Simultaneous Inhibition of PGE$_2$ and PGI$_2$ Signals Is Necessary to Suppress Hyperalgesia in Rat Inflammatory Pain Models

Ryusuke Sugita, 1 Harumi Kuwabara, 2 Kazufumi Kubota, 3 Kotaro Sugimoto, 2 Toshihiro Kiho, 4 Atsushi Tengeiji, 5 Katsuhiro Kawakami, 6 and Kohei Shimada 2

1 Cardiovascular-Metabolics Research Laboratories, Daiichi Sankyo Co., Ltd., Tokyo 140-8710, Japan
2 Frontier Research Laboratories, Daiichi Sankyo Co., Ltd., Tokyo 140-8710, Japan
3 Biological Research Laboratories, Daiichi Sankyo Co., Ltd., Tokyo 140-8710, Japan
4 Medicinal Chemistry Research Laboratories, Daiichi Sankyo Co., Ltd., Tokyo 140-8710, Japan
5 Venture Science Laboratories, Daiichi Sankyo Co., Ltd., Tokyo 140-8710, Japan
6 Global Project Management Department, Daiichi Sankyo Co., Ltd., Tokyo 140-8710, Japan

Correspondence should be addressed to Kohei Shimada; shimada.kohei.ma@daichisankyo.co.jp

Received 8 March 2016; Revised 30 May 2016; Accepted 5 June 2016

Academic Editor: Marc Pouliot

Prostaglandin E$_2$ (PGE$_2$) is well known as a mediator of inflammatory symptoms such as fever, arthritis, and inflammatory pain. In the present study, we evaluated the analgesic effect of our selective PGE$_2$ synthesis inhibitor, compound I, 2-methyl-2-[(cis-4-[(1-(6-methyl-3-phenylquinolin-2-yl)piperidin-4-yl]carbonyl amino)cyclohexyl] propanoic acid, in rat yeast-induced acute and adjuvant-induced chronic inflammatory pain models. Although this compound suppressed the synthesis of PGE$_2$ selectively, no analgesic effect was shown in both inflammatory pain models. Prostacyclin (PGI$_2$) also plays crucial roles in inflammatory pain, so we evaluated the involvement of PGI$_2$ signaling in rat inflammatory pain models using prostacyclin receptor (IP) antagonist, RO3244019. RO3244019 showed no analgesic effect in inflammatory pain models, but concomitant administration of compound I and RO3244019 showed analgesic effects comparable to celecoxib, a specific cyclooxygenase (COX-) 2 inhibitor. Furthermore, coadministration of PGE$_2$ receptor 4 (EP4) antagonist, CJ-023423, and RO3244019 also showed an analgesic effect. These findings suggest that both PGE$_2$ signaling, especially through the EP4 receptor, and PGI$_2$ signaling play critical roles in inflammatory pain and concurrent inhibition of both signals is important for suppression of inflammatory hyperalgesia.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs), including aspirin, indomethacin, diclofenac, and ibuprofen, are widely used in the treatment of inflammatory symptoms such as fever, arthritis, and inflammatory pain. These drugs reduce the production of prostaglandins by blocking both COX-1 and COX-2. COX-1 is constitutively expressed in various tissues and plays a role in homeostatic functions such as gastric epithelial cytoprotection and renal blood flow maintenance. Thus, traditional NSAIDs cause side effects to the gastrointestinal (GI) tract and renal function [1]. As a result, COX-2 selective inhibitors such as celecoxib, rofecoxib, and valdecoxib were developed. Although COX-2 inhibitors show an analgesic effect and fewer adverse effects on the GI tract, a cardiovascular hazard in patients with long-term use of these compounds has been reported. Eventually, all such compounds except for celecoxib were withdrawn from the market [2, 3].

Prostaglandins are synthesized through conversion of arachidonic acid to an unstable intermediate, prostaglandin H$_2$ (PGH$_2$), by COX-1 and COX-2. PGH$_2$ is further catalyzed to PGE$_2$, prostacyclin (PGI$_2$), prostaglandin F$_2\alpha$, and thromboxane A$_2$ (TXA$_2$) by PGE synthase (PGES), PGI synthase, prostaglandin F synthase, and thromboxane A synthase, respectively. Traditional NSAIDs suppress all prostanoids’ synthesis by inhibiting both COXs. On the other hand, COX-2 inhibitors selectively suppress COX-2-derived prostanoids which are mainly induced by inflammatory...
stimuli. Among all prostanoids, PGE\(_2\) is the most common prostanoid produced by a variety of cells and tissues and plays a pivotal role in inflammatory responses. There are three different PGES, cytosolic PGES (cPGES) and two microsomal PGES (mPGES-1 and mPGES-2). cPGES and mPGES-2 are constitutive enzymes, whereas mPGES-1 is induced by inflammatory stimuli [4–6]. Furthermore, many reports suggest that mPGES-1 plays an important role in inflammatory symptoms [7–9]; thus mPGES-1 is thought to be a therapeutic target alternative to traditional NSAIDs and COX-2 inhibitors. Presently, some mPGES-1 inhibitors have been discovered and the pharmacological profiles have been reported [10–12], suggesting the therapeutic potential of pharmacological inhibition of this enzyme for relieving inflammatory responses. On the other hand, PGI\(_2\) also plays an important role in inflammation and inflammatory pain. IP receptor-deficient mice showed a reduced writhing response in an acetic acid-induced acute inflammatory pain model [13, 14]. In addition, IP antagonists also showed anti-inflammatory and analgesic effects in rodent arthritis models [15, 16]. Thus, the IP receptor is thought to be another interesting target for anti-inflammatory and analgesic drug development. At this time, it remains inconclusive which action is a more promising target for an analgesic drug.

