figure 4 Regulation of IL-1β/TNF-α-stimulated NF-κB activation in SF by Erk and p38. SFs were incubated for 30 min ± 10 μM UO126 or SB203580, followed by overnight stimulation ± IL-1/TNF-α (each 20 ng ml^{-1}), as indicated above the images. NF-κB activation is indicated by accumulation of the NF-κB component p65 in the nucleus, imaged by immunofluorescence as described in Methods. Panel e: Quantitation of p65 nuclear accumulation (one-way repeated-measures ANOVA, $P<0.001$). Data shown are the mean ± s.e.m. of five experiments. (For pairwise comparisons, $^*P\leqslant 0.001$.)

to enhance MMP-1 secretion, SB203580 inhibited p65 translocation. Thus, both Erk and p38 positively regulate NF-κB activation.

We next tested the effects of 6MNA and nabumetone (Figure 5). 6MNA (150 μM) enhanced and nabumetone inhibited IL-1β/TNF-α-stimulated p65 translocation.

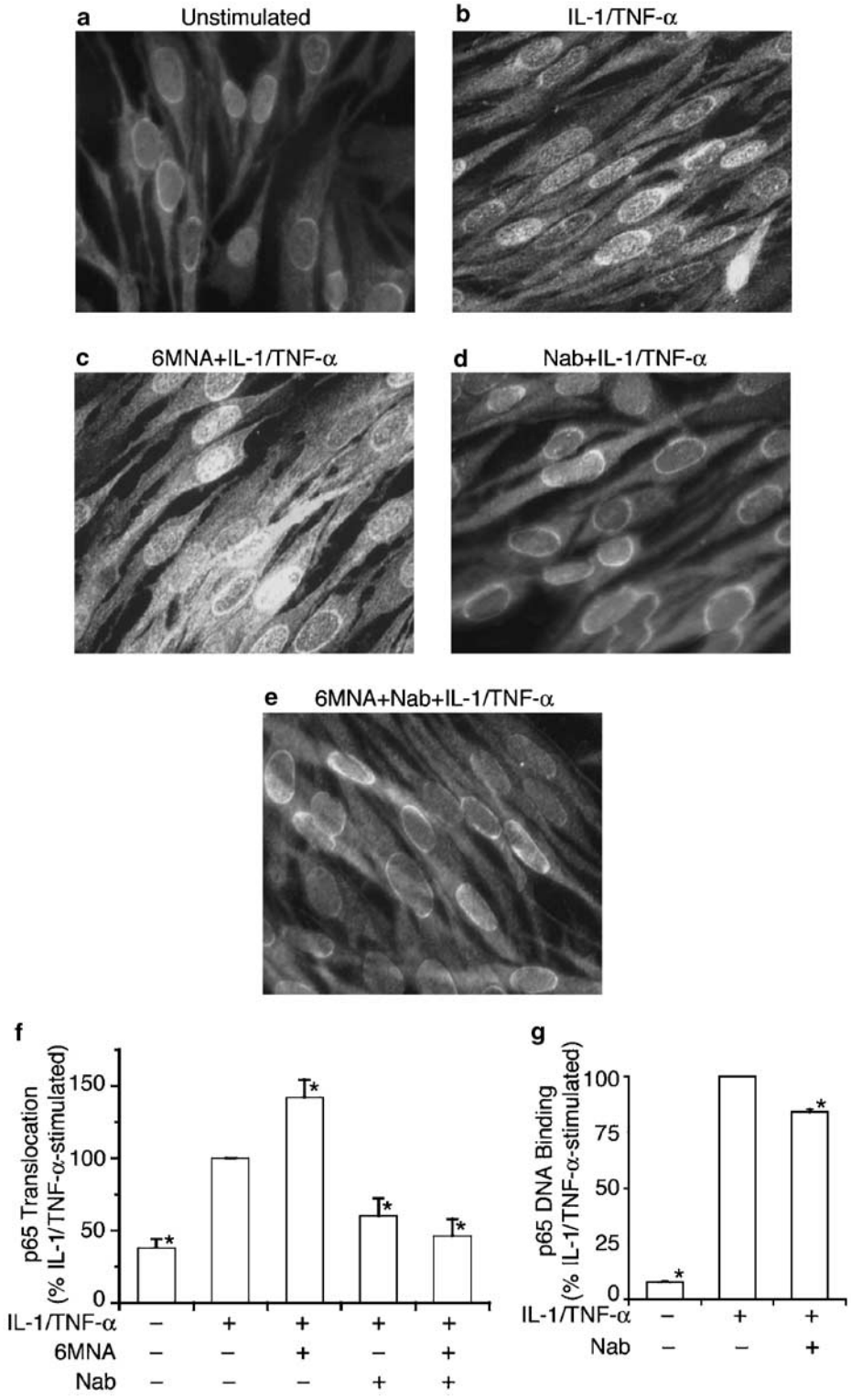

Figure 5 Regulation of IL-1β/TNF-α-stimulated NF-κB activation in SF by 6MNA and nabumetone. SF were incubated for 30 min ± 150 μM 6MNA, nabumetone or both, and stimulated overnight ± IL-1β/TNF-α, as indicated above the images. Panel f: Quantitation of p65 nuclear accumulation (one-way repeated-measures ANOVA, $P<0.001$). Panel g: Human RA SF were incubated overnight ± 150 μM nabumetone, stimulated for 6 h with IL-1β/TNF-α, and analyzed for p65 DNA binding as described in Methods (one-way repeated-measures ANOVA, $P<0.001$). Data shown are representative or the mean ± s.e.m. of seven (panels a–f) or three (panel g) experiments for each condition. (For pairwise comparisons, $^*P\leqslant0.013$ vs IL-1β/TNF-α stimulated.)

Coincubation of SF with 6MNA and nabumetone inhibited p65 translocation. 