Identification and Characterization of Novel Microsomal Prostaglandin E Synthase-1 Inhibitors for Analgesia


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Received August 25, 2015; accepted January 5, 2016

ABSTRACT

Prostaglandin (PG) E_2 plays a critical role in eliciting inflammation. Nonsteroidal anti-inflammatory drugs and selective inhibitors of cyclooxygenase, which block PGE_2 production, have been used as key agents in treating inflammation and pain associated with arthritis and other conditions. However, these agents have significant side effects such as gastrointestinal bleeding and myocardial infarction, since they also block the production of prostanooids that are critical for other normal physiologic functions. Microsomal prostaglandin E synthase-1 is a membrane-bound terminal enzyme in the prostanooid pathway, which acts downstream of cyclooxygenase 2 and is responsible for PGE_2 production during inflammation. Thus, inhibition of this enzyme would be expected to block PGE_2 production without inhibiting other prostanooids and would provide analgesic efficacy without the side effects. In this report, we describe novel microsomal prostaglandin E synthase-1 inhibitors that are potent in blocking PGE_2 production and are efficacious in a guinea pig monooiodoacetate model of arthralgia. These molecules may be useful in treating the signs and symptoms associated with arthritis.

Introduction

Prostaglandins (PGs) play critical physiologic roles in a variety of organ functions and serve as key mediators of inflammation, pain, and fever (Smith, 1989; Funk, 2001; Smyth et al., 2009). PGE_2, the most prominent prostanooid, is produced by sequential enzymatic reactions starting with the release of arachidonic acid from membrane glycerophospholipids by phospholipase A_2, followed by conversion to endoperoxide PGH_2 by either cyclooxygenase 1 or 2 (COX-1 or COX-2, respectively), and finally the isomerization of PGH_2 to PGE_2 by terminal prostaglandin E synthase (PGES). The intermediate PGH_2 also serves as a substrate for other synthases/isomerases leading to the production of thromboxane Tx A_2, PGI_2, PGD_2, and PGF_2a (Funk, 2001). The COX-1 isoenzyme is constitutively expressed and is responsible for the production of PGs that preserve the gastric mucosa, whereas the COX-2 is isoenzyme is induced in response to cytokines in inflammatory conditions such as rheumatoid arthritis and osteoarthritis (Sugimoto and Narumiya, 2007; FitzGerald, 2004; Smyth et al., 2009). Nonsteroidal anti-inflammatory drugs (NSAIDs) and COX-2 selective drugs provide symptomatic relief by blocking the production of PGE_2 through inhibition of both COX-1 and COX-2 or COX-2 alone (Rainsford, 2007). However, NSAIDs are associated with gastrointestinal bleeding as a result of the inhibition of constitutively produced PGE_2, whereas COX-2 selective inhibitors have been associated with increased thrombotic risk and myocardial infarction, which may be due to the inhibition of other inducible prostanooids such as PGI_2, which is required for proper cardiovascular function (FitzGerald and Patrono, 2001; Mukherjee et al., 2001; Cheng et al., 2002, 2006; Fries and Grosser. 2005; Wang et al., 2005, 2006, 2011; Grosser et al., 2006). Thus, a selective inhibition of PGE_2 without adversely affecting other prostanooids would be expected to provide anti-inflammatory and analgesic effects without the negative side effects.

Microsomal prostaglandin E synthases 1 and 2 (mPGES-1 and mPGES-2, respectively) are terminal enzymes in converting PGH_2 to PGE_2. mPGES-1 is expressed at low levels and is upregulated in a variety of inflammatory conditions, whereas mPGES-2 is constitutively expressed in a variety of tissues (Jakobsson et al., 1999; Tanikawa et al., 2002; Westmam et al., 2004; Samuelsson et al., 2007). Inhibition of mPGES-1 either by gene deletion or by pharmacological inhibition of activity demonstrates selective blockade of PGE_2 production and analgesic and anti-inflammatory activity (Treblino et al., 2003; Kamei et al., 2004; Xu et al., 2008; Mbalaviele et al., 2010; Abdul-Malik et al., 2013; Bahia et al., 2014; Korotkova and Jakobsson, 2014). These observations suggest that mPGES-1 plays a critical role in eliciting PGE_2-mediated inflammatory response and that blocking the enzyme activity is likely to

