MF498 [N-[(4-(5,9-Diethoxy-6-oxo-6,8-dihydro-7H-pyrrolo[3,4-g]quinolin-7-yl)-3-methylbenzyl)sulfonyl] -2-[(2-methoxyphenyl)acetamido], a Selective E Prostanoid Receptor 4 Antagonist, Relieves Joint Inflammation and Pain in Rodent Models of Rheumatoid and Osteoarthritis

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Received December 17, 2007; accepted February 19, 2008

ABSTRACT

Previous evidence has implicated E prostanoid receptor 4 (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 in mice lacking EP4, but not EP1-EP3, and the other showing no impact of EP4 antagonism on arthritis induced by collagens. Here, we assessed the effect of a novel and selective EP4 antagonist MF498 [N-[(4-(5,9-diethoxy-6-oxo-6,8-dihydro-7H-pyrrolo[3,4-g]quinolin-7-yl)-3-methylbenzyl)sulfonyl] -2-[(2-methoxyphenyl)acetamido] on inflammation in adjuvant-induced arthritis (AA), a rat model for rheumatoid arthritis (RA), and joint pain in a guinea pig model of iodoacetate-induced osteoarthritis (OA). In the AA model, MF498, but not the antagonist for EP1, MF266-1 [1-(5-{3-[2-(benzyloxy)iodoacetate-induced osteoarthritis (OA). In the AIA model, MF498, not EP1-EP3, and the other showing no impact of EP4 antagonism on arthritis induced by collagen. Here, we assessed the effect of a novel and selective EP4 antagonist MF498 [N-[(4-(5,9-diethoxy-6-oxo-6,8-dihydro-7H-pyrrolo[3,4-g]quinolin-7-yl)-3-methylbenzyl)sulfonyl] -2-[(2-methoxyphenyl)acetamido] on inflammation in adjuvant-induced arthritis (AA), a rat model for rheumatoid arthritis (RA), and joint pain in a guinea pig model of iodoacetate-induced osteoarthritis (OA). In the AA model, MF498, but not the antagonist for EP1, MF266-1 [1-(5-{3-[2-(benzyloxy)-5-chlorophenyl]-2-thiophenyl}[pyridin-3-yl)-2,2,2-trifluoroethane-1,1-diol] or EP3 MF266-3 [(2E)-N-[5-bromo-2-methoxyphenyl)sulfonyl]-3-[5-chloro-2-(2-naphthylmethyl)phenyl]acrylamide], inhibited inflammation, with a similar efficacy as a selective cyclooxygenase 2 (COX-2) inhibitor MF-tricyclic. In addition, MF498 was as effective as an nonsteroidal anti-inflammatory drug, diclofenac, or a selective microsomal prostaglandin E synthase-1 inhibitor, MF63 [2-(6-chloro-1H-phenanthro[9,10-d]imidazo[2,3]-yl)isoasophalnitrile], 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 assessed the renal effect of MF498 in a furosemide-induced diuresis model and demonstrated that the compound displayed a similar renal effect as MF-tricyclic [3-(3,4-difluorophenyl)-4-(4-(methylsulfonyl)phenyl)-2-(SH)-furanone], 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.

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 provide effective symptom relief for both OA and RA (Hochberg, 2005). Among the five major prostaglandins, namely, PGE₂, PGH₂, PGD₂, PGF₂α, and thromboxane, PGE₂ is the most abundant found in the synovial cavity of RA patients (Seppälä et al., 1985; Bertin et al., 1994), with levels that are significantly reduced by analgesic doses of NSAIDs (Seppälä et al., 1985; Bertin et al., 1994). In contrast, at least in RA, the concentrations of PGE₁, the other putative proinflammatory prostanoid, are not significantly altered by NSAIDs (Seppälä et al., 1985; Bertin et al., 1994). These observations suggest that PGE₂ may be the predominant prostaglandin in arthritis. In support of this view, PGE₂ has been shown to play a pivotal role in the development of joint inflammation and pain in animal models of arthritis. For instance, neutralizing PGE₂ with a specific antibody prevents the development of arthritis as effectively as NSAIDs in the AIA model (Portanova et al., 1996).