Recently, we have discovered quinoline derivatives which are orally available and have a potent inhibitory effect on PGE\(_2\) synthesis in rats. We have revealed that these compounds are highly selective for PGE\(_2\) synthesis inhibition [17, 18], and we have previously reported the antipyretic and anti-inflammatory profile of compound A in a rat LPS-induced pyrexia and adjuvant-induced arthritis model [17]. In the present study, we evaluated analgesic effects of another quinoline derivative, compound I, in acute and chronic inflammatory pain models. In addition, we investigated the function of PGE\(_2\) and PGI\(_2\) signaling in these models using our compound and an IP antagonist. Among all PGE\(_2\) receptors, EP4 plays an important role in inflammatory pain [19]; thus an EP4 antagonist was used in the present pain model.

2. Material and Methods

2.1. Animals. All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd. For the adjuvant-induced inflammatory pain model, male Lewis rats were purchased at the age of 5 weeks to 6 weeks from Charles River Japan Inc. (Kanagawa, Japan). For the yeast-induced inflammatory pain model, male Wistar-Imamichi rats were purchased at the age of 4 weeks to 5 weeks from the Institute for Animal Reproduction (Ibaraki, Japan). For the rat macrophage assay, male Sprague-Dawley rats were purchased at the age of 8 weeks from Japan SLC Inc. (Shizuoka, Japan). The animals were housed in an air-conditioned room with controlled temperature (23 ± 2°C) and humidity (55 ± 20%) and a 12 h light/dark cycle (light from 8:00 to 20:00). They were fed and given water ad libitum throughout the experimental period unless otherwise noted. They were acclimated for about 1 week before use.

2.2. Test Compounds. Compound I (2-methyl-2-[cis-4-[(1-(6-methyl-3-phenylquinolin-2-yl)piperidin-4-yl]carbonyl amino)cyclohexyl] propanoic acid) was synthesized at Medicinal Chemistry Research Laboratories, Daiichi Sankyo Co., Ltd. Detailed information of its chemical synthesis is described in the patent [18]. The chemical structure is shown in Figure 1. IP antagonist, RO3244019, (R)-3-phenyl-2-(5-phenyl-benzofuran-2-ylmethoxy carbonyl amino) propionic acid, and EP4 antagonist, CJ-023423, N-[2-[4-(2-ethyl-4,6-dimethyl-1H-imidazo[4,5-c]pyridin-1-yl)phenyl]ethyl]carbamoyl]-4-methylbenzenesulfonamide, were synthesized at Medicinal Chemistry Research Laboratories, Daiichi Sankyo Co., Ltd. Celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)pyrazol-1-yl]benzenesulfonyl amide) was purchased from Cayman Chemical Company (Ann Arbor, MI). All compounds were suspended in 0.5% methylcellulose 400 solution and sterilized (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and a volume of 5 mL/kg was administered orally to animals.