ABBREVIATIONS: COX, cyclooxygenase; EIA, enzyme immunoassay; FBS, fetal bovine serum; HEK-293, human embryonic kidney 293; IL, interleukin; LPS, lipopolysaccharide; MF-63, 2-6-chloro-1H-phenanthro[9,10-d]imidazol-2-yliisophthalonitrile; MIA, monooiodoacetate; mPGES, microsomal prostaglandin E synthase; NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin; PGE, prostaglandin E synthase; TNFα, tumor necrosis factor α; Tx, thromboxane.
provide analgesic and anti-inflammatory relief. We recently identified and described novel mPGES-1 inhibitors that are highly selective, potent, and orally available (Schiffer et al., 2016). In this report, we show that these molecules are selective in blocking PGE₂ production, while exhibiting no inhibition of other prostanoids, such as PGL₂ at the concentrations tested. We also show that the compounds are effective in reducing pain in a guinea pig model of knee joint pain.

Materials and Methods

mPGES-1 Inhibitors (Compounds 1 and 2 and Reference mPGES-1 Inhibitor MF-63). The synthesis of reference mPGES-1 inhibitor MF-63 [2-(6-chloro-1H-phenanthro-[9,10-j]imidazol-2-yl)isophthalonitrile] (Xu et al., 2008) and compounds 1 and 2 were described previously (Schiffer et al., 2016)

Human mPGES-1. Human mPGES-1 was purchased from Invitrogen (catalog no. 97002RG, clone ID 6314722; Invitrogen, Grand Island, NY) and was subcloned into pcDNA3.1 and transiently expressed in human embryonic kidney 293 (HEK-293) cells. Microsomes were prepared from cell pellets based on published methods (Ouellet et al., 2002; Thörén et al., 2003). In brief, cell pellets were sonicated in a buffer of 15 mM TRIS-HCl, pH 8.0, 0.25 mM sucrose, 0.1 mM EDTA, and 1 mM glutathione. The suspension was centrifuged at 5000 × g for 10 minutes at 4°C. The supernatant fraction was loaded into Beckman Quickseal tubes (342413; Beckman Coulter, Brea, CA) and centrifuged at 185,000 × g for 90 minutes at 4°C using a 70.1 Ti rotor. Pellets were resuspended in a buffer of 10 mM sodium phosphate, pH 7.0, 10% glycerol, 2.5 mM glutathione, and Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Final concentrations were 4.4 μg/ml microsomes and 1.69 μM PGH₂. All dilutions were made using the above buffer. After a 2.5-minute incubation at room temperature, 2.5 μl/well SnCl₂ in 0.5 N HCl was added to stop the reaction. PGE₂ was quantitated by standard liquid chromatography/mass spectrometry analysis.

Guinea Pig mPGES-1. Guinea pig mPGES-1 was cloned from interleukin (IL)-1stimulated 104C1 (CRL-1405; American Type Culture Collection, Manassas, VA) by 5′-RACE and was subcloned into pQXIN. HEK-293 cells were infected with the plasmid for 24 hours and then expanded under selection in Dulbecco’s Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F12) 3:1 (Invitrogen), all dilutions were made using the above buffer. After a 2.5-minute incubation at room temperature, 2.5 μl/well SnCl₂ in 0.5 N HCl was added to stop the reaction. PGE₂ was quantitated by standard liquid chromatography/mass spectrometry analysis.

Rat mPGES-1. Rat mPGES-1 cDNA was purchased from Open Biosystems (catalog no. MRN1768-99238049, clone ID 7456259; Open Biosystems, Huntsville, AL) and was subcloned into pQXIN. HEK-293 cells were infected with the plasmid for 24 hours and then expanded under selection in DMEM/F12 3:1 (Invitrogen), 10% fetal bovine serum (FBS), and 1 mg/ml G418. Cell pellets were processed into microsomes as described above. Activity was assessed as above with final concentrations of 15 μg/ml guinea pig mPGES-1 microsomes and 2 μM PGH₂. PGE₂ was measured by enzyme immunoassay (EIA) (500141; Cayman Chemical, Ann Arbor, MI) at a dilution of 1:1000.