6MNA in the absence of other stimuli did not induce p65 translocation (data not shown), consistent with our observation that Erk activation is necessary but not sufficient to activate NF-κB. We confirmed the ability of nabumetone to inhibit NF-κB in RA SF using a highly sensitive ELISA-based system that measures p65 binding to its promoter DNA (Figure 5g), although the effects were more modest than those seen in the p65 translocation assay. These data are consistent with a role for Erk in NF-κB activation, and with the ability of 6MNA and nabumetone to, respectively, enhance and inhibit NF-κB activation *via* effects on Erk.

To determine whether the effects of nabumetone and 6MNA on NF-κB in SF were generalizable to other cell types, we duplicated these experiments in chondrocytes, another critical cell type in the joint whose regulation is NF-κB-dependent (Sakai *et al*., 2001; Clancy *et al*., 2002; Liacini *et al*., 2002). IL-1β/TNF-α (each 20 ng ml^{-1}) stimulated p65 translocation in bovine chondrocytes, a well-established chondrocyte model (Patel *et al*., 1999). 6MNA enhanced and nabumetone inhibited IL-1β/TNF-α-stimulated p65 translocation. Thus, 6MNA and nabumetone, acting *via* NF-κB, may regulate potentially important effects on a number of different cell types in the inflammatory joint.

Effects of 6MNA and nabumetone on nitric oxide (NO) production

NO has been implicated in the regulation of Erk, NF-κB and MMPs in cell types including SF and chondrocytes (Tamura *et al*., 1996; Sasaki *et al*., 1998; Zhang *et al*., 2000; Gu *et al*., 2002; Jovanovic *et al*., 2002; Mendes *et al*., 2002; Zaragoza *et al*., 2002). We therefore hypothesized that 6MNA and nabumetone regulate Erk, NF-κB and MMP-1 in SF *via* the effects on NO generation. However, neither 6MNA nor nabumetone significantly affected total NO generation (Figure 6). Similar results were obtained using chondrocytes (data not shown). We further analyzed SF NO generation as nitrite or nitrate production; neither nitrite nor nitrate levels were affected by 6MNA or nabumetone (data not shown). Since neither 6MNA nor nabumetone affected NO generation, the effects of these agents on Erk activation, MMP-1 secretion and NF-κB activation must be independent of NO.

