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MF498, a selective EP4 antagonist, relieves joint inflammation and pain in rodent models of rheumatoid and osteoarthritis

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AIA, adjuvant-induced arthritis; CAIA, collagen antibody-induced arthritis; CIA, collagen-induced arthtitis; COX, cyclooxygenase; EP, E prostanoid receptor; MIA, monosodium iodoacetate; mPGES-1, microsomal PGE synthase 1; NSAID – non-steroidal anti-inflammatory drug; OA, osteoarthritis; RA, rheumatoid arthritis; PG, prostaglandin

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Abstract

Previous evidence has implicated EP4 in mechanical hyperalgesia induced by subplantar inflammation. However, its role in chronic arthritis remains to be further defined because previous attempts have generated two conflicting lines of evidence, with one showing a marked reduction of arthritis induced by a collagen antibody (CAIA) in mice lacking EP4 but not EP1-EP3, and the other showing no impact of EP4 antagonism on arthritis induced by collagen (CIA). Here, we assessed the effect of a novel and selective EP4 antagonist MF498 on inflammation in adjuvant-induced arthritis (AIA), a rat model for rheumatoid arthritis (RA), and joint pain in a guinea pig model of iodoacetate-induced osteoarthritis (OA). In the AIA model, MF498, but not the antagonist for EP1 MF266-1 or EP3 MF266-3, inhibited inflammation, with a similar efficacy as a selective COX-2 inhibitor MF-tricyclic. In addition, MF498 was as effective as an NSAID diclofenac or a selective mPGES-1 inhibitor MF63 in relieving OA-like pain in guinea pigs. When tested in rat models of gastrointestinal toxicity, the EP4 antagonist was well tolerated, causing no mucosal leakage or erosions. Lastly, we evaluated the renal effect of MF498 in a furosemide-induced diuresis model and demonstrated that the compound displayed a similar renal effect as a COX-2 inhibitor MF-tricyclic, reducing furosemide-induced natriuresis by ~50%. These results not only suggest that EP4 is the major EP receptor in both RA and OA but also provide a proof of principle to the concept that antagonism of EP4 may be useful for treatment of arthritis.

Introduction

Chronic joint inflammation and pain are the two main symptoms in patients with arthritis such as OA and RA. Joint inflammation begins with cartilage breakdown in OA whereas the process affects mainly the synovial membrane in RA (Gardner, 1994). Despite this difference, prostaglandins, primarily those derived from COX-2, are key mediators for inflammation and pain in both types of arthritis. Consequently, inhibition of COX-2 and prostaglandin synthesis by traditional NSAIDs and COX-2 inhibitors provides effective symptom relief for both OA and RA (Hochberg, 2005). Among the five major prostaglandins, namely, PGE_2, PGI_2, PGD_2, PGF_2 and thromboxane, PGE_2 is the most abundant found in the synovial cavity of RA patients (Bertin, et al., 1994; Seppala, et al., 1985), with levels that are significantly reduced by analgesic doses of NSAIDs (Seppala, et al., 1985; Bertin, et al., 1994). In contrast, at least in RA, the concentrations of PGI_2, the other putative pro-inflammatory prostanoid, are not significantly altered by NSAIDs (Seppala, et al., 1985; Bertin, et al., 1994). These observations suggest that PGE_2 may be the predominant prostaglandin in arthritis. In support of this view, PGE_2 has been shown to play a pivotal role in the development of joint inflammation and pain in animal models of arthritis. For instance, neutralizing PGE_2 with a specific antibody prevents the development of arthritis as effectively as NSAIDs in the AIA model (Portanova, et al., 1996). In addition, deletion of the gene for microsomal PGE synthase-1 (mPGES-1), a terminal PGE_2 synthase downstream of COX-2, protects mice from CIA similarly to COX-2 knockout (Trebino, et al., 2003; Myers, et al., 2000). More recently, we have demonstrated that inhibition of mPGES-1-dependent PGE_2 relieves joint pain in a guinea pig model of OA induced by iodoacetate (MIA) (Xu

et al., in preparation). Importantly, a selective EP4 antagonist has been shown to have COX-2 inhibitor-like analgesic efficacy in animal models of skin inflammation in the paw (Nakao, et al., 2007).

PGE_2 acts through four different EP receptors, namely EP1-4, to produce its biological actions. It is not clear which of these receptors mediates the pro-inflammatory effect of PGE_2. Identifying the key EP receptor in inflammation will not only improve our understanding of the pathogenesis of arthritis but also help find new molecular targets for the treatment of the disease. This effort, however, has been limited to mouse genetic studies because of the lack of selective antagonists for different EP receptors needed for rat studies. Two published studies using mice lacking the individual EP receptors have generated conflicting data concerning whether EP4 is the key EP receptor in arthritis. The first study shows that the deletion of EP4 but not EP1, 2 or 3 protects mice from arthritis induced by a collagen antibody (CAIA) (McCoy, et al., 2002). In contrast, the second study, in which the EP1, 2, 3 knockouts were also used but an EP4 antagonist was used as a surrogate for the EP4 knockout, demonstrates that none of the EP receptors have significant roles in murine arthritis induced by collagen (CIA) (Honda, et al., 2006). Thus, more work is needed to define the role of different EP receptors, especially that of EP4, in chronic joint inflammation. For this purpose, we determined the contribution of EP1, EP3 and EP4 to the development of joint inflammation in AIA using selective antagonists for these receptors. Our data show that the selective antagonist for EP4, MF498 or *N*-{[4-(5,9-diethoxy-6-oxo-6,8-dihydro-7-*H*-pyrrolo[3,4-*g*]quinolin-7-yl)-3-methylbenzyl]sulfonyl}-2-(2-methoxyphenyl)acetamide, but not that for EP1 (MF266-1) or EP3 (MF266-3), fully inhibited joint inflammation sensitive to the COX-2 inhibitor

MF-tricyclic in the model. Furthermore, the EP4 antagonist was as effective as NSAIDs in relieving pain in a guinea pig model of osteoarthritis. These findings suggest that EP4 is a major EP receptor in animal models of rheumatoid and osteoarthritis. Moreover, the EP4 antagonist displayed renal and gastrointestinal tolerability similar to those of a COX-2 inhibitor, inhibiting natriuresis in the kidney and causing no mucosal lesions in the gastrointestinal tract. Thus, EP4 antagonists hold promise for the treatment of arthritis.

Methods

Radioligand binding assays. Radioligand binding assays were carried out on membranes prepared from HEK293 (EBNA) clonal cell line expressing the human prostanoid receptor as described previously (Abramovitz, et al., 2000). Briefly, prostanoid receptor binding assays were performed in a final incubation volume of 0.2 ml in 10 mM MES/KOH (pH 6.0) (EP subtypes, FP and TP) or 10 mM HEPES/KOH (pH 7.4) (DP and IP), containing 1 mM EDTA, 30 mM $MnCl_2$ (EP1), 10 mM $MgCl_2$ (EP2, EP3, EP4 and TP), 2.5 (IP) or 10 mM $MnCl_2$ (DP and FP) and radioligand [0.5 to 1.25 nM [^{3}H]PGE_2 (181 Ci/mmol) for EP subtypes, 0.7 nM [^{3}H]PGD_2 (200 Ci/mmol) for DP, 0.95 nM [^{3}H]$PGF_{2\alpha}$ (170 Ci/mmol) for FP, 5 nM [^{3}H]iloprost (16 Ci/mmol) for IP and 1.8 nM [^{3}H]SQ-29548 (46 Ci/mmol) for TP. The reaction was initiated by addition of membrane protein (approximately 30-80 µg for EP1, 20-60 µg for EP2, 2-7 µg for EP3, 1-3 µg for rat EP3-I, 10-60 µg for EP4, 8-25 µg for rat EP4, 40-80 µg for FP, 20-30 µg for DP, 12-15 µg for IP, 5 µg for TP) from the 160,000 x g fraction. Ligands were added in dimethylsulfoxide (DMSO) which was kept constant at 1 % (v/v) in all incubations. Incubations were conducted on a mini-orbital shaker at room temperature for either 60