2.3. Rat Macrophage Assay. Rat peritoneal macrophages were prepared as described previously [20]. Briefly, 5% (w/v) peptone (BD Biosciences, Franklin Lakes, NJ) and 5% (w/v) starch (BD Biosciences) solution was intraperitoneally injected into male Sprague-Dawley rats at a dose of 5 mL/100 g body weight. Four days later, the rats were euthanized by cutting the carotid artery under anesthesia, and the peritoneal macrophages were collected. Macrophages were seeded in a 96-well plate at a density of 200,000 cells/well and pretreated with 100 μM aspirin (Sigma-Aldrich) at 37°C for 2 h in RPMI 1640 (Life Technologies) supplemented with 10% FBS and penicillin-streptomycin liquid (Life Technologies). The cells were washed twice to remove nonadherent cells and then preincubated at 37°C for 1 h with a compound or vehicle in 200 μL of RPMI medium. The cells were treated with 10 μg/mL lipopolysaccharide (LPS, lot number 0111:B4, Sigma-Aldrich) at 37°C and 5% CO\(_2\) for 24 h, and PGE\(_2\), 6-keto PGF\(_{1α}\) (stable metabolite of PGI\(_2\)), PGF\(_{2α}\), and TXB\(_2\) (stable metabolite of TXA\(_2\)) in the medium were measured by EIA (Assay Designs). Each experiment was performed in duplicate. The results of LPS(−), LPS(+), and celecoxib (positive control) were the same as our published paper [17] because this experiment was performed in the same plate.
2.4. Adjuvant-Induced Chronic Inflammatory Pain Model. Experiments were performed according to the method previously described [21] with slight modifications. Briefly, an adjuvant was prepared by suspending heat-killed dried M. butyricum (lot number 7047934, BD Biosciences) in dry-sterilized liquid paraffin (Wako Pure Chemical Industries Ltd.) and then sonicating with a Sonifier Cell Disruptor 200 (Branson Ultrasonic, Danbury, CT). The adjuvant (100 μg M. butyricum/0.05 mL/paw) was injected intradermally into the heels of the right hind footpads of male Lewis rats. Rats were fasted overnight on Day 18, and the pain response of the animals was examined by gently flexing the tarsotibial joint of the uninjected foot 5 times at intervals of 2 s to 3 s on Day 19. Animals squeaking at every flexion were defined as pain-positive. The pain-positive rats were then randomly divided into groups and test compounds or the vehicle was orally administered (Figure 3(a)). The pain response was examined at 1, 2, 3, and 4 h after administration by flexing the uninjected foot 5 times in the same way, and the number of instances of squeaking was recorded as the pain score. The score of noninjected animals was 0 all the time in this experiment. After the final measurement, animals were euthanized and the left (uninjected) hind paws were immediately removed and weighed. The paws were homogenized with a Polytron homogenizer at 4°C and then sonicated with a Sonifier Cell Disruptor 200 (Branson Ultrasonic, Danbury, CT). The adjuvant (100 μg M. butyricum/0.05 mL/paw) was injected intradermally into the heels of the right hind footpads of male Lewis rats. Rats were fasted overnight on Day 18, and the pain response of the animals was examined by gently flexing the tarsotibial joint of the uninjected foot 5 times at intervals of 2 s to 3 s on Day 19. Animals squeaking at every flexion were defined as pain-positive. The pain-positive rats were then randomly divided into groups and test compounds or the vehicle was orally administered (Figure 3(a)). The pain response was examined at 1, 2, 3, and 4 h after administration by flexing the uninjected foot 5 times in the same way, and the number of instances of squeaking was recorded as the pain score. The score of noninjected animals was 0 all the time in this experiment. After the final measurement, animals were euthanized and the left (uninjected) hind paws were immediately removed and weighed. The paws were snap frozen with liquid nitrogen and stored at −80°C until use. The paws were then crushed and a Cryo-Press (Microtec Co., Ltd., Chiba, Japan) and homogenized with a Polytron homogenizer at 4°C for 60 s four times the volume of each paw of phosphate buffered saline (PBS) with 10 mM EDTA and 100 μM indomethacin. The homogenate was centrifuged at 1,560 ×g for 15 min at 4°C, and 1 mL of the supernatant fraction was further centrifuged at 9,500 ×g for 5 min at 4°C. The supernatant was then stored at −20°C until the measurement of prostanoid contents. Paws of noninjected animals were used as a control. Prostanoids were measured with respective EIA kit (Cayman Chemicals) according to the manufacturer’s instructions.

2.5. Yeast-Induced Acute Inflammatory Pain Model. Experiments were performed according to the method previously described with slight modification [22]. Briefly, male Wistar-Izmami rats weighing from 60 g to 80 g were fasted overnight and 0.1 mL of 20% suspension of dead Brewer’s yeast (Sigma) was injected intradermally into their right hind footpads. A hypernociceptive stimulation was produced by applying 250 g of pressure to the inflamed paw twice at 16 h and 18 h after yeast injection by a balance pressure device (Ugo-Basile, Italy). A constant increase in pressure was applied to the inflamed paw 18.5 h after the yeast injection and the pain threshold was determined by measuring the pressure in g at the time when the animal began to cry. Animals with a pain threshold between 60 g and 120 g were selected for assays and test compounds or vehicle was immediately administered to them orally (n = 6). Then the pain threshold at 1, 2, 3, and 4 h after dosing was measured. After the final measurement, the animals were euthanized and inflamed paws were collected. The supernatants of homogenized paw samples were prepared as described above. About 2 cm of each spinal cord including the lumbar segment was also harvested and supernatants were prepared as described above. Prostanoids were measured with a respective EIA kit (Cayman Chemical) according to the manufacturer’s instructions.

2.6. Statistical Analysis. In a macrophage assay, data are expressed as the mean ± SD and other data are expressed as the mean ± SEM. In vitro experiments, IC50 values were derived from four point titrations. In the inflamed tissue assay, the percent inhibition of prostanoid content by a compound was calculated by the following equation: 1 − [(prostanoid content of a compound treated animal) − (a prostanoid content of a normal group)] / [(prostanoid content of a vehicle-treated group) − (prostanoid content of a normal group)] × 100. ID50 values were calculated based on linear regression lines obtained from the percent inhibitions and the logarithmic values of the doses by the least squares method. The statistical analysis for the prostanoid content was performed by Dunnett’s test, otherwise by Steel’s test for multiple comparisons.

3. Results

3.1. Compound I Suppresses PGE2 Production Selectively in Rat Macrophages. To elucidate the inhibitory profile of our compound, production of prostaglandins was evaluated in a rat macrophage assay system. In this assay, LPS stimulation induced the production of PGE2, 6-keto PGF1α, PGF2α, and TXB2. Among them, the amount of PGE2 was the highest (Figure 2(a)). Compound I suppressed the production of PGE2 in a dose-dependent manner and its IC50 was 1.9 nM (Figure 2(b)). The production of 6-keto PGF1α and PGF2α was not suppressed (Figures 2(c) and 2(d)), whereas TXB2 production was accelerated (Figure 2(e)). Celecoxib suppressed all kinds of prostanoid synthesis (Figure 2(f)).

