

Research Article

Selective mPGES-1 Inhibitor Ameliorated Adjuvant-Induced Arthritis in the Rat Model

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Endogenous prostaglandin E_2 (PGE₂) plays an important role in maintaining the homeostasis conditions. However, the overexpression of PGE₂ in response to various inflammatory stimulations is an important target of anti-inflammatory drugs. Both inducible COX-2 (cyclooxygenase-2) and mPGES-1 (microsomal prostaglandin E_2 synthase-1) enzymes are responsible for the inflammatory overexpressed PGE₂ production. Among them, mPGES-1 is regarded as a more promising ideal target for anti-inflammatory drugs without the gastrointestinal and cardiovascular side effects. As our continuous research for the discovery of novel mPGES-1 inhibitors, we have characterized MPO-0144 as a selective mPGES-1 inhibitor with a selectivity index of >270 over COX-1 and >25 over COX-2, respectively. Herein, we evaluated the anti-inflammatory effect of MPO-0144 in an adjuvant-induced arthritis rat model. MPO-0144 attenuated the inflammatory responses without severe gastrointestinal side effects and organ toxicities. These overall data suggest a possibility that MPO-0144 downregulates PGE₂ production by potent mPGES-1 and weak COX-2 inhibitory activities, thus attenuating the paw swelling in AIA (adjuvant-induced arthritis) rat models. MPO-0144 also exhibited favorable ADMET profiles. However, MPO-0144 did not show any inhibitory effects on human mPGES-1 enzyme at a high concentration. Therefore, MPO-0144 represents a valuable pharmacological tool for the study of regulation of inducible mPGES-1 in an inflammatory arthritis rat model.

1. Introduction

Inflammation is one of the complex biological responses of body tissues to harmful stimuli such as pathogens, injured cells, or irritants. It is also a process by which body's immune systems protect our body from these stimuli [1, 2]. There are two main types of inflammation: acute and chronic [3–5]. As compared to acute inflammation characterized by 5 cardinal signs such as pain, redness, loss of function, swelling, and heat, chronic inflammation is associated with various diseases such as cancer, heart disease, diabetes, asthma, arthritis, and Alzheimer's disease [5].

Prostaglandin E_2 (PGE₂) plays an important role in the physiology of the mammalian body but is a principal mediator of inflammation [6]. In cells exposed to diverse harmful stimuli, excess of arachidonic acid is converted into upregulated PGE_2 via the coupled action of both inducible cyclooxygenase-2 (COX-2) and terminal PGE synthase, particularly microsomal PGE synthase-1 (mPGES-1) [7]. Therefore, nonsteroidal anti-inflammatory drugs (NSAIDs) and selective cyclooxygenase-2 (COX-2) inhibitors reduce PGE₂ production to relieve inflammation. However, these inhibitors have adverse effects such as the gastrointestinal bleeding of NSAIDs and the risk of serious cardiovascular events of COX-2 inhibitors [8, 9]. Thus, selective inhibition of mPGES-1, which is the terminal enzyme responsible for the conversion of PGH₂ into PGE₂, has been suggested as a safer therapeutic strategy without affecting the normal production of other prostaglandins related to the body's homeostasis [10, 11]. Despite the high number of inhibitors identified so far [12], however, only a few mPGES-1 inhibitors such as GRC-27864 (zaloglanstat) and GS-248 (vipoglanstat) have been tested in humans (Figure 1) [13, 14].

Continuing our research for the discovery of novel mPGES-1 inhibitors, we recently reported that phenylsulfonyl hydrazide derivative MPO-0144 inhibited LPSinduced PGE₂ production (IC₅₀:41.77 nM) in RAW 264.7 cells via the inhibition of murine mPGES-1 enzyme (IC₅₀:1.16 nM) as shown in Figure 1 [15–17]. MPO-0144 exhibited *in vitro* and *in vivo* strong neuroprotective effects in the animal model of Parkinson's disease via this mechanism of action [17]. Based on *in vitro* and *in vivo* results, herein, we investigated the anti-inflammatory properties of MPO-0144 in adjuvant-induced arthritis (AIA) rodent models.

2. Materials and Methods

2.1. Synthesis of MPO-0144. The synthetic method of phenylsulfonyl hydrazide derivative MPO-0144 was recently reported by our group [17].

2.2. Animals. Sprague-Dawley (SD) male rats (180–200 g) were obtained from Orient Bio Inc. (Seongnam-si, Korea) and maintained under constant conditions (temperature: $20 \pm 2^{\circ}$ C, humidity: 40–60%, and light/dark cycle: 12 h). All animal experiments were managed according to the university guideline of Animal Care and Use approved by the Ethical Committee at Kyung Hee University (KHUASP(SE)-18–032).