min (EP1, EP2, EP3, DP, TP and IP) or 120 min (EP4), 90 min at 30°C (FP) or 60 min at 37°C (rat receptors). The binding assay was terminated by rapid filtration through a 96-well Unifilter GF/C (Canberra Packard) prewetted in assay incubation buffer without EDTA (at 4°C) using a Tomtec Mach III 96-well semi-automated cell harvester. The filters were washed with 3 to 4 ml of the same buffer and dried under vacuum for 30 to 60 min at 55°C. The residual radioactivity bound to the individual filters was determined by scintillation counting with addition of 25 µL of Ultima Gold F (Canberra Packard) using a 1450 MicroBeta Wallac scintillation counter. Specific binding, which was calculated by subtracting nonspecific binding from total binding, accounted for 85 to 90% of the total binding and was linear with respect to the concentrations of radioligand and protein used. Total binding represented 5 to 10% of the radioligand added to the incubation media.

Cell-based functional assays

PGE_2 induced-aequorin luminescence assay for measuring EP1 antagonist activity. The aequorin luminescence assay was performed as described previously (Ungrin, et al., 1999). In short, HEK293 cells co-expressing hEP1 receptor and aequorin were first incubated with coelenterazine, an aequorin co-factor, for 1 h. Next, the cells were plated in a 96-well plate (50000 cells/well) containing PGE_2 with or without the antagonist. Upon stimulation of the hEP1 receptor by PGE_2, the cells release intracellular calcium which binds to aequorin, resulting in emission of luminescence. The emission was recorded for 30 s (peak 1) and then 0.1% Triton X-100 was added to the wells (20 µl/well) to trigger the release of residual luminescence from the non-reacted aequorin (peak 2). Fractional luminescence, an index of EP1 activity, was calculated using the

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formula: area under peak or AUP 1/(AUP 1 + AUP 2). Fractional luminescence was used to calculate EC_{50} of PGE_2 stimulated aequorin or EP1 activity by employing a modified version of the Levenberg-Marquardt four-parameter curve-fitting algorithm (LDAM software). Kb was calculated by using Schild plot.

EP3 agonist sulprostone-induced cAMP accumulation assay for measuring EP3 antagonist activity. The assay was performed using human erythroleukemia cells (ATCC HEL 92.1.7) (2×10^5 cells/well) that endogenously express the EP3 receptor in HBSS containing 100 μM RO-20174 and 15 μM forskolin (0.2 ml/well). MF266-3 (0–3×10^{-6} M) was added to the incubation mixture in DMSO (0.5% final concentration), the reaction was initiated by adding sulprostone (Biomol) (0–3×10^{-5} M in 0.5% DMSO), and incubated at 37 °C for 10 min. The reaction was terminated by boiling the samples for 3 min and cAMP was measured by the cAMP SPA assay (RPA556, Amersham).

PGE_2-induced cAMP accumulation assay for measuring EP4 antagonist activity. HEK293 cells stably expressing human EP4 receptor were grown to 95% confluence and then harvested by incubation in enzyme-free cell dissociation buffer, followed by centrifugation at 300 x g for 6 min. The cells were washed with PBS, centrifuged as above, resuspended at a final concentration of 2.5 x 10^6 cells/ml in 25 mM HEPES, pH 7.4, HBSS containing isobutylmethylxanthine (IBMX) at 0.5 mM. 2.5 x 10^5 cells were pre-incubated (10 min, 37°C) with 0 to 1 x 10^{-5}M of MF498 in DMSO (1% final concentration) and were subsequently challenged with 0.3 nM PGE_2, in the absence or presence of 10% human serum, at 37ºC for 10 min in a final assay volume of 0.2 ml. The reactions were stopped by boiling for 3 min and the samples were centrifuged at 1400 x g for 10 min. The supernatant was collected and the cAMP generated was assayed

using a cAMP SPA kit (RPA556, Amersham) according to the manufacturers' instructions.

Experimental animals. All procedures used for the in vivo experiments were approved by the Animal Care Committee at the Merck Frosst Centre for Therapeutic Research (Kirkland, QC, Canada) and were performed according to guidelines established by the Canadian Council on Animal Care. The animals used for the present studies were male Sprague-Dawley rats and Hartley guinea pigs obtained from Charles River Laboratories (St-Constant, QC, Canada). All test compounds with the exception of diclofenac (Sigma Aldrich Canada) were synthesized by the Medicinal Chemistry Department, Merck Research Laboratories. Experiments involving the use of ^{51}Cr-EDTA were conducted in strict accordance with regulations and guidelines established by the Merck Frosst Radiation Safety Committee as well as the Canadian Nuclear Safety Commission.

Adjuvant-induced arthritis. Complete Freund's adjuvant, namely 0.6 mg *M. butyricum* (Difco Labs, Detroit, MI) in 50 µl mineral oil, was injected subplantarly in the left hind paw of rats (n≥7). A group of animals (n=3) was injected subplantarly with saline and used as non-arthritic (non-AIA) controls. Volume of both the injected (primary) and non-injected (secondary) paw was measured at day 0, 7 and 18 following complete Freund's adjuvant injection. On day 7, animals were randomly grouped based on their increase in paw volume from baseline (Δpaw volume) and administered test compounds or vehicle. Test compounds were administered orally at various doses as indicated in figure 1C between day 9 and 18. Vehicle was administered to a group of 7 animals which were used as vehicle controls. The saline-injected animals also received

vehicle administration. Paw measurement was performed in a double-blinded fashion after the initiation of drug treatment. The change in paw volume (Δpaw volume) between day 18 and day 0 was used as an index of inflammatory edema. Percent inhibition was calculated by comparing Δpaw volume among different groups using the following formula.

% inhibition = 100 - (drug treatment – non-AIA) / (vehicle control - non-AIA) * 100

The ED_{50}, which is the 50% inflection point of the dose response curve, was calculated using Prism 4.0. The ED_{50} corresponds to ID_{30} (dose for 30% inhibition of paw swelling) and ID_{50} in the primary and secondary paws, respectively.

EP4 agonist-induced hyperalgesia in guinea pigs. Male Hartley guinea pigs weighing 175-200 g received an intraplantar injection (left hindpaw) of a selective EP4 agonist L-902688 (40 ng in 50 µl of saline) after an overnight fasting. Control animals received an equivalent volume of saline into the left hindpaw. The EP4 antagonist (formulated in 0.5% methylcellulose) was orally dosed 2 h before injection of the EP4 agonist. Measurements of nociception were obtained using a dynamic plantar aesthiometer (Ugo Basile, Comerio, Italy). The paw withdrawal latency was recorded and used as an index for sensitivity to the thermal stimulus.

MIA-induced shoulder joint pain in guinea pigs. While under anesthesia (2 – 3% isoflurane), guinea pigs weighing 175-200g received an intra-articular injection (into the left shoulder joint) of 50 µl of MIA (1 to 10 mg/ml) or saline. The right shoulder joint of each animal received 50 µl of 0.9% sterile saline. Immediately following injection, animals were removed from anesthesia and placed in a recovery cage until

righting reflexes returned. Once awake, animals were returned to their home cages. Weight bearing on each forelimb was measured using an incapacitance tester at various time points after MIA injection. Three individual measurements lasting 4 s each were taken for each animal at each of the time points. The average for each side was used to calculate the ratio of weight bearing between the two sides (L/R ratio), an index of pain. On day 7, animals that had received an injection of MIA showed a significant decrease in left/right weight bearing ratio (L/R) from 1 to less than 0.5. By comparison, animals that received an intra-articular injection of saline displayed a L/R of approximately 1, similar to that of naïve animals. Animals with a L/R ratio of $\geq$ 0.7 were considered non-responders and were removed from the study. Animals were orally dosed with either vehicle or a test compound at the indicated doses and incapacitance measurements taken 6 h later. In order to perform the measurements in a double-blinded fashion, the animals were randomized and assigned new codes. The percentage of incapacitance was calculated based on the formula $(\Delta L/R_{compound}-\Delta L/R_{-MIA})/(\Delta L/R_{veh}-\Delta L/R_{-MIA}) \times 100$, where ΔL/R is the decrease in L/R from the baseline.