3.2. Compound I Selectively Reduces PGE2 Production but Shows No Analgesic Effect in Adjuvant-Induced Chronic Inflammatory Pain Models. In order to investigate the analgesic effect of compound I, an adjuvant-induced chronic inflammatory pain model was used. Chronic pain was well-established 19 days after adjuvant injection and the pain continued throughout the measurement. While celecoxib (10 mg/kg) reduced the pain score from 5 ± 0 to 2.1 ± 0.7 at 4 h after dosing, compound I showed little or no effect (Figure 3(a)). After the last measurement, paw tissues were harvested and the content of PGE2 and 6-keto PGF1α was measured. PGE2 production was increased from 0.3 ± 0.08 ng/paw (noninjected animal) to 9.3 ± 1.8 ng/paw by adjuvant injection, whereas production of 6-keto PGF1α was almost unchanged (from 9.5 ± 1.1 ng/paw to 10.0 ± 1.0 ng/paw). Compound I selectively reduced PGE2 production with an ID50 value of less than 1 mg/kg (Figure 3(b)), whereas celecoxib reduced both PGE2 and 6-keto PGF1α in inflamed tissue. Inhibition activity of PGE2 was almost the same level between 30 mg/kg of compound I and 10 mg/kg of celecoxib (Figure 3(b)).

3.3. Compound I Shows No Analgesic Effect in Yeast-Induced Acute Inflammatory Pain Models. A yeast-induced acute inflammatory pain model is frequently used for evaluation of
Figure 2: Induction of prostanoids synthesis and inhibitory profile of compound A in rat peritoneal macrophages. (a) The production of PGE$_2$, 6-keto PGF$_{1\alpha}$, PGF$_{2\alpha}$, and TXB$_2$ with or without LPS treatment. (b) to (e) Dose-dependent effects of compound A on the production of PGE$_2$ (b), 6-keto PGF$_{1\alpha}$ (c), PGF$_{2\alpha}$ (d), and TXB$_2$ (e) in rat macrophages. (f) Dose-dependent effects of celecoxib on the production of PGE$_2$, 6-keto PGF$_{1\alpha}$, PGF$_{2\alpha}$, and TXB$_2$ in rat macrophages. Results are shown as the mean ± SD (in duplicate). A representative result from reproduced experiments is shown.
analgesic effect of NSAIDs, so we used this model to assess the analgesic effect of our compound. After the injection of yeast, production of PGE$_2$ and 6-keto PGF$_{1\alpha}$ was increased in both the inflamed paw (from 1.1 ± 0.02 ng/paw to 30.9 ± 2.6 ng/paw and from 4.0 ± 0.4 ng/paw to 11.9 ± 2.0 ng/paw, resp.) and spinal cord (from 0.08 ± 0.02 ng/tissue to 0.37 ± 0.04 ng/tissue and from 0.2 ± 0.02 ng/tissue to 0.32 ± 0.04 ng/tissue, resp.). Compound I selectively reduced PGE$_2$ synthesis in a dose-dependent manner in both inflamed paw and spinal cord with an ID$_{50}$ value of 2.9 mg/kg and 4.2 mg/kg, respectively (Figures 4(a) and 4(b)). However, this compound showed no analgesic effect (Figure 4(c)). On the other hand, 5 mg/kg of celecoxib reduced both PGE$_2$ and 6-keto PGF$_{1\alpha}$ in inflamed paw and spinal cord (Figures 4(a) and 4(b)) and showed an analgesic effect 2 h and 3 h after administration (Figure 4(c)). Pain threshold of noninjected animal is about 300 g in this model (data not shown), and so celecoxib improved inflammatory hyperalgesia almost to normal level.

3.4. Concomitant Administration of Compound I and IP Antagonist Showed Analgesic Effect. To identify the reason why our compound did not relieve inflammatory pain, we investigated whether other prostanoids contribute to the present pain models. Since PGI$_2$ is also reported to play a role in inflammatory pain, we tested the analgesic effect of an IP antagonist RO3244019 in the inflammatory pain models. In the adjuvant-induced pain model, RO3244019 showed no analgesic effect. On the other hand, concomitant administration of compound I and RO3244019 showed an equivalent analgesic effect to celecoxib (Figure 5(a)). Furthermore, in the yeast-induced inflammatory pain model, concomitant administration of these two compounds improved the pain threshold at a level comparable to celecoxib, whereas RO3244019 or compound I alone showed no analgesic effect (Figure 5(b)).

3.5. Concomitant Administration of EP4 Antagonist and IP Antagonist Also Showed Analgesic Effect in Adjuvant-Induced Inflammatory Pain Model. Among PGE$_2$ receptors, EP4 is reported to play an important role in inflammatory pain [19]. Thus, we evaluated the effect of EP4 antagonist CJ-023423 in an adjuvant-induced inflammatory pain model. As shown in Figure 6, EP4 antagonist and IP antagonist showed little or no effect on the pain scores, whereas concomitant administration of these two compounds significantly improved them.