COX-2 inhibitor MF-tricyclic in the model. Furthermore, the EP₄ antagonist was used as a surrogate for the EP₄ knockout, demonstrating that none of the EP receptors has significant antagonist activity. The first study shows that the deletion of EP₄ but not EP₁, 2, or 3 protects mice from arthritis induced by a collagen antibody (HEK293-EbNA) expressing the human prostaglandin receptor as described previously (Abramovitz et al., 2000). In brief, prostaglandin 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 MnCl₂ (EP₁), 10 mM MgCl₂ (EP₂, EP₃, EP₄, and TP), and 2.5 (IP) or 10 mM MnCl₂ (DP and FP) and radioligand [0.5 to 1.25 nM [³H]PGE₂ (181 Ci/mmol)] for EP subtypes, 0.7 nM [³H]PGD₂ (200 Ci/mmol) for DP, 0.95 nM [³H]PGF₁α (170 Ci/mmol) for FP, 5 nM [³H]iloprost (16 Ci/mmol) for IP, and 1.8 nM [³H]HSQ-29548 (46 Ci/mmol) for TP. The reaction was initiated by the 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, and 5 µg for TP) from the 160,000 g fraction. Ligands were added in dimethyl sulfoxide (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, DP, TP, and IP) or 120 min (EP₄) or 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 Industries, Meriden, CT) prewetted in assay incubation 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 Industries) using a 1450 MicroBeta Wallac scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). 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 Industries) using a 1450 MicroBeta Wallac scintillation counter (PerkinElmer Life and Analytical Sciences). 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.

Materials and Methods

Radioligand Binding Assays

Radioligand binding assays were carried out on membranes prepared from human embryonic kidney 293-Epstein-Barr virus-encoded nuclear antigen cell line (HEK293-EBNA) expressing the human prostaglandin receptor as described previously (Abramovitz et al., 2000). For this purpose, we determined the role of different EP receptors, especially that of EP₄, in the development of joint inflammation and pain in animal models of arthritis. For instance, neutralizing PGE₂ 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 prostaglandin E synthase-1 (mPGES-1), a terminal PGE₂ synthase downstream of COX-2, protects mice from collagen-induced arthritis similarly to COX-2 knockout mice (Myers et al., 2000; Trebino et al., 2003). More recently, we have demonstrated that inhibition of mPGES-1-dependent PGE₂ relieves joint pain in a guinea pig model of OA induced by iodoacetate (MIA) (D. Xu, S. Rowland, P. Clark, A. Giroux, B. Côte, S. Guiral, M. Salem, Y. Ducharme, R. Friesen, N. Métot, et al., manuscript in preparation). It is noteworthy that 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).

PGE₂ acts through four different EP receptors, namely EP₁–₄, to produce its biological actions. It is not clear which of these receptors mediates the proinflammatory effect of PGE₂. 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. However, this effort 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 (McCoy et al., 2002). In contrast, the second study, in which the EP₁, 2, and 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 has significant roles in murine arthritis induced by collagen-induced arthritis (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, but not that for EP₁ (MP266-1) or EP₃ (MP266-3), 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.

Cell-Based Functional Assays

**PGE₂ 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 coexpressing hEP₁ receptor and aequorin were first incubated with coelenterazine, an aequorin cofactor, for 1 h. Next, the cells were plated in a 96-well plate (50,000 cells/well) containing PGE₂ with or without the antagonist. Upon stimulation of the hEP₁ receptor by PGE₂, the cells release intracellular calcium that 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 nonreacted aequorin (peak 2). Fractional luminescence, an index of EP₁ activity, was calculated using the formula: area under peak or AUP 1/AUP 1 + AUP 2. Fractional luminescence was used to calculate EC₅₀ of PGE₂ stimulated aequorin or EP₁ activity by employing a modified version of the Levendez-Marquardt four-paramater curve-fitting algorithm (LDAM software, Merck Frosst Informatics Group, Kirkland, QC, Canada). The EC₅₀ was calculated by using Schid plot.
EP3 Agonist Sulprostone-Induced cAMP Accumulation Assay for Measuring EP3 Antagonist Activity. The assay was performed using human erythrocyte leukemia cells (ATCC HEL 92.1.7) (2 × 10⁶ cells/well) that endogenously express the EP3 receptor in Hanks’ balanced salt solution 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), and 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 scintillation proximity assay using the RPA556 kit from GE Healthcare Bio-Sciences (Piscataway, NJ).