Furosemide-induced diuresis and natriuresis in rats. Diuresis studies were conducted using male Sprague-Dawley rats weighing 150-175 g. Animals were subcutaneously implanted with mini-osmotic pumps (model 2ML1, ALZET, Cupertino, CA, USA) filled with furosemide (Sigma-Aldrich, Oakville, ON, Canada) dissolved in 60% PEG200 (12 mg/day). Furosemide was delivered at a rate of 10 µl/h for 7 days. The mini-osmotic pumps were positioned in a pocket constructed in the subcutaneous tissue just below the back scapular region. For the control group, 60% PEG200 was infused by the same method. MF-tricyclic and MF498 were each orally dosed for 5 days (twice

daily and once daily, respectively). Animals were placed in metabolism cages immediately following the final oral dosing (day 5) to collect urine over a 24 h period. In addition to tap water, rats received a separate solution containing 0.9% NaCl and 0.1% KCl for the duration of the experiment, beginning 1 day before implantation of the mini-osmotic pumps. Urine electrolytes were measured using a Vitros 350 chemistry analyzer (Ortho-Clinical Diagnostics, Markham, ON, Canada).

Quantitative analysis of RNA in the kidney. Whole kidney lysates were prepared for reverse transcription and real-time quantitative PCR analysis of RNA for COX-2, mPGES-1, EP and IP receptors. Briefly, the mRNA from individual samples was isolated using the mRNA Express Kit from RNAture according to the manufaturer's instructions. The reverse transcription reaction was performed immediately after removal of the wash buffer using the TaqMan reverse transcription reagent kit (Applied Biosystems). The synthesized cDNAs were analyzed by PCR amplification using the TaqMan PCR master mix (Applied Biosysytems) and the appropriate pre-developped mix of primers and probes purchased from Applied Biosystems. Each probe was labeled with a fluorescent reporter dye, FAM (6-carboxyfluorescein), at the 5' end and a quencher dye, TAMRA (6-carboxytetramethylrhodamine), at the 3'end. The quantification of the QPCR was performed in User Bulletin 2 of ABI Prism 7700 Sequence Detection System (PE Biosystems). The results are expressed as fold of increase over the vehicle control.

Acute mucosal erosions in rats. Rats weighing 250-300 g were fasted overnight before oral administration of either vehicle (0.5% methylcellulose) or test compounds. Animals were sacrificed 3 h post dose and the stomach from each animal was harvested

for the assessment of mucosal erosion. Whole stomachs were stretched and examined under a microscope using a 1 x lens.

Urinary excretion of ^{51}Cr-EDTA as a measure of gastrointestinal integrity in rats. Rats weighing 250-300 g received daily oral administration of MF498 for 5 days or Indomethacin for 3 days. Rats were fasted overnight before the final compound dosing and orally dosed with ^{51}Cr-EDTA (10 μCi in 2 ml) (Draximage, Montreal, QC, Canada) 5 min after the final compound dosing. Immediately following oral gavage of ^{51}Cr-EDTA, animals were placed in metabolism cages for a 24 h collection of urine. Levels of ^{51}Cr-EDTA were measured using a gamma radiation counter and normalized to total volume of urine excreted.

Statistical analysis. One-factor comparison was made using one way analysis on variance (ANOVA) plus Dunnett's post-tests. Two-factor comparison was made using two way ANOVA plus Bonferroni's post-tests. Data are shown as mean ± SEM.

Results

Potency, selectivity and oral bioavailability of MF266-1, MF266-3 and MF498. MF266-1, MF266-3 and MF498 are selective antagonists for EP1, EP3 and EP4, respectively. The chemical structures of the three compounds are shown in figure 1A. The discovery and synthesis of MF266-1 and MF266-3 have been described previously (Ducharme, et al., 2005; Juteau, et al., 2001), whereas those of MF498 will be reported soon (Burch et al, in preparation). The binding affinity of the compounds for recombinant human prostanoid receptors was examined in a standard binding assay. The three compounds MF266-1, MF266-3 and MF498 displayed strong binding affinity for

the EP1, EP3 and EP4 receptors, respectively, with a Ki of 3.8, 0.8 and 0.7 nM, and a high degree of selectivity over other EP receptors (Ki > 1 μM). The compounds also displayed a relatively good selectivity over other prostanoid receptors, although all three compounds had only moderate selectivity for TP (Table 1). To determine the antagonist activities of the compounds, we examined their effects on EP ligand-induced biochemical responses. Specific assays used were PGE_2-induced aequorin-mediated luminescence for EP1, and sulprostone- or PGE_2-induced cAMP accumulation for EP3 and EP4, respectively. In the PGE_2-induced aequorin-dependent luminescence assay, in which EP1-mediated calcium release was indirectly measured by quantifying calcium sensitive aequorin-dependent luminescence, PGE_2 caused a dose-dependent increase in the emission of luminescence with an EC_{50} of 7.1 nM. Addition of the antagonist MF266-1 resulted in a rightward shift of the dose-response curve with a marked increase of the EC_{50} to 281 nM and a calculated Kb value of 14.9 nM for functional EP1 inhibition (Fig. 1B). In the case of EP3, sulprostone cause a dose-dependent accumulation of cAMP in HEK293 cells with an EC_{50} of 1.07 nM. Addition of MF266-3 caused a rightward shift of the sulprostone-cAMP dose response curve and increases in the EC_{50} values in a dose-dependent manner, yielding a Kb of 4.5 nM for functional EP3 inhibition (Fig. 1C). The functional antagonist activity of MF498 was determined in a similar cAMP accumulation assay but in HEK293 cells stimulated with PGE_2 at a single concentration of 0.3 nM. In the assay, MF498 inhibited PGE_2-stimulated cAMP accumulation in a dose-dependent manner with IC_{50} values of 1.7 and 17 nM in the absence and presence of 10% serum, respectively (Fig. 1D). In addition to activity on the human receptors, MF266-1, MF266-3 and MF498 also showed potent binding activity on the respective rat EP receptors, with

Ki values of 15, 2.0 and 0.7 nM, respectively on the rat EP1, EP3 and EP4 (data not shown). In vivo pharmacokinetic studies in this species showed excellent bioavailability ranging from 46% for MF266-1, 65% for MF266-3 to 100% for MF498, resulting in plasma concentrations of 5.5, 2.6 and 1.2 μM, respectively, at 6 h after oral dosing at 20 mg/kg. The 6 h plasma concentrations were significantly higher than the Ki values of the compounds for the target rat receptors (data not shown).