2.3. Adjuvant-Induced Arthritis (AIA) Mode. AIA was set up by injection of Freund's complete adjuvant containing *Mycobacterium butyricum* into the right tibiotarsal ankle joint (10 mg/mL, 100 μ L per rat) [18]. MPO-0144 (10 mg/kg, p.o.) and indomethacin (2.5 mg/kg, p.o.) were orally administered at the time of adjuvant injection on day 0 for 14 days. Paw volumes were measured by using a plethysmometer just before induction of AIA and every third day for 14 days. Paw volumes were individually normalized as percentages of change from their values at day 0 and then averaged for each treatment group. On the last day, animals were euthanized. The rat plasma and stomach were obtained and freshly frozen (-80°C) for the biochemical analysis.

2.4. COX-1 and COX-2 Inhibitory Assay. MPO-0144 was tested for its inhibitory activities against COX-1 and COX-2 enzymes at four concentrations (0.1, 1, 10, and 100 μ M) using the COX (ovine/human) inhibitor screening assay kit (Item No. 560131, Cayman Chemical, Ann Arbor, MI, USA) in a cell-free assay according to the manufacturer's instructions [19].

2.5. Gastric Mucosal Injury Test in AIA Rats. The rats were sacrificed, their stomachs were removed, and the gastric mucosa was photographed using a digital camera. The area

of the lesions was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Ulcer scoring in the indomethacin-treated AIA mice (n = 8) was determined using the procedures described by [20]. Briefly, stomach lesion width (mm) and length (mm) were determined using a vernier caliper.

2.6. Determination of Plasma Glutamic Oxaloacetic Transaminase (GOT), Glutamic Pyruvic Transaminase (GPT), Troponin I, and Blood Urea Nitrogen (BUN) in AIA Rats. For biochemical analysis, blood was centrifuged at 4,000 rpm for 5 min, and then, the supernatant was analyzed using LC-MS/MS for the following parameters: GOT, GPT, troponin I, and BUN [21–23].

2.7. Determination of pKa and LogP. These values were evaluated according to potentiometric acid-base titration using Sirius T3 (Pion Inc, Riverside, UK). For pKa, the pH, when the ionized state and the neutral state are the same at a 50% ratio from the titration curve, estimated by acid-base titration in the pH range of 2.0-12.0 was measured. LogP was measured for the partition coefficient by acid-base titration using the same method as pKa in a dual-phase solvent system of octanol and water [24].

2.8. Determination of Solubility. The solubility of MPO-0144 was determined by the following methods. Stock solutions of MPO-0144 were prepared at 10 mM in 5% DMSO (dimethyl sulfoxide) and then diluted in 99% phosphate-buffered saline (PBS, pH 7.4) buffer. As a result, the diluted compound had a final concentration of 100 μ M. The volume of the test compound in a 96-well plate was set to be 250 μ L, and the solubility was measured by NEPHELOstar®, which is a fully automated laser-based microplate nephelometer that measures forward light scattering [25].

2.9. Determination of Permeability (PAMPA). The stirring double-sink PAMPA (parallel artificial membrane permeability) method patented by pION Inc (Billerica, MA) was employed to determine the permeability of MPO-0144 via PAMPA passive diffusion [26].

2.10. P-Glycoprotein (P-Gp) Inhibition Assay. MPO-0144 was evaluated for inhibitory potential toward P-gp by using the simplified *in vitro* assay for P-gp substrates based on P-gp-overexpressing LLC-PK1-MDR1 monolayer cells: $10 \,\mu$ M of MPO-0144, $5 \,\mu$ M of quinidine (substrate), and $50 \,\mu$ M of verapamil (reference inhibitor) [27].

2.11. Human CYP450 Inhibitory Assay. MPO-0144 was screened for inhibitory potential toward five CYP450 isozymes using Promega P450-GloTM Screening System (Madison, WI, USA) according to the manufacturer's protocol [28]. The following known compounds were used as positive controls for the inhibition assay: CYP1A2 (α -naphthoflavone, 10 μ M), CYP2C9 (sulfaphenazole,



FIGURE 1: mPGES-1 inhibitors under development and biological activity of MPO-0144.

10 μ M), CYP2C19 (amitriptyline, 100 μ M), CYP2D6 (quinidine, 10 μ M), and CYP3A4 (ketoconazole, 10 μ M) [29].