Human mPGES-2. Human mPGES-2 was obtained from Open Biosystems (catalog no. MH8101 L-14465, clone ID 3946495) and was subcloned into pET21d base vector. It was expressed in BL21CDE3 cells. The enzyme was purified by nickel affinity and size exclusion chromatography. Activity was assessed in a buffer of 100 mM KPO₄, pH 7.0, with 1 mM dithiothreitol. Final concentrations were 10 μg/ml human mPGES-2 and 2 μM PGP1. PGE₂ was measured by EIA (500141; Cayman Chemical) at a dilution of 1:1000.

COX-1 and COX-2 Activity Assay. COX activity was assessed using a commercially available kit utilizing ovine COX-1 and human COX-2 (560131; Cayman Chemical). Enzyme-Inhibitor Reversibility Studies by a Rapid Dilution Assay. A rapid dilution assay was performed as previously described to determine whether the inhibitor binding to the enzyme was reversible (Copeland, 2005). Briefly, human mPGES-1 was diluted into buffer at 100× its usual assay concentration. Compounds (or dimethylsulfoxide) were added to the enzyme at 10× their respective IC₅₀ values and incubated for 30 minutes at room temperature. PGH₂ was diluted into buffer to give a 2 μM final concentration and a volume equal to 100× the enzyme plus inhibitor volume was added to initiate the reaction. At 20-second intervals, SnCl₂ was added to stop the reactions. PGE₂ was measured by EIA (500141; Cayman Chemical) at a dilution of 1:1000.

A549 Epithelial Carcinoma Cell Assay. Human epithelial lung carcinoma cell line A549 was purchased from American Type Culture Collection (CCL-185) and was maintained in Kighn’s F12 plus 10% FBS in 5% CO₂. For assay, cells were plated at 40,000/well in 96-well Falcon plates (353072, Corning Incorporated, Corning, NY), 24 hours prior to treatment. Compounds were diluted in dimethylsulfoxide and were added at 1 μl/well (n = 2), to give seven concentrations each. Cells were pretreated for 30 minutes at 37°C, 5% CO₂. Recombinant human IL-1α (R&D Systems, Minneapolis, MN) was added to give 0.2 ng/ml final. The treatment period was 18 hours. The conditioned medium was assayed for levels of PGE₂, PGF₁α, TXB₂, and 6-keto PGF₁α by EIA (Cayman Chemical). The IC₅₀ values were calculated using GraphPad Prism nonlinear regression sigmoidal dose response curve fitting (GraphPad Software Inc., La Jolla, CA). Data are the means ± S.D. of the indicated number of determinations.

Human Whole Blood Assay. Blood was collected from normal volunteer donors into sodium heparin vacutainer tubes (BD, Franklin Lakes, NJ). Donors had not taken NSAIDₐs aspirin, celecoxib, or glucocorticoids within 2 weeks of the donation. Blood was distributed into deep-well polystyrene plates and compounds were added. The blood was pretreated at 37°C, 5% CO₂, in a humidified atmosphere, loosely covered, for 30 minutes; lipopolysaccharide (LPS) (serotype 0111:B4; Sigma-Aldrich, St. Louis, MO) was then added to give a final concentration of 100 μg/ml. The plates were incubated for 20–24 hours, loosely covered, at 37°C, 5% CO₂, in a humidified atmosphere, on an orbital shaker at 100 rpm. The plates were sealed tightly with silicone cap mats and were chilled on ice for 1 hour, then centrifuged at 1800 × g, 10 minutes, 4°C, in an Eppendorf 5810R centrifuge (Eppendorf, Hauppage, NY). Plasma was removed from the cell layer and transferred to v-bottom polystyrene plates. One hundred microliters was quantitatively transferred to Costar cluster tube blocks (4411, Corning Incorporated, Corning, NY) and 400 μl/well methanol/internal standard standard was added. Liquid phase extraction was performed using Waters HLB 30-mg/bed 96-well plates (Waters, Milford, MA), which were subjected to liquid chromatography–tandem mass spectrometry analysis. Calibration curves were obtained by plotting the peak area ratio PGE₂, PGF₁α, TXB₂, and respective internal standard against the concentration. A weighted (1/concentration) least-squares regression analysis was used to obtain a linear equation over the range of the calibration. The IC₅₀ values were calculated using GraphPad Prism nonlinear regression sigmoidal dose response (variable slope), with a fitted top of less than 1.5× the LPS control and a fitted bottom between zero and 1.5× the reference standard. Data are the geometric means ± S.D. of determinations from six donors.