EP4, but not EP1 or EP3, mediates inflammation in arthritis. To determine the contribution of the three EP receptors to joint inflammation, we examined the effects of the selective antagonists for these receptors and a benchmark selective COX-2 inhibitor MF-tricyclic (Rowland, et al., 2007) on paw swelling, an index of inflammation, in the rat AIA model. Subplantar injection of complete Freund's adjuvant (CFA) caused swelling in the injected or primary paw shortly (~ 6 h) after the injection. Swelling in the primary paw then worsened with time and resulted in an average increase in paw volume of 2.1 ± 0.2 ml (n= 7) over the baseline value of ~1 ml on day 18 post CFA injection. In contrast to that in the primary paw, swelling in the secondary paw was not evident until ~10 days after the injection, but then progressed with time and resulted in a ~50% increase in paw volume over the baseline on day 18. The effects of test compounds on paw swelling were examined by using post treatment beginning 9 days after CFA injection with daily oral administration. The benchmark compound MF-tricyclic, at the dose of 6 mg/kg/day, attenuated swelling in both the primary and secondary paw, by 65% (Fig. 2A) and 92% (Fig.2B), respectively, when compared with the vehicle. MF498 displayed a similar anti-inflammatory effect as MF-tricyclic in both the primary (Fig. 2A) and secondary (Fig.2B) paw with a maximum inhibition of ~70% and ~100% (n=17),

respectively, indicating that the two compounds were equally efficacious. MF498, however, was much more potent than MF-tricyclic, displaying an ED_{50} of 0.02 mg/kg/day over 1 mg/kg/day for MF-tricyclic in the primary paw (Fig. 2C) and 0.01 mg/kg/day over 0.3 mg/kg/day in the secondary paw (data not shown). In contrast, both the EP1 antagonist MF266-1 and the EP3 antagonist MF266-3 failed to inhibit swelling in either paw at doses up to 10 mg/kg/day (Fig. 2C, primary paw) (data for seconday paw not shown). These data indicate that MF498 fully abrogates inflammation that is sensitive to COX-2 inhibitors.

EP4 mediates inflammatory pain in arthritis. Having demonstrated that EP4 was the predominant prostanoid receptor mediating joint inflammation, we then assessed the role of the receptor in inflammatory joint pain. Animals with AIA, although suffering from pain, are not suited for pain assessment by using weight bearing because of polyarthritis. To better assess analgesia for joint pain, we used a guinea pig model of mono-osteroarthritis induced by MIA, which was directly injected into the joint cavity of the left shoulder. Guinea pigs were used to compare MF498 with a selective mPGES-1 inhibitor MF63, which was active on the guinea pig but not the rat enzyme. To determine whether MF498 was active in the guinea pig in the absence of receptor binding data, we first examined the effect of the compound on thermal hyperalgesia induced by a highly selective EP4 agonist L-902688. L-902688 is a potent agonist for human EP4 receptor with a Ki of 0.3 nM and is highly ($\geq$ 4,000 fold) selective over other EP and prostanoid receptors (Young, et al., 2004). L-902688, when injected at the amount of 80 ng into the left forepaw, resulted in a marked reduction of the paw withdrawal latency, indicative of hyperalgesia, from 6.7 ± 0.3 to 3.8 ± 0.4 seconds. MF498, when dosed 2 h before the

injection of L-902688 attenuated the agonist-induced hyperalgesia in a dose-dependent manner, completely blocking the hyperalgesic response at 30 mg/kg, indicating that the compound is suited for pharmacological assessment in the guinea pig (Fig 3A). Next we assessed the effect of MF498 on joint pain in the MIA model and compared it with that of diclofenac and MF63. Joint pain was measured indirectly by recording weight bearing ratio between the injected or left forelimb and the non-injected or right forelimb (L/R). The baseline L/R ratio in naïve animals or in those whose shoulder joint was injected with saline was between 1.0 and 1.2. The L/R ratio decreased in a dose- and time-dependent manner, as a result of joint inflammatory pain, after the injection of MIA into the synovial cavity of the left shoulder. The lowest L/R ratio of ~0.5 was measured between day 3 and 7 at the doses of 7.5 and 10 mg/joint and was significantly lower than that of the baseline ~1.2 ($p<0.01$) (Fig. 3B). The analgesic effect of the test compounds were subsequently assessed in animals with established osteoarthritic pain 7 days after MIA injection. MF-tricyclic, when administered at the doses of 1.5, 5 and 15 mg/kg, reversed the decrease of L/R ratio in a dose-dependent manner, with a maximum of 76% reversal. Diclofenac, at the dose of 10 mg/kg, displayed a slightly better efficacy, relieving pain by 91% (Fig. 3C). MF498, at the dose of 30 mg/kg, the maximally efficacious dose in this species, reversed the pain response similarly to both the NSAID diclofenac (10 mg/kg) and the mPGES-1 inhibitor MF63 (Cote, et al., 2007) (100 mg/kg) (Fig. 3D). Together, the data demonstrate that antagonism of EP4 is as effective as NSAIDs and mPGES-1 inhibitors in relieving joint pain in arthritis.

Antagonism of EP4 inhibits natriuresis. To evaluate the impact of EP4 antagonism on sodium and fluid excretion, we examined the effect of the compound on

furosemide-induced diuresis and natriuresis. Furosemide caused a significant increase in COX-2 and mPGES-1 ($p < 0.05$), a decrease in EP3 and EP4 ($p<0.05$) and no significant changes in EP1, EP2 and IP mRNA levels (data not shown) in the rat kidney 7 days after being continuously infused at the rate of 2.5 mg/kg/hour into the peritoneum (Fig. 4A). Furosemide also caused robust diuresis and natriuresis, as demonstrated by a significant increase in urine and sodium output over baseline obtained in animals infused with 60% PEG200 (Fig. 4B and C). MF-tricyclic, when dosed orally at 10 mg/kg daily during furosemide infusion, attenuated furosemide-induced diuresis and natriuresis by 58% and 76%, respectively, when compared with the vehicle ($p<0.05$). MF498, at the doses of 0.1, 1 and 10 mg/kg, inhibited both diuresis and natriuresis in a dose-dependent manner, achieving a maximal reduction of 40% and 52%, respectively, relative to vehicle at the dose of 10 mg/kg ($p<0.05$). The inhibitory effects of MF498 were lower than those of MF-tricyclic but the difference was not statistically significant ($p>0.05$) (Fig. 4B and C).

EP4 antagonism does not affect the integrity of gastrointestinal mucosa. To assess the effect of EP4 antagonists on the integrity of gastrointestinal mucosa, we tested MF498 in two standard rodent models that were used for assessing the gastrointestinal toxicity of traditional NSAIDs and COX-2 inhibitors. In the first model, the ability of MK498 and indomethacin to cause mucosal lesions in the stomach was assessed by examining mucosal erosions. Mucosal erosions were noted in the stomach of all 5 rats treated with indomethacin 3 h following an oral dose of the compound at 10 mg/kg whereas no such lesions were seen in animals treated with vehicle or MK498 at doses up to 30 mg/kg (Fig. 5A; 30 mg/kg). We then assessed whether MF498 caused gastrointestinal mucosal leakage of ^{51}Cr-EDTA into the blood circulation by quantifying

the percentage of total radioactivity excreted into urine 24 h after orally dosing the radioactive compound. The NSAID indomethacin caused a significant 4-fold increase over vehicle in the urinary ^{51}Cr-EDTA levels after being dosed at 5 mg/kg for 3 days ($p<0.05$). In contrast, MF498, at the doses ranging from 1 to 30 mg/kg for 5 days, and MF-tricyclic (30 mg/kg/day, 5 days), did not significantly increase the urinary excretion of ^{51}Cr-EDTA when compared with vehicle treatment (Fig. 5B). The data demonstrate that MF498, at doses expected to fully block the EP4 receptor, does not compromise the mucosal integrity in the gastrointestinal tract.