PGE₂-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 centrifugation at 3000g for 6 min. The cells were washed with phosphate-buffered saline, centrifuged as above, resuspended at a final concentration of 2.5 × 10⁶ cells/ml in 25 mM HEPES, pH 7.4. Hanks’ balanced salt solution containing isobutylmethylxanthine at 0.5 mM. Cells (2.5 × 10⁶) were preincubated (10 min, 37°C) with 0 to 1 × 10⁻⁵ M MF498 in DMSO (1% final concentration) and were subsequently challenged with 0.3 nM PGE₂ in the absence or presence of 10% human serum at 37°C 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 1400g for 10 min. The supernatant was collected, and the cAMP generated was assayed using a cAMP scintillation proximity assay kit (RPA556; GE Healthcare Bio-Sciences) 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 of the test compounds, with the exception of diclofenac (Sigma-Aldrich, Oakville, ON, Canada), were synthesized by the Medicinal Chemistry Department at Merck Frosst Research Laboratories (Kirkland, QC, Canada). Experiments involving the use of ⁵¹Cr-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 (CFA), namely 0.6 mg of Mycobacterium butyricum (Difco, Detroit, MI) in 50 µl of mineral oil, was injected subplantarily in the left hind paw of rats (n ≥ 7). A group of animals (n = 3) was injected subplantarily with saline and used as nonarthritic (nonAIA) controls. Volume of both the injected (primary) and noninjected (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 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. Percentage inhibition was calculated by comparing Δpaw volume among different groups using the formula: % inhibition = [100 – (drug treatment – nonAIA)/(vehicle control – nonAIA)] × 100.

The ED₅₀, which is the 50% inflection point of the dose-response curve, was calculated using Prism 4.0 (GraphPad Software Inc., San Diego, CA). The ED₅₀ corresponds to ID₅₀ (dose for 30% inhibition of paw swelling) and ID₅₀, in the primary and secondary paws, respectively.

EP4 Agonist-Induced Hyperalgesia in Guinea Pigs

Male Hartley guinea pigs weighing 175 to 200 g received an intraplantar injection (left hindpaw) of a selective EP4 agonist L-902868 (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 agonist (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 aesthesiometer (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 to 200 g received an intra-articular injection (into the shoulder joint) of 50 µl of MIA (1–10 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 incapacitation 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 naive animals. Animals with a L/R ratio of ≥0.7 were considered nonresponders and were removed from the study. Animals were orally dosed with either vehicle or a test compound at the indicated doses, and incapacitation measurements were taken 6 h later. To perform the measurements in a double-blinded fashion, the animals were randomized and assigned new codes. The percentage of incapacitation was calculated based on the formula (ΔL/R-compound – ΔL/R-MIA)/(ΔL/R-compound – ΔL/R-MIA) × 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 to 175 g. Animals were subcutaneously implanted with mini-osmotic pumps (model 2ML1; ALZET, Cupertino, CA) filled with furosemide (Sigma-Aldrich) 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. In brief, the mRNA from individual samples was isolated using the mRNA Express Kit from RNeasy according...
to the manufacturer’s instructions. The reverse transcription reaction was performed immediately after removal of the wash buffer using the TaqMan reverse transcription reagent kit (Applied Biosystems, Foster City, CA). The synthesized cDNAs were analyzed by PCR amplification using the TaqMan PCR master mix (Applied Biosystems), and the appropriate predeveloped mixture of primers and probes was 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 quantitative polymerase chain reaction was performed in User Bulletin 2 of ABI Prism 7700 Sequence Detection System (Applied Biosystems). The results are expressed as fold of increase over the vehicle control.

Acute Mucosal Erosions in Rats

Rats weighing 250 to 300 g were fasted overnight before oral administration of either vehicle (0.5% methylcellulose) or test compounds. Animals were sacrificed 3 h after 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 10x lens.

Urinary Excretion of $^{51}$Cr-EDTA as a Measure of Gastrointestinal Integrity in Rats

Rats weighing 250 to 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 plus Dunnett’s post-tests. Two-factor comparison was made using two-way analysis on variance plus Bonferroni’s post-tests. Data are shown as mean ± S.E.M.

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 have been described previously (Juteau et al., 2001; Ducharme et al., 2005; Burch et al., 2008). 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 $K_i$ of 3.8, 0.8, and 0.7 nM, and a high degree of selectivity over other EP receptors ($K_i > 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...
and increases in emission of luminescence with an EC50 of 7.1 nM. Addition of luminescence, PGE2 caused a dose-dependent increase in the measured by quantifying calcium sensitive aequorin-dependent assay, in which EP1-mediated calcium release was indirectly inhibited PGE2-stimulated cAMP accumulation in a dose-de-
centrations of 5.5, 2.6, and 1.2 nM, 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 CFA caused swelling in the injected or primary paw shortly (0.2 ml) of the antagonists for recombinant human prostanoid receptors

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<td>MF266-1</td>
<td>3.8**</td>
<td>5165</td>
<td>1373</td>
<td>3837</td>
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<td>MF266-3</td>
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<td>0.8**</td>
<td>5987</td>
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<td>834</td>
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<td>MF498</td>
<td>&gt;9500</td>
<td>2100</td>
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<td>580</td>
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** Significantly different from other prostanoid receptors (P < 0.001).