Intra-Articular Injection of LPS plus Tumor Necrosis Factor α. To assess the ability of mPGES-1 inhibitors to inhibit prostaglandin production in the knee, a guinea pig model was used. Male guinea pigs weighing around 300 g were first dosed subcutaneously with either vehicle (95% capex and 5% N-methyl-2-pyrrolidine (NMP), 50 mg/kg MF-63, or 30 mg/kg dicyclofenac (an NSAID used as a positive control). One hour postdose, animals were injected with either 50 μl saline into both right and left knees or with 100 μg LPS (L2630 strain 0111:B4; Sigma) plus 50 ng tumor necrosis factor α (TNFα) (5053-TG-025; R&D Systems) in 50 μl saline into both knees. Six hours after intra-articular injection, knee joints were lavaged to collect synovial fluid, and the fluid was measured for PGE₂, PGF₁α, and PGF₂α levels using EIA kits from Cayman Chemical.
**Monoiodoacetate (MIA) Pain Model.** To assess pain efficacy, male Hartley guinea pigs (Charles River Laboratories, Burlington, MA) weighing approximately 200–250 g were used. To induce pain, the right knee of each guinea pig was injected with 0.3 mg MIA in 50 μl saline and the left knee with 50 μl saline. To test the efficacy of compounds, guinea pigs were either dosed 5 days (compound 1) or 9 days (compound 2) after MIA injection with vehicle (10% Cremaphor EL in saline), 30 mg/kg diclofenac (NSAID-positive control), two doses of compound 1 (50 or 75 mg/kg), or two doses of compound 2 (10 or 50 mg/kg). All dosing was subcutaneous at a dose volume of 5 ml/kg and the group size was six. Dose group was randomly assigned to each animal and dosing staggered by 10 minutes for each guinea pig. Pain was measured 4 hours postdosing via incapacitance testing. This test measures the difference in hind paw weight bearing between the

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**Fig. 1.** Characterization of mPGES-1. (A) HEK-293 cells were transfected with cDNA for human mPGES1; the microsomal and cytosolic fractions were separated as described in the Materials and Methods. An aliquot was subjected to gel electrophoresis, followed by immunoblot analysis with anti–mPGES-1 antibody (Cayman Chemical). Lane 1, starting material-cell homogenate; lane 2, low-speed supernatant fraction; lane 3, cytosolic fraction; and lane 4, microsomal fraction. (B) The microsomal fraction was assayed for mPGES-1 activity using various concentrations of substrate (PGH₂) and the PGE₂ was quantitated by liquid chromatography–tandem mass spectrometry analysis.

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**Fig. 2.** Chemical structure of mPGES-1 inhibitors. Compounds 1 and 2 are newly described mPGES-1 inhibitors. Celecoxib, rofecoxib (COX-2 inhibitors), and MF-63 (a reference mPGES-1 inhibitor) were used in some experiments.
The in vitro enzyme activity assay was used to identify and optimize novel chemical scaffolds as inhibitors. Various concentrations of compound 1 (A) or compound 2 (B) were first mixed with mPGES-1 enzyme (microsomal preparation) followed the addition of substrate PGH₂. After a 2.5-minute incubation at room temperature, the reaction was stopped by the addition of SnCl₂. The product PGE₂ was measured by liquid chromatography–tandem mass spectrometry.

**Results**

**Preparation and Characterization of mPGES-1 Enzyme.** To develop a reproducible mPGES-1 activity assay, HEK-293 cells were transiently transfected with human mPGES-1 cDNA and microsomal and cytosolic fractions were evaluated for mPGES-1 expression using immunoblot analysis. The results (Fig. 1A) demonstrate that the microsomal fraction contained mPGES-1. The untransfected cells contained no basal mPGES-1 protein. The microsomal fraction was used to determine enzyme activity.