Discussion

Lack of selective antagonists has been one of the major hurdles for elucidating the role of various EP receptors in inflammation. In recent years, our laboratories have developed selective antagonists for EP1, EP3 and EP4. The antagonists reported here are potent at binding to their target receptors with Ki values in the nanomolar range and highly selective over other prostanoid receptors. All three compounds behave as functional antagonists on the corresponding receptors in cell-based assays. These properties are generally similar or superior to those of similar compounds reported in the literature. Specifically, MF266-1 is more selective toward TP (60 fold) than the EP1 antagonist GW848687 (30 fold) (Giblin, et al., 2007); MF266-3 shows better selectivity over EP4 (>7000 fold) than that of the reported EP3 receptor antagonist ONO-AE3–240 (250 fold with a Ki for EP4 = 58 nM) (Amano, et al., 2003); and MF498 has a higher affinity for the EP4 receptor (0.7 vs. 13 nM) and better bioavailability than CJ-023,423 (100% vs. 4.6%) (Nakao, et al., 2007). Furthermore, the antagonists used in the present

study also have the desired potency and pharmacokinetic properties in rats and hence are suitable for studying the role of the target receptors in the rat AIA model.

AIA is an autoimmune-driven polyarthritis characterized by inflammation in the synovial membrane, causing joint swelling and pain similar to those noted in RA. AIA has been used for the proof-of-concept studies on COX inhibitors and therefore, is a relevant model for studying the role of different EP receptors in arthritis (Chan, et al., 1999). In this model, MF-tricyclic and other COX-2 inhibitors that were reported previously significantly alleviate joint inflammation with similar efficacy to that of NSAIDs (Chan, et al., 1995; Chan, et al., 1999), suggesting that as in RA, pro-inflammatory prostaglandins are primarily derived from COX-2 in this model. PGE_2 is the predominant prostanoid mediator in the AIA model because blockade of its activity with an antibody completely prevents NSAID-sensitive joint swelling (Portanova, et al., 1996). In addition, genetic blockade of COX-2 or mPGES-1, two key enzymes for PGE_2 synthesis, also protects against the development of autoimmune arthritis (Myers, et al., 2000; Trebino, et al., 2003). In support of PGE_2 as the key prostaglandin in AIA, we have demonstrated that an EP4 antagonist is as effective as a COX-2 inhibitor in suppressing joint inflammation. More importantly, our observation pinpoints EP4 as the principal EP receptor for PGE_2 in this process. We have not been able to address the role of EP2 in the present study because a selective antagonist for the receptor is still lacking. But the full inhibition of COX-2-dependent inflammation by an EP4 antagonist in AIA suggests that the role of EP2 in this paradigm may be either insignificant or redundant with that of EP4. Nevertheless, our data are in close agreement with those from a previous study using mice lacking individual EP receptors, which demonstrate that the

deletion of EP4 but not EP1-3 protects the animals from arthritis induced by a collagen antibody (McCoy, et al., 2002). Taken together, the evidence summarized above suggests that COX-2, mPGES-1 or PGE_2, and EP4 are essential for the development of autoimmune arthritis in both mice and rats. Thus, COX-2, mPGES-1, PGE_2 and EP4 may constitute a pro-inflammatory prostanoid axis in auto-immune arthritis. The relevance of this axis in human RA is supported by the well documented role of COX-2 as well as the existing biochemical evidence on the tissue levels of PGE_2, mPGES-1 and EP4. For instance, PGE_2 is a major prostaglandin in RA patients and is sensitive to NSAID treatment (Bertin, et al., 1994; Seppala, et al., 1985). COX-2, mPGES-1 and EP4 are expressed in the inflamed synovial tissues (Westman, et al., 2004; Yoshida, et al., 2001; Korotkova, et al., 2005) or in human synoviocytes stimulated with interleukin-1β, a major cytokine in RA (Yoshida, et al., 2001). Importantly, the expression profiles of COX-2, mPGES-1 and EP4 in human tissues or cells are similar to those in rat AIA (Claveau, et al., 2003; Kurihara, et al., 2001). Together, these lines of evidence suggest that COX-2, mPGES-1 and EP4 are key contributors from the prostanoid synthesis and signaling pathway to joint inflammation in both AIA and RA.

In addition to having a pro-inflammatory role, EP4 has been shown to be important in mediating the action of PGE_2 in inflammatory pain because antagonism of EP4 results in an analgesic effect on pain associated with cutaneous inflammation in the paw induced by PGE_2, carrageenan (Nakao, et al., 2007) or adjuvant (Lin, et al., 2006; Nakao, et al., 2007). In agreement with these findings, we have demonstrated here that stimulation of EP4 by a selective agonist causes a strong hyperalgesic response in the guinea pig paw. More importantly, we have demonstrated that a selective EP4 antagonist

is as efficacious as COX-2 or mPGES-1 inhibitors in relieving chronic joint pain in the guinea pig model of OA induced by MIA, indicating that as in cutaneous inflammatory pain, EP4 also plays a major role in mediating OA-like arthritic pain that is largely dependent on COX-2 as well as mPGES-1.

EP4 mediates some of the PGE_2 activities in the gastrointestinal tract, such as promoting the duodenal secretion of $HCO3^-$ (Aoi, et al., 2004; Larsen, et al., 2005), gastric mucus production (Takahashi, et al., 1999), and protects gastric epithelial cells from apoptosis (Hoshino, et al., 2003), suggesting that EP4 antagonism might have a negative impact on the gastrointestinal tract. However, previous evidence has shown that a selective EP4 antagonist CJ-42794 is well tolerated by the gastrointestinal tract (Takeuchi, et al., 2007). In agreement with this evidence, the selective EP4 antagonist used in the present study did not cause gastric mucosal erosions after acute dosing. More importantly, the compound did not increase mucosal leakage, a more sensitive measure of gastrointestinal toxicity, after subchronic administration. In addition, no lesions were noted upon gross examination in the stomachs of AIA rats chronically treated with the compound (data not shown). Together, these findings demonstrate that antagonism of EP4 is as well tolerated by the gastrointestinal tract as the selective inhibition of COX-2.

PGE2 also has important biological functions in the kidney such as regulating renal bloodflow, sodium and water excretion (Villa, et al., 1997). Inhibition of prostaglandin synthesis by NSAIDs and COX-2 inhibitors can result in fluid retention and other renal-based adverse effects, especially under certain disease conditions in which renal function becomes more dependent on prostaglandins (Curtis, et al., 2004). Furosemide-induced natriuresis and diuresis represents a useful model for assessing the

impact of PGE_2 suppression on sodium and fluid retention because of the increased renal dependency on COX-2-derived PGE_2 (Nusing, et al., 2005). EP receptors, notably EP4, have been shown to be important for furosemide-induced natriuresis in mice (Nusing, et al., 2005). In agreement with this finding, we have demonstrated that antagonism of the receptor by MF498 results in significant anti-natriuretic and anti-diuretic effects similarly to the COX-2 inhibitor MF-tricyclic. Together, these findings indicate that EP4 mediates most of the natriuretic action of PGE_2, which may be derived from COX-2 and mPGES-1, both of which are induced by furosemide. An EP4 antagonist may have a comparable renal profile as traditional NSAIDs and COX-2 inhibitors. It deserves mention that the renal effects of these drugs occur only rarely and are readily manageable in the clinic (Curtis, et al., 2004). What is more important is whether the anti-natriuretic effect may lead to the development of hypertension. Although the potential effect of EP4 antagonists on blood pressure remains to be determined, current evidence suggests that EP2 as well as IP may play a greater role than EP4 in the regulation of blood pressure because deletion of the gene for these receptors renders mice more susceptible to salt-induced hypertension (Kennedy, et al., 1999; Francois, et al., 2005).

In summary, we have demonstrated that a selective EP4 antagonist fully abrogates COX-2-dependent arthritic inflammation and pain, indicating that EP4 mediates the action of PGE_2 in both processes. Although PGI_2 has also been shown to play an important role in arthritis (Pulichino, et al., 2006), the results presented here demonstrate that an EP4 antagonist is capable of producing NSAID-like anti-inflammatory and analgesic efficacy. In addition, the EP4 antagonist is well tolerated by the gastrointestinal tract and has a similar anti-natriuretic effect as a COX-2 inhibitor in the

kidney. Unlike COX-2 inhibitors, EP4 antagonists do not suppress PGI_2, which possesses potent vasodilatory and anti-thrombotic activities, and may be cardioprotective (Fitzgerald, 2004; Francois, et al., 2005; Wang, et al., 2006). Together, our findings suggest that EP4 antagonists may represent a novel class of therapeutic agents for the treatment of chronic arthritis.