2.12. Human Ether-à-Go-Go-Related Gene (hERG) Channel Assay. The hERG channel current was measured by using the 384-IonWorks Barracuda automated patch clamp system (Molecular Devices, USA) [30]. Experiments were carried out at room temperature on HEK293 cells $(2 \sim 4 \times 10^{6} \text{ cells})$ stably expressing the hERG (Kv11.1) potassium channel, which were distributed in 384 wells. The following solutions were used during patch-clamp recording: the external solution was 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose, and pH 7.4; the internal solution was 140 mM KCl, 2 mM MgCl₂, 10 mM HEPES, 5 mM EGTA, and pH 7.4. The following voltage protocol was used for HEK-hERG normal current: holding at -80 mV, 100 ms at -40 mV, 1,000 ms at +50 mV, and finally 2 s at -50 mV. Various concentrations of MPO-0144 were then added to 384 wells, and the voltage pulse was reapplied after 5 min. The resultant hERG channel current was measured to obtain the IC₅₀ value of MPO-0144.

2.13. Ames Test (Salmonella typhimurium Reverse Mutation Assay). The Ames test of MPO-0144 against Salmonella typhimurium TA98 with/without the rat liver S9 fraction was performed using Ames MPFTM 98/100 Microplate Format Mutagenicity Assay Kit (Xenometrix, Swiss) [31]: 2nitrofluorene (2-NF, $2\mu g/mL$) and 2-aminoacridine (2-AA; $0.5\mu g/mL$) were used as positive controls (mutagens), and DMSO (solvent) was used as a negative control. In addition, the Ames test of MPO-0144 against Salmonella *typhimurium* TA100 with/without the rat liver S9 fraction was also performed using Ames MPFTM 98/100 Microplate Format Mutagenicity Assay (Xenometrix, Swiss): 4-nitro-quinoline-*N*-oxide (4-NQO, $0.1 \,\mu$ g/mL) and 2-aminoacridine (2-AA, $1.25 \,\mu$ g/mL) were used as positive controls (mutagens), and DMSO (solvent) was used as a negative control.

2.14. Acute Oral Toxicity Testing In Vivo. The acute oral toxicity test was carried out in ICR mice (9 weeks old, each group n = 6, 3 male and 3 female mice) at doses of 5.5, 17.5, 55, 175, 550, and 1,000 mg/kg p.o. of MPO-0144 (total volume = 10 mL: DMSO 20% and olive oil 80%). The animals were observed for signs of toxicity for 2 days. As no dead animals were observed up to 1,000 mg/kg, the 2nd toxicity test was performed at two high doses of 1,000 and 2,000 mg/kg (n = 6, 3 male and 3 female mice). The animals were continuously observed for signs of toxicity for 7 days. Observations were conducted twice daily, including morality, injury, and abnormal behavior. Besides, body weights for all mice were recorded on study days 1, 3, 5, and 7. On study day 7, all the animals were sacrificed.

2.15. Statistical Analysis. Results are presented as the means \pm SD of triplicate experiments. In the animal study, the data were expressed as the means \pm SD (n=8). Statistically significant values were compared using one-way ANOVA and Dunnett's post hoc test. *P* values less than 0.05 were considered significant. All statistics were performed using GraphPad Prism 5 statistical software (GraphPad Software Inc., CA, USA).



FIGURE 2: The evolution of arthritis (paw volume) of AIA rats following the treatment of vehicle, indomethacin (2.5 mg/kg), and MPO-0144 (10 mg/kg), respectively, once daily for 14 days. Rate of increase (%) = (paw volume (each day)-paw volume (day 0))/(paw volume (day 0)) × 100. Values are means ± SEM of n = 8 animals for each group. ^{###} P < 0.001 vs. the control group (normal rat); *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the AIA group.



FIGURE 3: Effects of MPO-0144 on mPGES-1, COX-1, and COX-2 enzymes. (a) Inhibitory activity of MPO-0144 against mPGES-1 activity in IL-1 β (1 ng/mL)-stimulated RAW 264.7 macrophage cells. MK886 (10 μ M) was used as a positive control of mPGES-1 inhibition [17]. (b) The inhibition activity of MPO-0144 against ovine COX-1 enzyme. sc-560 (100 nM) was used as a positive control of COX-1 inhibition. (c) The inhibition activity of MPO-0144 against human recombinant COX-2 enzyme. Dup-697 (100 nM) was used as a positive control of COX-2 inhibition. This IC₅₀ value (IC₅₀ = 0.03 μ M) was obtained by the extrapolation method. Values are indicated as the mean ± SEM (*n* = 3). Statistically significant values were compared using one-way ANOVA and Dunnett's post hoc test. **P* < 0.05; ***P* < 0.001 vs. the vehicle group. All statistics were performed using GraphPad Prism 5 statistical software (GraphPad Software Inc., CA, USA).