The synthase activity was determined based on the ability of mPGES-1 (microsomal preparation diluted in phosphate buffer, pH 7) to convert PGH₂ (substrate) to PGE₂. A representative example of the effects of various substrate concentrations on PGE₂ production is shown in Fig. 1B. The Kₘ value (13.2 μM) of the enzyme activity is comparable to the value of 14 μM reported elsewhere (Ouellet et al., 2002).

**Identification of Novel and Selective mPGES-1 Inhibitors.** The in vitro enzyme activity assay was used to identify and optimize novel chemical scaffolds as inhibitors. The structures of two optimized molecules (compounds 1 and 2) representing two different scaffolds, MF-63 (a reference mPGES-1 inhibitor) and celecoxib, are shown in Fig. 2. Both compounds 1 and 2 (Fig. 3) demonstrate full efficacy (100% inhibition) and concentration-dependent inhibitory activity against mPGES-1 enzyme with IC₅₀ values of 0.241 ± 0.0085 μM and 0.00094 ± 0.00059 μM, respectively. For comparison, the IC₅₀ value of the reference mPGES-1 inhibitor was 0.005 ± 0.003 μM (data not shown). Compounds 1 and 2 show very little activity against isolated mPGES-2, COX-1, or COX-2. Furthermore, the COX-2 selective inhibitor (celecoxib) and nonselective NSAIDs (ibuprofen and diclofenac) show no activity against mPGES-1 at the concentration tested. To determine relevant animal species for evaluation as a disease model, the compounds were also tested against mPGES-1 from guinea pig and rat. The inhibitors showed potent activity versus guinea pig mPGES-1 but very poor activity versus rat mPGES-1 at the concentration tested. A summary of enzyme activities of the mPGES-1 inhibitors as well as celecoxib is shown in Table 1. These results establish that the molecules are potent and selective.

We also assessed whether the compounds bound to the enzyme in a reversible fashion. A rapid dilution method was used to assess the nature of binding. mPGES-1 was incubated with concentrations of the inhibitors that were 10-fold higher than their respective IC₅₀ values. These solutions were then diluted 100-fold with the substrate solution resulting in inhibitor concentrations of one-tenth of their IC₅₀ values. As shown in Fig. 4, the dilution resulted in a recovery of the enzyme activity over time. The results indicate that both compounds were reversible inhibitors.

**Selective Inhibition of mPGES-1 in IL-1β–Stimulated A549 Cells.** We next evaluated whether the mPGES-1 inhibitors were effective in blocking PGE₂ production in cells in response to an inflammatory stimuli. The A549 human

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**TABLE 1**

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>IC₅₀ Value</th>
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<tr>
<td></td>
<td>Compound 1</td>
</tr>
<tr>
<td>Human mPGES-1</td>
<td>0.241 ± 0.0085 (n = 6)</td>
</tr>
<tr>
<td>Guinea pig mPGES-1</td>
<td>0.511 (n = 1)</td>
</tr>
<tr>
<td>Rat mPGES-1</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Human mPGES-2</td>
<td>&gt;100</td>
</tr>
<tr>
<td>COX-1</td>
<td>9% inhibition at 100 μM</td>
</tr>
<tr>
<td>COX-2</td>
<td>16% inhibition at 30 μM</td>
</tr>
</tbody>
</table>
epithelial carcinoma cell line produces a variety of prostanoids (PGE2, PGI2, PGF2α, and PGD2) in response to IL-1 (Thorén and Jakobsson, 2000). Initially, we compared the effects of the reference mPGES-1 inhibitor (MF-63) and a COX-2 selective inhibitor (rofecoxib) on various prostanoids produced by IL-1 treated with A549 cells. A549 cells were pretreated with various concentrations of compounds for 30 minutes, followed by IL-1β treatment for an additional 18 hours, and the conditioned media were analyzed for various prostanoids using EIA. The COX-2 selective inhibitor rofecoxib blocked the production of all prostanoids (PGE2, PGI2, PGF2α, and PGD2) in a dose-dependent manner. The reference mPGES-1 inhibitor (MF-63) blocked PGE2 production (Fig. 5), but demonstrated varying levels of increase in other prostanoids, suggesting shunting toward these molecules. We next evaluated effects of compounds 1 and 2 on IL-1β–treated A549 cells on PGE2 and PGL2 under similar conditions. The results (Fig. 6) demonstrate that mPGES1 inhibitors and celecoxib blocked PGE2 production in a concentration-dependent manner. Compounds 1 and 2 demonstrated IC50 values of 0.87 ± 0.42 M and 0.012 ± 0.006 M, respectively. The mPGES-1 inhibitors also caused a 2- to 3-fold increase in PGI2 levels, demonstrating a shunting toward other prostanoids. In comparison, celecoxib, a COX-2 selective inhibitor, blocked the production of both prostanoids. These results established that mPGES-1 inhibitors were effective in blocking IL-1β–stimulated PGE2 production and that the inhibitory effects were selective to PGE2 synthesis.