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Legends for figures

Fig. 1. Functional inhibition of the EP1, EP3 and EP4 receptors by MF266-1, MF266-3 and MF498 in cell-based assays. (A). Chemical structure of MF266-1, MF266-3, and MF498. (B). Inhibition of PGE_2-stimulated aequorin-mediated emission of luminescence by MF266-1 in HEK293 cells co-expressing the EP1 receptor and aequorin, as demonstrated by the rightward shift of the dose-response curve in the presence of MF266-1 (500 nM), resulting in an increase of the EC_{50} from 7.1 (no antagonist, solid squares) to 281 nM (solid triangles) (n = 3, duplicate points; $p<0.01$ comparing vehicle to MF266-1). (C). Dose-dependent inhibition of sulprostone-induced cAMP accumulation in human erythroleukemia cells (HEL92.1.7) by MF266-3 as demonstrated by the rightward shift of the sulprostone-induced luminescence dose-response curves at increasing doses of MF266-3, with an EC_{50} of 1.07, 3.41, 7.59 and 23.7 nM, respectively at 0 (no antagonist, solid squares), 10 (solid triangles), 30 (inverted solid triangles) and 100 nM (solid diamonds) of the antagonist (n = 1 in triplicate). (D). Dose-dependent inhibition of PGE_2-induced cAMP accumulation by MF498 in HEK293 cells stably expressing the human EP4 receptor in 0 (solid squares; $IC_{50} = 1.7 \pm 0.9$ nM) and 10% (solid triangles; $IC_{50} = 17.4 \pm 11.9$ nM) serum (n = 3, single points; $p<0.01$ comparing presence and absence of serum).

Fig. 2. Inhibition of paw swelling by MF498 but not by MF266-1 or MF266-3 in rats with AIA. (A). Inhibitory effects of MF498 and MF-tricyclic in the primary paw. **, $p<0.01$ vs. vehicle (0 mg/kg/day). (B). Inhibitory effects of the same compounds in the

secondary paw. **, $p<0.01$ vs. vehicle (0 mg/kg/day); n= at least 8 animals per treatment. (C). Dose-response curves for the primary paw for MF498 (squares), MF-tricyclic (triangles), MF266-1 (diamonds), and MF266-3 (round dots). n= at least 6 animals per treatment.

Fig. 3. Analgesic effects of MF498 on EP4 agonist-induced thermal hyperalgesia and MIA-induced OA-like joint pain. (A). Dose-dependent inhibition of thermal hyperalgesia in guinea pigs induced by the selective EP4 agonist L-902688. (B). Time course and dose response relationship of MIA-induced incapacitance in the guinea pig. (C). Reversal of MIA-induced incapacitance by MF-tricyclic and diclofenac. (D). Analgesic effects of MF498, MF63 and diclofenac on MIA-induced incapacitance. **, $p<0.01$ vs. vehicle (0 mg/kg/day) (A, C and D) or baseline (B); n= at least 5 animals per treatment.

Fig. 4. Effect of MF498 on furosemide-induced diuresis. (A). Fold of increase in the expression of COX-2, mPGES-1 and prostanoid receptor mRNA in the kidneys of rats infused with furosemide for 7 days over those infused with PEG300. *, $p<0.05$ (t-test); n= at least 5 animals per treatment. (B). Inhibition of furosemide-induced diuresis by MF498 and MF-tricyclic. Shown on the Y axis is the average urine volume in milliliter (ml) over 24 h normalized over body weight in grams (ml/g/24hr). (C). Inhibition of furosemide-induced natriuresis by MF498 and MF-tricyclic. *, $p<0.05$ vs. vehicle (0 mg/kg/day); n = 8 animals per treatment (B and C).

Fig. 5. Lack of gastrointestinal toxic effects by MF498 in rats. (A). Erosions were noted in the stomach from animals treated with indomethacin (10 mg/kg) (middle) but not vehicle (left) or MF498 (30 mg/kg) (right). (B). Lack of effect by MK498 and MF-tricyclic on gastrointestinal mucosal leakage of ^{51}Cr-EDTA. Urinary excretion of ^{51}Cr-

EDTA within a 24 h period was determined by measuring radioactivity in urine samples collected after the last dose of test compounds. A significant increase in urinary ^{51}Cr-EDTA radioactivity was noted with indomethacin but not with MF498. **, $p<0.05$ vs. vehicle (0 mg/kg/day); n = 5 animals per treatment.

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Table 1. Binding affinity (Ki) of the antagonists for recombinant human prostanoid receptors (nM)

	EP1	EP2	EP3	EP4	DP	TP	FP	IP
MF266-1	**3.8** **	5165	1373	3837	>13900	226	20465	11965
MF266-3	1182	>96900	**0.8** **	5987	1182	834	25190	4644
MF498	>9500	2100	3900	**0.7** **	1400	580	1600	>6800