4. Discussion

PGE$_2$ plays a critical role in inflammatory symptoms such as fever, edema, or inflammatory pain. Traditional NSAIDs or COX-2 inhibitors are widely used to treat these symptoms. We previously reported that a novel selective PGE$_2$ synthesis inhibitor shows antipyretic and anti-inflammatory effects
Figure 4: Analgesic effect of compound I and celecoxib in yeast-induced acute inflammatory pain in rats. (a) Effect of compound I and celecoxib on inflamed paw prostanoids' production in yeast-induced acute inflammatory pain model. Inflamed paw was obtained after the last measurement of pain threshold. (b) Effect of compound I and celecoxib on lumbar area of spinal cord prostanoid production in yeast-induced acute inflammatory pain model. Spinal cord was obtained after the last measurement of pain threshold. (c) Time course of pain threshold. Male Wistar-Imamichi rats received a single, right hind-paw intradermal injection of dead Brewer's yeast (20%/0.1 mL/paw). After 18.5 h, compounds or 0.5% MC were administered orally to animals with established hyperalgesia (pain threshold: 60–120 g). Pain response was examined 1, 2, 3, and 4 h after administration. Results are shown as the mean ± SEM (n = 6/group). The statistical analysis was performed by Dunnett's test and Steel's test for prostanoids' content ((a) and (b)) and pain threshold (c), respectively. *p < 0.05; **p < 0.01 for compound treated versus 0.5% MC (0 mg/kg) treated animals.
In the present study, we evaluated the analgesic potential of our compound in inflammatory pain models. In a rat macrophage assay, compound I selectively and strongly inhibited LPS-induced PGE\textsubscript{2} production. On the other hand, this compound did not change the production of 6-keto PGF\textsubscript{1\alpha} and PGF\textsubscript{2\alpha} and enhanced the production of TXB\textsubscript{2}. This may be the result of the redirection of PGH\textsubscript{2} metabolism from PGE\textsubscript{2} to TXB\textsubscript{2}. Furthermore, mPGES-1 is critically involved in the synthesis of PGE\textsubscript{2} induced by LPS [23]. Taken together, compound I probably inhibits mPGES-1. This inhibitory profile was similar to that of another of our compounds as reported previously [17]. Other papers reported that mPGES-1 deficiency or a pharmacological inhibition of mPGES-1 also shows the prostanoids’ redirection [24–27]. However, the inhibitory profile against each prostanoid differs among them [28], suggesting that reactivity may differ depending on the expression profile of terminal synthases in target cells or tissues.

Previous reports advocate that the inhibition of PGE\textsubscript{2} synthesis is necessary to relieve the inflammatory hyperalgesia [7, 8, 10, 12]. Thus, we investigated whether our compound can relieve hyperalgesia in rat inflammatory pain models which were well-established in evaluating traditional NSAIDs and COX-2 inhibitors. In an adjuvant-induced chronic pain model, adjuvant injection induced the production of PGE\textsubscript{2}. Compound I suppressed the production of PGE\textsubscript{2}, whereas 6-keto PGF\textsubscript{1\alpha} synthesis was not significantly changed and rather tended to be increased at a high dose. The maximum dose (30 mg/kg) of compound I suppressed PGE\textsubscript{2} synthesis to almost the same degree as celecoxib. However, celecoxib, but not our compound, showed an analgesic effect in this model. Unlike compound I, celecoxib also suppressed 6-keto PGF\textsubscript{1\alpha} synthesis, suggesting that only the inhibition of PGE\textsubscript{2} is insufficient to relieve inflammatory hyperalgesia. In a yeast-induced acute inflammatory pain model, PGE\textsubscript{2} and 6-keto PGF\textsubscript{1\alpha} production in inflamed paw were increased. Because intraspinal PGE\textsubscript{2} has been shown to act on central sensitization in an acute arthritis model [29], we measured prostaglandins’ production in spinal cord tissue. In this model, PGE\textsubscript{2} and 6-keto PGF\textsubscript{1\alpha} content were increased in the spinal cord. Compound I suppressed PGE\textsubscript{2} production selectively in both inflamed paw and spinal cord, and the maximum dose of compound I suppressed PGE\textsubscript{2} production almost to the same degree as celecoxib. However, while celecoxib showed a strong analgesic effect, compound I showed no effect. Celecoxib suppressed both PGE\textsubscript{2} and 6-keto PGF\textsubscript{1\alpha} in both tissues, further suggesting the contribution of other prostanoid signals to inflammatory pain.
Among prostanoids, PGI₂ is also known as a mediator of inflammation and inflammatory pain. To assess the involvement of PGI₂ signaling in inflammatory pain models, the IP antagonist, RO3244019, was used as reported previously [30]. In the adjuvant-induced chronic inflammatory pain model, single administration of RO3244019 showed no analgesic effect. On the other hand, concomitant administration of compound I and RO3244019 improved the pain score as well as or more than celecoxib, and the same result was obtained in the yeast-induced acute inflammatory pain model. These results strongly suggest that both PGE₂ and PGI₂ signals are sufficient to induce inflammatory hyperalgesia and inhibition of only one prostaglandin signal is insufficient to relieve the pain. In the adjuvant-induced inflammatory pain model, the amount of 6-keto PGF₁α was not increased by adjuvant injection, indicating that normally existing PGI₂ also acts as a mediator of inflammatory pain. Although the exact mechanism remains to be clarified, the adjuvant injection may increase IP expression followed by accelerating IP signal in this model. Thus, PGI₂ signal may contribute to inflammatory pain only in inflammatory condition. Another study demonstrated that pain behavior is not reduced in mPGES-1 knockout mice in a zymosan-evoked hyperalgesia model, and it is suggested that other prostanoids may compensate the loss of PGE₂ synthase in mPGES-1-deficient mice [31]. In the present study, the same compensation may occur in PGE₂ selectively suppressed mice by compound I. On the other hand, not only PGE₂ but also other prostaglandins contribute to peripheral and central sensitization. For example, intrathecal injection of prostaglandin D₂ induced hyperalgesia [32]. Although the precise mechanism is still unknown, PGF₂α also induces allodynia [33]. Although our results suggest that the inhibition of PGE₂ and PGI₂ synthesis is enough to improve inflammatory hyperalgesia, further investigation for the involvement of other prostanoids in inflammatory pain may be needed.