Selective Inhibition of PGE2 Production in LPS-Stimulated Human Whole Blood In Vitro. Previous studies with NSAIDs and coxibs have shown a correlation...
between the in vitro human whole blood IC₈₀ value and the plasma concentration achieved in vivo at clinically efficacious doses, thereby providing a basis for using biochemical potency to predict analgesic efficacy (Huntjens et al., 2005). Therefore, we compared the activity of mPGES-1 inhibitors against the standard of care, celecoxib, in human whole blood in vitro. Compounds were added to freshly collected human blood obtained from normal volunteers who had not consumed any anti-inflammatory drugs during the past 2 weeks. LPS (100 μg/ml) was added 30 minutes after the addition of the compounds; after a 24-hour incubation at 37°C, the prostanoids secreted into the plasma were quantified using liquid chromatography–tandem mass spectrometry. The results (Fig. 7) show that compound 1, compound 2, and celecoxib blocked PGE₂ production in a concentration-dependent manner. The IC₈₀ values were 0.792 ± 0.267 μM, 0.015 ± 0.009 μM, and 0.551 ± 0.490 μM, respectively. Once again, clear differences were observed between mPGES-1 inhibitors and celecoxib on other prostanoids. Whereas celecoxib blocked the production of both PGF₂α and TxB₂, mPGES-1 inhibitors showed no inhibitory activity against either of these prostanoids at the concentration tested. These results further demonstrate that the two mPGES-1 inhibitors selectively blocked PGE₂ production in whole human blood cells, with compound 2 being more potent than celecoxib.

mPGES-1 Inhibitors Were Efficacious in a Guinea Pig MIA Model of Pain. We next wanted to evaluate whether the mPGES-1 inhibitors were efficacious in an...
animal model of pain that is known to be at least partially mediated though PGE2 (Park et al., 2014). Previous studies have suggested that a reference mPGES-1 inhibitor was effective in a guinea pig MIA model of pain (Xu et al., 2008). Since compounds 1 and 2 did not inhibit rat mPGES-1 but were effective against guinea pig mPGES-1 (Table 1), as a first step we evaluated whether the reference mPGES-1 inhibitor (MF-63) was effective in blocking PGE2 production in the knee joints of guinea pigs injected with TNFα plus LPS. Preliminary studies established that the optimal inflammation was achieved by a combination of TNFα and LPS (data not shown). The guinea pigs were given either diclofenac (30 mg/kg) or MF-63 (50 mg/kg) by subcutaneous injection 1 hour prior to intra-articular injection of LPS plus TNFα. The joint fluid was collected by lavage with saline 6 hours after TNFα plus LPS injection and the lavage fluids were analyzed for PGE2, PGI2, and PGF2α levels. The results demonstrate that the LPS plus TNFα stimulation of PGE2 was blocked in animals dosed with the reference mPGES-1 inhibitor or diclofenac (Fig. 8). Whereas diclofenac-treated animals showed suppression of other prostanoids (PGI2 and PGF2α), the reference mPGES-1 inhibitor treatment inhibited only PGE2. These results demonstrate that the selectivity of mPGES-1 was also observed in vivo at least for the reference inhibitor.