**, Significantly different from other prostanoid receptors (p<0.001)

Fig 1

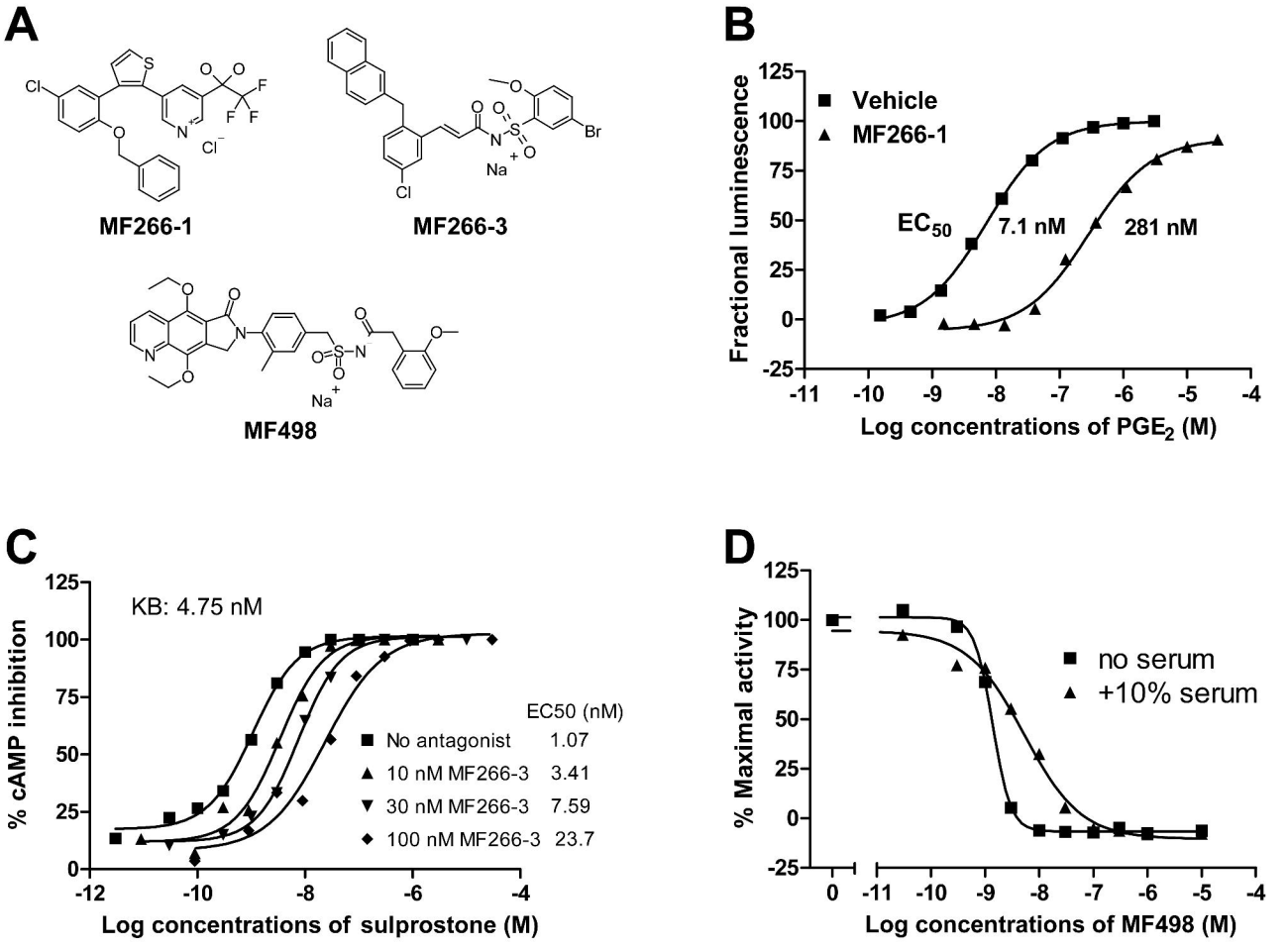

A
MF266-1
MF266-3
MF498
B
Vehicle
MF266-1
EC50
7.1 nM
281 nM
Fractional luminescence
Log concentrations of PGE2 (M)
C
KB: 4.75 nM
% cAMP inhibition
EC50 (nM)
No antagonist 1.07
10 nM MF266-3 3.41
30 nM MF266-3 7.59
100 nM MF266-3 23.7
Log concentrations of sulprostone (M)
D
% Maximal activity
no serum
+10% serum
Log concentrations of MF498 (M)

Fig 2

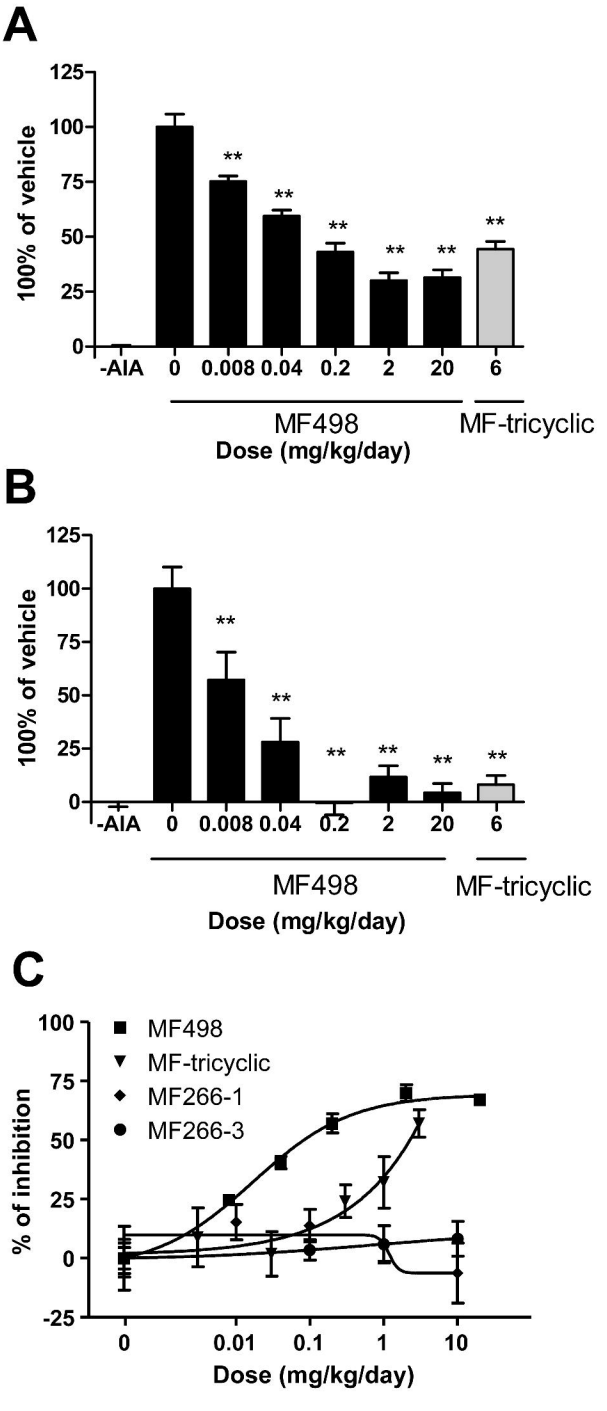

A
125
100
75
50
25
0
100% of vehicle
**
-AIA
0
0.008
0.04
0.2
2
20
6
MF498
MF-tricyclic
Dose (mg/kg/day)
B
125
100
75
50
25
0
100% of vehicle
**
-AIA
0
0.008
0.04
0.2
2
20
6
MF498
MF-tricyclic
Dose (mg/kg/day)
C
100
75
50
25
0
-25
% of inhibition
MF498
MF-tricyclic
MF266-1
MF266-3
0
0.01
0.1
1
10
Dose (mg/kg/day)