We next assessed which of the EP receptors is important to inflammatory pain. Among all EP receptors, EP4 is predominantly induced in DRG neurons by adjuvant injection and mainly contributes to inflammatory pain hypersensitivity [19]. In addition, blockade of the EP4 receptor shows anti-inflammatory and analgesic effects in inflammation models [34, 35]. Thus, EP4 antagonist CJ-023423, which was reported to show high selectivity for the EP4 receptor and reverse inflammation and inflammatory pain [35, 36], was used to assess the contribution of this subtype to our inflammatory pain model. However, single treatment of CJ-023423 did not improve the pain score in the present adjuvant-induced inflammatory pain model. In the previous paper, carrageenan-induced mechanical hyperalgesia model and adjuvant-induced weight bearing deficit model were used as acute and chronic inflammatory pain models, respectively [35]. They performed the test 2 days after adjuvant injection when arthritis was not established yet, whereas we measured the pain score 19 days after adjuvant injection when arthritis was fully established. Because EP4 expression is induced in DRG neuron from 2 to 7 days after adjuvant injection [19], EP4 contributes to inflammation and inflammatory pain at that period and EP4 antagonist shows analgesic effect [35]. However, in a later phase when arthritis is fully established, it is uncertain whether EP4 still mainly contributes to inflammation. In the present study, coadministration of CJ-023423 and RO3244019 reversed adjuvant-induced inflammatory pain, suggesting that not only the EP4 but also the IP signal contributes to inflammatory hyperalgesia in such a late phase. Although contributions of other EP receptors were not investigated, this result suggests that the EP4 receptor dominantly contributes to the PGE₂ signal in the chronic inflammatory pain model.

In summary, we evaluated the analgesic effect of a PGE₂ selective inhibitor, but it showed no effect in rat acute and chronic inflammatory pain models. On the other hand, dual inhibition of PGE₂ and PGI₂ signals relieved the pain behavior at a level comparable to COX-2 inhibition, suggesting that both PGE₂ and PGI₂ signals play important roles in inflammatory pain. Among PGE₂ receptors, the EP4 receptor dominantly contributes to PGE₂ signaling during inflammatory hyperalgesia.

Abbreviations

COX: Cyclooxygenase
DMEM: Dulbecco’s modified Eagle’s medium
The authors declare that they have no competing interests.

Competing Interests
The authors declare that they have no competing interests.