FIGURE 4: Analysis of MPO-0144 side effects. All data were collected from AIA rats, which had been treated with MPO-0144 for 14 days. (a) Representative images of gastric lesions in the corpus of the stomach following different treatments, and the blue-colored arrows indicated the indomethacin-induced gastric hemorrhagic regions (top). Ulcerative score of the indomethacin-treated group = 3.5 ± 0.6 , which are means \pm SD of n = 8 animals (bottom); (b) level of biomarkers representing liver, kidney, and heart toxicities. Values are means \pm SD of n = 8 animals for each group. Statistically significant values were compared using one-way ANOVA and Dunnett's post hoc test. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the vehicle group. All statistics were performed using GraphPad Prism 5 statistical software (GraphPad Software Inc., CA, USA).

3. Results and Discussion

To evaluate the antiarthritic effects of MPO-0144, we investigate its therapeutic effects in the AIA rat model. AIA was set up by injection of Freund's complete adjuvant containing Mycobacterium butyricum into the right tibiotarsal ankle joint [18]. The induction of arthritis significantly enhanced the arthritic paw edema from the first day onwards and attained maximum edema at 14 days. MPO-0144 (10 mg/kg) or indomethacin (2.5 mg/kg, a positive control) treatment significantly attenuated AIA-induced the paw swelling as displayed in Figure 2. At the incipient stage, the treatment with MPO-0144 exhibited a lower inhibitory activity than indomethacin but almost the same potency to indomethacin in suppressing paw swelling from the 11th day. This observation showed that MPO-0144-treated rats did not develop severe arthritis, indicating that MPO-0144 exhibited potential immune-modulating activity.

In order to evaluate the selectivity of MPO-0144 for mPGES-1, MPO-0144 was assayed for its potential inhibitory activities against COX-1 and COX-2, respectively, together with each positive control such as sc-560 and Dup-697 using enzyme immunoassay (EIA) kits in a cell-free assay. As seen in Figures 3(b) and 3(c), MPO-0144 moderately inhibited COX-1 ($IC_{50} = 0.83 \mu M$) but significantly inhibited COX-2 ($IC_{50} = 0.03 \,\mu$ M, which value was calculated by the extrapolation method). Compared with its highest inhibition against rat mPGES-1 enzyme in Figure 3A ($IC_{50} = 1.16$ nM, which value was previously reported by our group [16]), nevertheless, MPO-0144 was selective for the mPGES-1 enzyme over both COX-1 (selectivity index: >700) and COX-2 (selectivity index: >25). These overall data suggest a possibility that MPO-0144 could downregulate PGE₂ production by the combined inhibition of potent mPGES-1 and weak COX-2, thus attenuating the paw swelling in AIA rat models. Interestingly, MPO-0144 did not show any inhibitory effects on human mPGES-1 enzyme prepared from IL-1 β -stimulated A549 human lung cancer cells at a high concentration of 1 μ M (the data were not shown here) [32].

In order to evaluate the acute toxicity of MPO-0144 in the GI tract, we also investigated the real morphological data obtained by dissecting the stomach interior of each group. No hemorrhagic lesions were observed in the MPO-0144treated group compared with indomethacin-treated groups in the gastric mucosa (ulcerative score: 3.5 ± 0.6) as shown in Figure 4(a). To estimate whether the MPO-0144-treated group has any adverse effects on their organs, we have measured plasma parameters such as GOT/GPT (liver injury marker), BUN (kidney function marker), and troponin I

pKa LogP			Value		
LogP			2.96		
			6.77		
Absorption Solubility	Equ	uilibrium solubility 4M (4 08 + 0 78a/m1)		Kinetic solubility in 5% DN 342 9+1 5M (186 3+0 8e	SO SO
$PAMPA^{a}$		Third (1.00 - 0.00 pg/mild) LogPe	$(cm/sec) = -4.19 \pm 0.02$ (me	$\frac{1}{2}$	(
P-glycoprotein		c	2.13% inhibition at 10 μ M	×	
	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4
Metabolism CIF450	21.5%	51.9%	52.4%	4.1%	47.8%
hERG			$7.37 \mu M$		
Toxicity Ames	TA96 N	8/TA100 without S9 Jegative/negative		TA98/TA100 with S9 Negative/negative	
Acute toxicity ^c		0	LD50 => 2,000 mg/kg d	0 0	

of MPO-01.
properties
ADMET
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TABLE

(sulfaphenazole, 94.3% @10 μ M), CYP2C19 (amitriptyline, 88.9% @100 μ M), CYP2D6 (quinidine, 95.7% @10 μ M), and CYP3A4 (ketoconazole, 96.8% @10 μ M). ^cOral rat acute toxicity. ^dALD (approximate lethal dose).