Fig 3

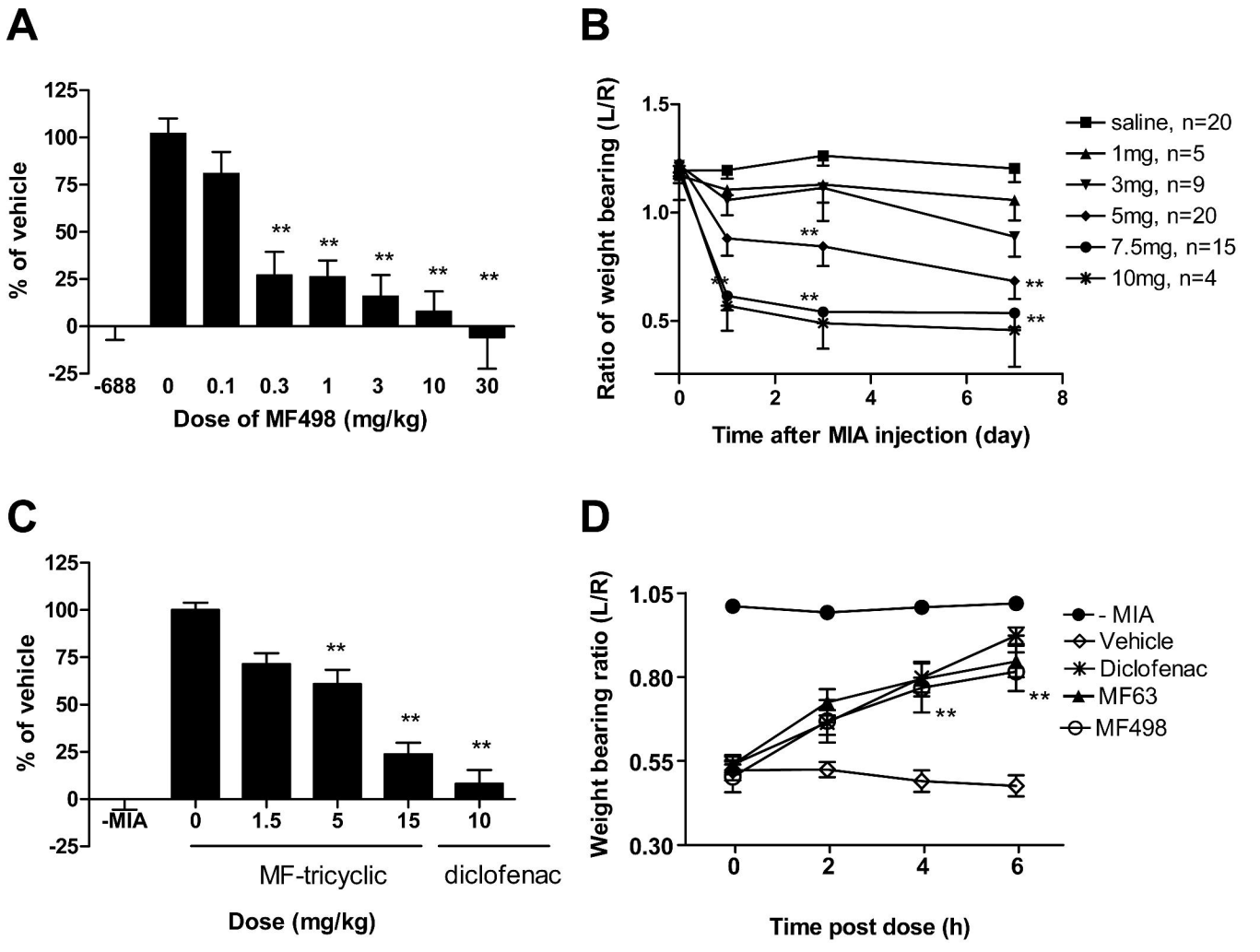

A
% of vehicle
125
100
75
50
25
0
-25
**
-688
0
0.1
0.3
1
3
10
30
Dose of MF498 (mg/kg)
B
Ratio of weight bearing (L/R)
1.5
1.0
0.5
0
2
4
6
8
Time after MIA injection (day)
saline, n=20
1mg, n=5
3mg, n=9
5mg, n=20
7.5mg, n=15
10mg, n=4
C
% of vehicle
-MIA
MF-tricyclic
diclofenac
Dose (mg/kg)
D
Weight bearing ratio (L/R)
1.05
0.80
0.55
0.30
Time post dose (h)
- MIA
Vehicle
Diclofenac
MF63
MF498

Fig 4

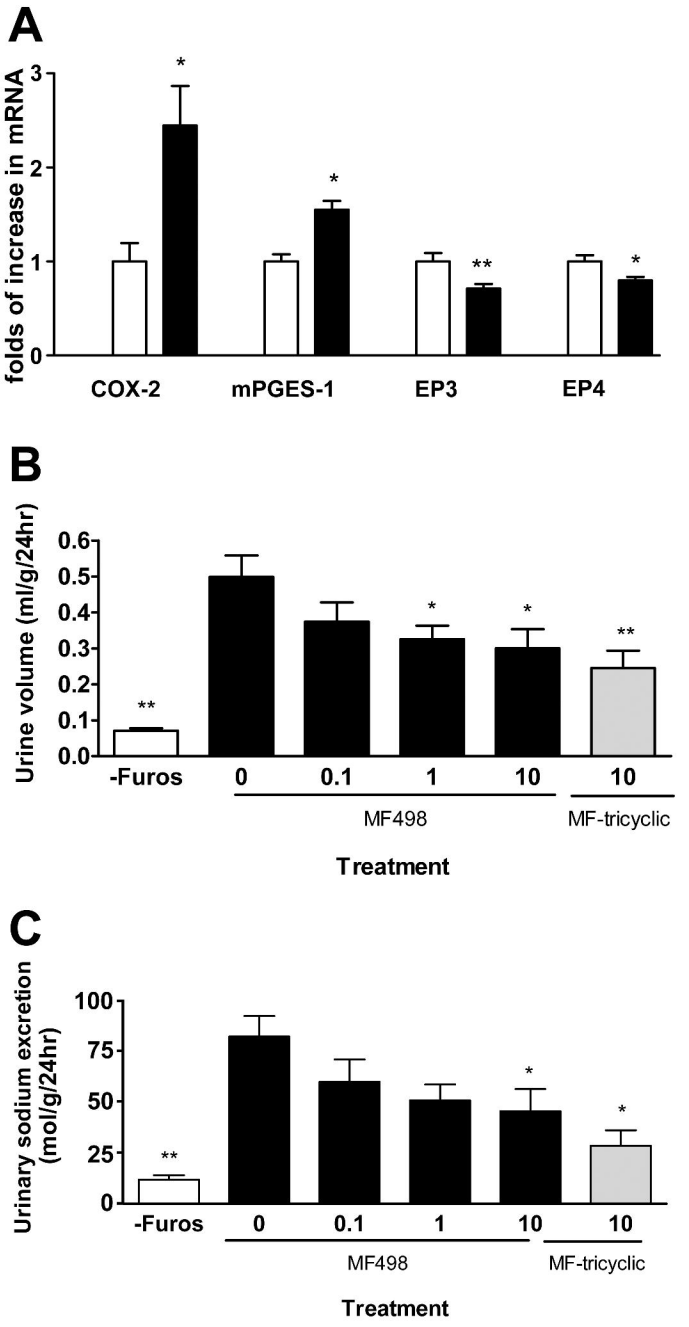
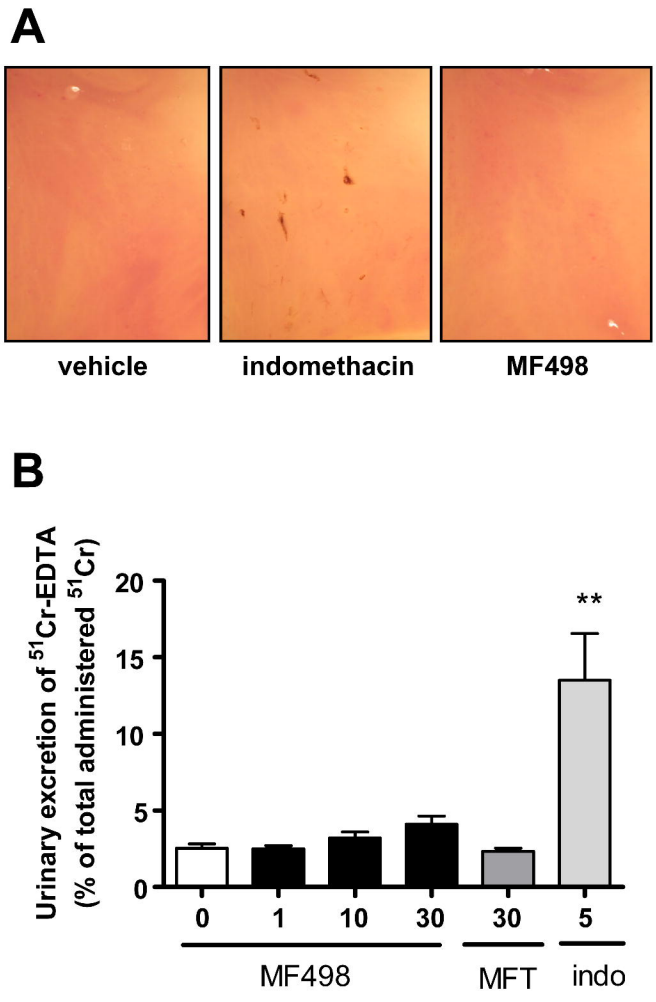

A
folds of increase in mRNA
0
1
2
3
*
*
**
*
COX-2
mPGES-1
EP3
EP4
B
Urine volume (ml/g/24hr)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
**
*
*
**
-Furos
0
0.1
1
10
10
MF498
MF-tricyclic
Treatment
C
Urinary sodium excretion (mol/g/24hr)
0
25
50
75
100
**
*
*
-Furos
0
0.1
1
10
10
MF498
MF-tricyclic
Treatment

Fig 5


A
vehicle
indomethacin
MF498
B
Urinary excretion of 51Cr-EDTA
(% of total administered 51Cr)
20
15
10
5
0
**
0
1
10
30
30
5
MF498
MFT
indo