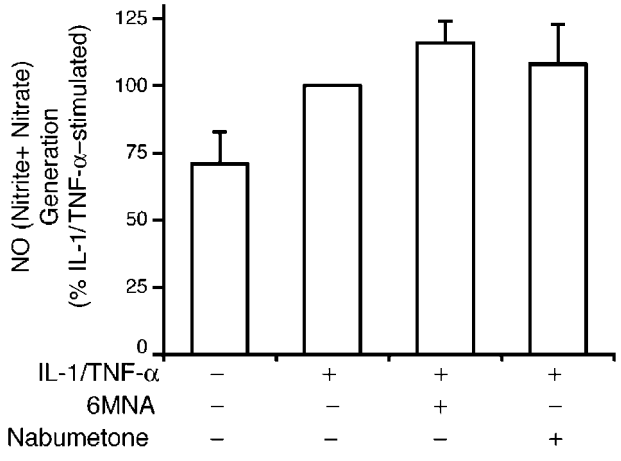

Figure 6 6MNA and nabumetone do not regulate SF NO production. SF were incubated ±150 μM 6MNA or nabumetone for 30 min, stimulated overnight ± IL-1β/TNF-α (each 20 ng ml^{-1}), and supernatants assayed for NO (One way repeated measures ANOVA, $P = 0.086$). Data are the mean ± s.e.m. of 3 experiments.

Discussion

Our data indicate that nabumetone has anti-inflammatory effects on SF. Nabumetone inhibited activation of Erk, an MAP kinase that regulates both SF proliferation and the secretion of MMP-1, but not MMP-13 (Pillinger *et al*., 2003). Consistent with its effects on Erk, nabumetone inhibited MMP-1 but not MMP-13 secretion from SF. Although nabumetone inhibited PG synthesis, its effects on Erk and MMP-1 were COX-independent, since they were not duplicated by 6MNA, a recognized COX inhibitor. Nabumetone also inhibited activation of NF-κB, an important transcriptional regulator of inflammation. In some cell types, Erk regulates NF-κB (Birkenkamp *et al*., 2000; Garcia-Garcia *et al*., 2001). We did not determine whether nabumetone inhibits SF NF-κB *via* Erk. However, we confirmed that specific Erk inhibition resulted in inhibition of NF-κB. Thus, it is likely that nabumetone acts on SF NF-κB, in whole or part, *via* effects on Erk.

Interestingly, nabumetone shares its COX-independent effects with acetyl and sodium salicylate, which at millimolar concentrations inhibit Erk (Schwenger *et al*., 1996; Pillinger *et al*., 1998; 2003) and NF-κB (Kopp & Ghosh, 1994; Alpert *et al*., 1999; Alpert & Vilcek, 2000), as well as MMP-1 secretion (Pillinger *et al*., 2003) from SF and other cells. Whether salicylates and nabumetone share common mechanisms of action remains to be determined. As NO regulates Erk, NF-κB and MMPs in a number of cell types, we tested the hypothesis that nabumetone acts on SF *via* regulation of NO generation. However, nabumetone did not affect NO levels in our studies, ruling out a role for NO in the regulation of SF by nabumetone. The ability to inhibit Erk, MMP-1 secretion and NF-κB activation suggests that nabumetone may have distinct and potentially important anti-inflammatory effects that have not been previously recognized.