References
[1] M. J. S. Langman, J. Weil, P. Wainwright et al., “Risks of bleeding peptic ulcer associated with individual non-steroidal anti-inflammatory drugs,” The Lancet, vol. 343, no. 8905, pp. 1075–1078, 1994.
[2] R. S. Bresalier, R. S. Sandler, H. Quan et al., “Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial,” The New England Journal of Medicine, vol. 352, no. 11, pp. 1092–1102, 2005.
[3] N. A. Nussmeier, A. A. Whelton, M. T. Brown et al., “Complications of the COX-2 inhibitors parecoxib and valdecoxib after cardiac surgery,” The New England Journal of Medicine, vol. 352, no. 11, pp. 1081–1091, 2005.
[4] P.-J. Jakobsson, S. Thorén, R. Morgenstern, and B. Samuelsson, “Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target,” Proceedings of the National Academy of Sciences of the United States of America, vol. 96, no. 13, pp. 7220–7225, 1999.
[5] M. Murakami, H. Naraba, T. Tanioka et al., “Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2,” The Journal of Biological Chemistry, vol. 275, no. 42, pp. 32783–32792, 2000.
[6] T. Tanioka, Y. Nakatani, N. Semmyo, M. Murakami, and I. Kudo, “Molecular identification of cytosolic prostaglandin E2 synthase that is functionally coupled with cyclooxygenase-1 in immediate prostaglandin E2 biosynthesis,” The Journal of Biological Chemistry, vol. 275, no. 42, pp. 32775–32782, 2000.
[7] C. E. Trebino, J. L. Stock, C. P. Gibbons et al., “Impaired inflammatory and pain responses in mice lacking an inducible prostaglandin E synthase,” Proceedings of the National Academy of Sciences of the United States of America, vol. 100, no. 15, pp. 9044–9049, 2003.
[8] D. Kamei, K. Yamakawa, Y. Takegoshi et al., “Reduced pain hypersensitivity and inflammation in mice lacking microsomal prostaglandin E synthase-1,” The Journal of Biological Chemistry, vol. 279, no. 32, pp. 33684–33695, 2004.
[9] D. Claveau, M. Sirinyan, J. Guay et al., “Microsomal prostaglandin E synthase-1 is a major terminal synthase that is selectively up-regulated during cyclooxygenase-2-dependent prostaglandin E2 production in the rat adjuvant-induced arthritis model,” The Journal of Immunology, vol. 170, no. 9, pp. 4738–4744, 2003.
[10] D. Xu, S. E. Rowland, P. Clark et al., “MF63 [2-(6-chloro-1H-phenanthro[9,10-d]imidazo[2-yl]-isophthalonitrile], a selective microsomal prostaglandin E synthase-1 inhibitor, relieves pyrexia and pain in preclinical models of inflammation,” Journal of Pharmacology and Experimental Therapeutics, vol. 326, no. 3, pp. 754–763, 2008.
[11] D. Xu, S. E. Rowland, P. Clark et al., “MF63 [2-(6-chloro-1H-phenanthro[9,10-d]imidazo[2-yl]-isophthalonitrile], a selective microsomal prostaglandin E synthase-1 inhibitor, relieves pyrexia and pain in preclinical models of inflammation,” Journal of Pharmacology and Experimental Therapeutics, vol. 326, no. 3, pp. 754–763, 2008.
[12] D. Xu, S. E. Rowland, P. Clark et al., “MF63 [2-(6-chloro-1H-phenanthro[9,10-d]imidazo[2-yl]-isophthalonitrile], a selective microsomal prostaglandin E synthase-1 inhibitor, relieves pyrexia and pain in preclinical models of inflammation,” Journal of Pharmacology and Experimental Therapeutics, vol. 326, no. 3, pp. 754–763, 2008.
[13] D. Xu, S. E. Rowland, P. Clark et al., “MF63 [2-(6-chloro-1H-phenanthro[9,10-d]imidazo[2-yl]-isophthalonitrile], a selective microsomal prostaglandin E synthase-1 inhibitor, relieves pyrexia and pain in preclinical models of inflammation,” Journal of Pharmacology and Experimental Therapeutics, vol. 326, no. 3, pp. 754–763, 2008.
[14] D. Xu, S. E. Rowland, P. Clark et al., “MF63 [2-(6-chloro-1H-phenanthro[9,10-d]imidazo[2-yl]-isophthalonitrile], a selective microsomal prostaglandin E synthase-1 inhibitor, relieves pyrexia and pain in preclinical models of inflammation,” Journal of Pharmacology and Experimental Therapeutics, vol. 326, no. 3, pp. 754–763, 2008.
[15] D. Xu, S. E. Rowland, P. Clark et al., “MF63 [2-(6-chloro-1H-phenanthro[9,10-d]imidazo[2-yl]-isophthalonitrile], a selective microsomal prostaglandin E synthase-1 inhibitor, relieves pyrexia and pain in preclinical models of inflammation,” Journal of Pharmacology and Experimental Therapeutics, vol. 326, no. 3, pp. 754–763, 2008.
[16] D. Xu, S. E. Rowland, P. Clark et al., “MF63 [2-(6-chloro-1H-phenanthro[9,10-d]imidazo[2-yl]-isophthalonitrile], a selective microsomal prostaglandin E synthase-1 inhibitor, relieves pyrexia and pain in preclinical models of inflammation,” Journal of Pharmacology and Experimental Therapeutics, vol. 326, no. 3, pp. 754–763, 2008.
[17] D. Xu, S. E. Rowland, P. Clark et al., “MF63 [2-(6-chloro-1H-phenanthro[9,10-d]imidazo[2-yl]-isophthalonitrile], a selective microsomal prostaglandin E synthase-1 inhibitor, relieves pyrexia and pain in preclinical models of inflammation,” Journal of Pharmacology and Experimental Therapeutics, vol. 326, no. 3, pp. 754–763, 2008.
[18] D. Xu, S. E. Rowland, P. Clark et al., “MF63 [2-(6-chloro-1H-phenanthro[9,10-d]imidazo[2-yl]-isophthalonitrile], a selective microsomal prostaglandin E synthase-1 inhibitor, relieves pyrexia and pain in preclinical models of inflammation,” Journal of Pharmacology and Experimental Therapeutics, vol. 326, no. 3, pp. 754–763, 2008.
[19] D. Xu, S. E. Rowland, P. Clark et al., “MF63 [2-(6-chloro-1H-phenanthro[9,10-d]imidazo[2-yl]-isophthalonitrile], a selective microsomal prostaglandin E synthase-1 inhibitor, relieves pyrexia and pain in preclinical models of inflammation,” Journal of Pharmacology and Experimental Therapeutics, vol. 326, no. 3, pp. 754–763, 2008.
[20] D. Xu, S. E. Rowland, P. Clark et al., “MF63 [2-(6-chloro-1H-phenanthro[9,10-d]imidazo[2-yl]-isophthalonitrile], a selective microsomal prostaglandin E synthase-1 inhibitor, relieves pyrexia and pain in preclinical models of inflammation,” Journal of Pharmacology and Experimental Therapeutics, vol. 326, no. 3, pp. 754–763, 2008.
[21] D. Xu, S. E. Rowland, P. Clark et al., “MF63 [2-(6-chloro-1H-phenanthro[9,10-d]imidazo[2-yl]-isophthalonitrile], a selective microsomal prostaglandin E synthase-1 inhibitor, relieves pyrexia and pain in preclinical models of inflammation,” Journal of Pharmacology and Experimental Therapeutics, vol. 326, no. 3, pp. 754–763, 2008.
[22] D. Xu, S. E. Rowland, P. Clark et al., “MF63 [2-(6-chloro-1H-phenanthro[9,10-d]imidazo[2-yl]-isophthalonitrile], a selective microsomal prostaglandin E synthase-1 inhibitor, relieves pyrexia and pain in preclinical models of inflammation,” Journal of Pharmacology and Experimental Therapeutics, vol. 326, no. 3, pp. 754–763, 2008.
S. Uematsu, M. Matsumoto, K. Takeda, and S. Akira, “Lipopolysaccharide-dependent prostaglandin E\textsubscript{2} production is regulated by the glutathione-dependent prostaglandin E\textsubscript{2} synthase gene induced by the toll-like receptor 4/MyD88/NF-IL6 pathway,” The Journal of Immunology, vol. 168, no. 11, pp. 5811–5816, 2002.