To better assess analgesia for joint pain, we used a guinea pig model of mono-osteoarthritis 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 (≥4000-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 s. 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). We next 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 noninjected or right forelimb (L/R). The baseline L/R ratio in naive 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 days 3 and 7 at the doses of 0.38 and 0.5 mg/joint and was significantly lower than that of the

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 use of weight bearing because of polyarthritus. To better assess analgesia for joint pain, we used a guinea pig model of mono-osteoarthritis 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 (≥4000-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 s. 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). We next 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 noninjected or right forelimb (L/R). The baseline L/R ratio in naive 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 days 3 and 7 at the doses of 0.38 and 0.5 mg/joint and was significantly lower than that of the
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/day 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% PEG2000 (Fig. 4, B 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, 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. 4, B 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 stomachs of all five rats treated with indomethacin 3 h after 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 51Cr-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 51Cr-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 51Cr-EDTA 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 K_i values in the nanomolar range and highly selective over other prostanoid receptors. All three com-
pounds 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 versus 13 nM) and better bioavailability than CJ-023,423 (100 versus 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, 1999), suggesting that, as in RA, proinflammatory prosta
glandins are primarily derived from COX-2 in this model. PGE2 is the predominant prostaglandin 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 PGE2 synthesis, also protects against the development of autoimmune arthritis (Myers et al., 2000; Trebino et al., 2003). In support of PGE2 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 PGE2 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. However, 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 PGE2 and EP4 are essential for the development of autoimmune arthri-

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 versus vehicle (0 mg/kg/day) (A, C, and D) or baseline (B); n = at least 5 animals per treatment.
tis in both mice and rats. Thus, COX-2, mPGES-1, PGE₂, and EP4 may constitute a proinflammatory prostanoid axis in autoimmune 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 PGE₂, mPGES-1, and EP4. For instance, PGE₂ is a major prostaglandin in RA patients and is sensitive to NSAID treatment (Seppälä et al., 1985; Bertin et al., 1994). COX-2, mPGES-1, and EP4 are expressed in the inflamed synovial tissues (Yoshida et al., 2001; Westman et al., 2004; Korotkovka et al., 2005) or in human synoviocytes stimulated with interleukin-1β, a major cytokine in RA (Yoshida et al., 2001). It is noteworthy that the expression profiles of COX-2, mPGES-1, and EP4 in human tissues or cells are similar to those in rat AIA (Kurihara et al., 2001; Claveau et al., 2003). 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 proinflammatory role, EP4 has been shown to be important in mediating the action of PGE₂ in inflammatory pain because antagonism of EP4 results in an analgesic effect on pain associated with cutaneous inflammation in the paw induced by PGE₂, 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 PGE₂ activities in the gastrointestinal tract, such as promoting the duodenal secretion of HCO₃⁻ (Aoi et al., 2004; Larsen et al., 2005) and gastric...
mucous 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 blood flow, 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 represent a useful model for assessing the impact of PGE2 on sodium and fluid retention because of the increased renal dependence on COX-2-derived PGE2 (Nüsing et al., 2005). EP receptors, notably EP4, have been shown to be important for furosemide-induced natriuresis in mice (Nüsing et al., 2005). In agreement with this finding, we have demonstrated that antagonism of the receptor by MF498 results in significant antinatriuretic and antidiuretic effects similarly to the COX-2 inhibitor MF-tricyclic. Together, these findings indicate that EP4 mediates most of the natriuretic action of PGE2, 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 antinatriuretic 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 PGE2 in both processes. Although PGI2 has also been implicated in inflammation and pain, indicating that EP4 mediates the action of PGE2, which may be derived from COX-2 and mPGES-1, both of which are induced by furosemide. 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 antinatriuretic effect as a COX-2 inhibitor in the kidney. Unlike COX-2 inhibitors, EP4 antagonists do not suppress PGL2, which possesses potent vasodilatory and antithrombotic 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.

Acknowledgments

We thank Bernard Côté and Michel Gallant for the ligand synthesis of MF266-1 and MF266-3, as well as our colleagues from the Department of Comparative Medicine for technical assistance with the in vivo studies.

References


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