(heart attack marker) levels using EIA kits. As shown in Figure 4(b), the levels of all biomarkers were not significantly affected compared to those in the vehicle-treated AIA group.

We also explored the brief drug-like properties of MPO-0144 through the in vitro ADMET study using the reported standard protocol [33]. Preliminary results of the in vitro ADMET study are listed in Table 1. With regard to absorption of compounds, the evaluated parameters showed that MPO-0144 has a pK_a value of 2.96 (an ionized form exists at physiological pH) and a logP value of 6.77, which resulted in its high hydrophobicity, lower water solubility, and medium membrane permeability as shown in Table 1. MPO-0144 showed a little inhibitory effect (2.13% inhibition) on P-glycoprotein (gp) at $10 \,\mu$ M concentration, not causing P-gp-mediated drug-drug interaction (DDI) compared with verapamil as a positive control (99.74% at $10\,\mu$ M). The inhibitory activity of MPO-0144 against CYP450 was also evaluated at $10 \,\mu$ M concentration. MPO-0144 showed a little or no inhibitory effect on CYP1A2 and CYP2D6 enzymes (21.5 and 4.1% inhibition, respectively) but could moderately inhibit the other three CYP isozymes (~50% inhibition). Therefore, MPO-0144 had a relatively favorable profile on CYP isoforms involved in the drug-drug interaction. In the hERG inhibition, we observed an intermediate range of activity $(1 \mu M < IC_{50} < 10 \mu M)$ of hERG potassium channels possibly corresponding to cardiotoxicity with an IC₅₀ value of $7.37 \,\mu$ M. In the acute toxicity study, a single administration of MPO-0144 was performed orally to the ICR rats at the single maximum doses of 1,000 (1st test) and 2,000 mg/kg (2nd test), and they were then monitored for possible side effects, mortality, or behavioral changes up to 2 and 7 days, respectively. It was observed that MPO-0144 did not cause any behavioral alterations at a high dose of 2,000 mg/kg. No mortality was recorded for the period of 7 days, and after necropsy, no macroscopic pathological alterations caused by MPO-0144 were found in all mice, indicating that the lethal dose of MPO-0144 is above 2,000 mg/kg in rats (LD₅₀: >2,000 mg/ kg). We also screened for potential mutagenic activities of MPO-0144 using the Ames test. An Ames test was carried out on two strains of Salmonella typhimurium (TA98 and TA100) in the absence and presence of metabolic activation (S9). As a result, MPO-0144 showed no mutagenic activity regardless of metabolic activation in both tester strains. These overall ADMET profiles disclosed that MPO-0144 can be considered to be less harmful compound for further in vivo therapeutic studies.

4. Conclusions

Based on *in vitro* inhibitory activity of MPO-0144 against PGE_2 production, we evaluated its anti-inflammatory effects using AIA rat models *in vivo*. The oral treatment of MPO-0144 could strongly suppress arthritic paw edema in AIA rat models, while it did not affect the plasma levels of biomarkers representing liver, kidney, and heart toxicities. On the other hand, MPO-0144 exhibited the selectivity of mPGES-1 over both COX-1 (SI: >270) and COX-2 (SI: >25). These overall data suggest a mode of action that

MPO-0144 could downregulate PGE₂ production by potent mPGES-1 and weak COX-2 inhibitory activities, thus reducing arthritis in rat models. In addition, MPO-0144 demonstrated favorable ADMET profiles. However, MPO-0144 did not show any inhibitory effects on human mPGES-1 enzyme at a high concentration. Therefore, MPO-0144 represents a valuable pharmacological tool for the study of the regulation of inducible mPGES-1 in the only inflammatory arthritis rat model. Now, we are performing the optimization study for the discovery of new compounds with inhibitory potency on both human and murine mPGES-1.

Data Availability

The data used to support the findings of this research study are included within the article. However, further data may be obtained from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Min Ji Kim, Choi Kim, and Ja Yeon Lee performed the synthesis of MPO-0144. Hwi-Ho Lee and Kyung-Sook Chung performed the *in vivo* experiments. Kyung-Tae Lee and Jae Yeol Lee received the fund, analyzed the experimental data, and wrote the manuscript.

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