P. Leclerc, H. Idborg, L. Spahiu et al., “Characterization of a human and murine mPGES-1 inhibitor and comparison to mPGES-1 genetic deletion in mouse models of inflammation,” Prostaglandins and Other Lipid Mediators, vol. 107, pp. 26–34, 2013.

S. U. Monrad, F. Kojima, M. Kapoor et al., “Genetic deletion of mPGES-1 abolishes PGE\textsubscript{2} production in murine dendritic cells and alters the cytokine profile, but does not affect maturation or migration,” Prostaglandins Leukotrienes and Essential Fatty Acids, vol. 84, no. 3-4, pp. 113–121, 2011.

A. Bruno, L. Di Francesco, I. Coletta et al., “Effects of AF3442 [N-(9-ethyl-9H-carbazol-3-yl)-2-(trifluoromethyl)benzamide], a novel inhibitor of human microsomal prostaglandin E synthase-1, on prostanoid biosynthesis in human monocytes in vitro,” Biochemical Pharmacology, vol. 79, no. 7, pp. 974–981, 2010.

C. E. Trebino, J. D. Eskra, T. S. Wachtmann, J. R. Perez, T. J. Carty, and L. P. Audoly, “Redirection of eicosanoid metabolism in mPGES-1-deficient macrophages,” The Journal of Biological Chemistry, vol. 280, no. 17, pp. 16579–16585, 2005.

R. Sugita, K. Kubota, K. Sugimoto et al., “A novel selective prostaglandin E\textsubscript{2} synthesis inhibitor relieves pyrexia and arthritis in Guinea pigs inflammatory models,” Journal of Pharmacological Sciences, vol. 130, no. 2, pp. 128–135, 2016.

A. Ebersberger, B. D. Grubb, H. L. Willingale, N. J. Gardiner, J. Nebe, and H.-G. Schaible, “The intraspinal release of prostaglandin E\textsubscript{2} in a model of acute arthritis is accompanied by an up-regulation of cyclo-oxygenase-2 in the spinal cord,” Neuroscience, vol. 93, no. 2, pp. 775–781, 1999.

J. S. Cefalu, Q. M. Zhu, A.-C. Eggers et al., “Effects of the selective prostacyclin receptor antagonist RO3244019 on the micturition reflex in rats,” Journal of Urology, vol. 178, no. 6, pp. 2683–2688, 2007.

C. Brenneis, O. Coste, R. Schmidt et al., “Consequences of altered eicosanoid patterns for nociceptive processing in mPGES-1-deficient mice,” Journal of Cellular and Molecular Medicine, vol. 12, no. 2, pp. 639–648, 2008.

R. Uda, S. Horiguchi, S. Ito, M. Hyodo, and O. Hayaishi, “Nociceptive effects induced by intrathecal administration of prostaglandin D\textsubscript{2}, E\textsubscript{2}, or F\textsubscript{2\alpha} to conscious mice,” Brain Research, vol. 510, no. 1, pp. 26–32, 1990.

T. Minami, R. Uda, S. Horiguchi, S. Ito, M. Hyodo, and O. Hayaishi, "Alloodynia evoked by intrathecal administration of prostaglandin F2 alpha to conscious mice," Pain, vol. 50, no. 2, pp. 223–229, 1992.

P. Clark, S. E. Rowland, D. Denis et al., "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)acetamide], a selective E prostanoid receptor 4 antagonist, relieves joint inflammation and pain in rodent models of rheumatoid and osteoarthritis,” The Journal of Pharmacology and Experimental Therapeutics, vol. 325, no. 2, pp. 425–434, 2008.

K. Nakao, A. Murase, H. Ohshiro et al., "CJ-023,423, a novel, potent and selective prostaglandin EP4 receptor antagonist with antihyperalgesic properties," Journal of Pharmacology and Experimental Therapeutics, vol. 322, no. 2, pp. 686–694, 2007.

T. Okumura, Y. Murata, K. Taniguchi, A. Murase, and A. Nii, "Effects of the selective EP4 antagonist, CJ-023,423 on chronic inflammation and bone destruction in rat adjuvant-induced arthritis,” Journal of Pharmacy and Pharmacology, vol. 60, no. 6, pp. 723–730, 2008.