In contrast to nabumetone, 6MNA had potentially undesirable effects on SF. Exposure of SF to 6MNA resulted in increased Erk phosphorylation and MMP-1 secretion. These responses depended upon PGE depletion, and were similar to the effects seen with other COX inhibitors (Dayer *et al*., 1976; Pillinger *et al*., 2003). 6MNA enhancement of MMP-1 secretion was mediated through Erk, since Erk inhibition blocked 6MNA-stimulated MMP-1 secretion.

6MNA also enhanced IL-1β/TNF-α-stimulated NF-κB activation. Since IL-1β/TNF-α stimulation of NF-κB was Erk-dependent, 6MNA enhancement of NF-κB was consistent with its ability to enhance Erk. However, our data do not exclude the possibility that 6MNA regulates NF-κB through pathways other than Erk. Since Erk activity was necessary but not sufficient for NF-κB activation, and since 6MNA in the absence of other agents stimulated Erk but not NF-κB, it was not possible to test whether Erk inhibitors could modulate the effect of 6MNA on NF-κB in the absence of cytokine stimuli. As in the case of nabumetone, the effects of 6MNA on Erk, MMP-1 and NF-κB were not mediated by NO, since 6MNA did not affect NO generation by SF.

Taken together, these data suggest that the proinflammatory effects of COX inhibitors may be more profound than generally appreciated. Indeed, E PGs reduce arthritis and other forms of inflammation in both rat and mouse models (Zurier & Quagliata, 1971; Zurier *et al*., 1977; Rossetti *et al*., 1994; Bandeira-Melo *et al*., 2000). Prior studies suggest that selective COX-2 inhibition offers no advantage over non-selective NSAIDs *vis-a-vis* Erk activation, MMP secretion and NF-κB (He *et al*., 2002; Pillinger *et al*., 2003). On the other hand, the ability of nabumetone to inhibit Erk phosphorylation, NF-κB activation and MMP secretion, together with its ability to reverse the effects of 6MNA, suggests that nabumetone may modulate the undesirable effects of COX inhibition.

One question of clinical importance is whether the concentrations of nabumetone needed to produce the observed effects are extant in SF *in vivo*. The concentrations of 6MNA and nabumetone tested, 50 and 150 μM, were in the range of 6MNA concentrations reported in the synovial fluid and serum, respectively (Miehlke *et al*., 1990), and also in the range of reported IC_{50}'s for 6MNA for COX-1 ($\approx 40\,\mu$M) and COX-2 ($\approx 150\,\mu$M) (Davies, 1997; Warner *et al*., 1999). Pharmacokinetic studies indicate that nabumetone is hepatically converted to 6MNA and that concentrations of nabumetone in the bloodstream are very low (Haddock *et al*., 1984; Torre *et al*., 1987; Blower, 1992; Hyneck, 1992; Davies, 1997). However, only one study has addressed nabumetone concentrations in the joint. Miehlke *et al*. (1990) measured synovial fluid concentrations of nabumetone after 4 days of treatment to be approximately $21 \pm 15\,\mu g\,l^{-1}$, or in the nanomolar range. Thus, the concentrations of nabumetone we tested were as much as 5×10^4 higher than those observed in synovial fluid. Whether nabumetone accumulates in the synovium itself was not determined in Miehlke's studies, and the fact that nabumetone (in contrast to 6MNA) is a nonacidic, hydrophobic molecule suggests that it may more readily accumulate in tissues than in body fluids. Ishiwata *et al*. (2003) have recently reported that nabumetone accumulates in the gastric lining in an animal model and reverses gastric damage induced by both 6MNA and indomethacin, suggesting that tissue persistence of unmetabolized nabumetone may contribute to the relatively benign gastric safety profile of this agent (Melarange *et al*., 1992; Helfgott, 1994; Basson *et al*., 2001; Morgan *et al*., 2001). The identification of nabumetone as an effective, nontoxic, *in vitro* inhibitor of Erk, MMP secretion and NF-κB activation suggests that its clinical effects deserve further investigation, and that nabumetone may serve as a model compound in the development of other inhibitors of these aspects of inflammation and arthritis.

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