We next evaluated the ability of mPGES-1 inhibitors to block the pain resulting from joint injury caused by the intra-articular injection of MIA. Previous studies have established that the injection of MIA into the knee joint of rats and guinea pigs produces an acute inflammatory insult, joint degeneration, and pain (Schwartz et al., 1981; Williams and Thonar, 1989; Pomonis et al., 2005; Malfait et al., 2013). The pain resulting from the joint injury can be measured via differential weight bearing of the hind legs using an incapacitance tester. To evaluate the analgesic efficacy, MIA-injected guinea pigs were dosed with vehicle, mPGES-1 inhibitors at the indicated doses, or 30 mg/kg of the NSAID diclofenac (vehicle saline). The pain was measured using incapacitance testing 4 hours postdosing. The mPGES-1 inhibitors (compounds 1 and 2) and diclofenac significantly inhibited pain versus vehicle, with the 75-mg/kg dose of compound 1 and the 50-mg/kg dose of compound 2 being significantly different from both the low doses of the respective compounds and diclofenac (P < 0.05, Dunnett’s test with Bonferroni correction for comparison between groups; Fig. 9). These results establish that the mPGES-1 inhibitors were effective in a guinea pig model of pain.

**Discussion**

mPGES-1 is a terminal enzyme induced during inflammation, is responsible for the production of PGE2, and is a potential target for effective analgesic and anti-inflammatory activity without causing side effects (Trebino et al., 2003; Xu et al., 2008; Mbalaviele et al., 2010; Abdul-Malik et al., 2013; Bahia et al., 2014; Korotkova and Jakobsson 2014). Here, we describe and characterize novel mPGES-1 inhibitors that are potent, selective, and effective in a guinea pig model of pain. The two molecules exemplified in this study are potent against human, dog, and guinea pig mPGES-1 enzymes and bind to the human enzyme in a reversible manner. They are highly selective and show no discernible activity versus mPGES-2, COX-1, and COX-2 enzymes. Both molecules are effective in blocking PGE2 production in IL-1–stimulated A549 cells, as well as in LPS-stimulated human
whole blood. Finally, they demonstrate efficacy in a guinea pig MIA model of pain.

NSAIDs and COX-2 inhibitors have been extensively used to treat the inflammation and pain associated with rheumatoid arthritis and osteoarthritis but show significant side effects. Specifically, the cardiovascular side effects have been suggested to be due to a general blockage of all prostanoids (FitzGerald and Patrono, 2001; Mukherjee et al., 2001; Fries and Grosser, 2005; Wang et al., 2005; Grosser et al., 2006). TxA2, a COX-1–mediated product produced in platelets, is critical in vasoconstriction and platelet aggregation. Conversely, COX-2–derived PGI2, produced in vascular smooth muscle cells and endothelial cells, is a vasodilator and inhibits platelet activation. Coxibs modulate the prothrombotic TxA2 production only marginally, decrease the production of antithrombotic PGI2, and create an alteration in the TxA2/PGI2 ratio that favors the prothrombotic status (FitzGerald and Patrono, 2001; Mukherjee et al., 2001; Fries and Grosser, 2005; Wang et al., 2005; Grosser et al., 2006). Because of this cardiovascular liability, some coxibs have been withdrawn from the market (FitzGerald and Patrono, 2001; FitzGerald, 2003). Thus, a significant need exists in developing safer alternatives to coxibs and NSAIDs.

mPGES-1 is an inducible integral membrane protein and acts as the terminal enzyme downstream of COX enzymes in producing PGE2 from the intermediate PGH2. This enzyme is normally coexpressed with COX-2 at very low levels in most tissues, is induced by various inflammatory signals (e.g., IL-1 and TNFα), and is upregulated in synovial tissue, cartilage, and chondrocytes of patients with osteoarthritis and rheumatoid arthritis (Tanioka et al., 2000; Stichtenoth et al., 2001; Yamagata et al., 2001; Kojima et al., 2002, 2004; Lazarus et al., 2002; Claveau et al., 2003; Li et al., 2005). Two other enzymes, mPGES-2 and cytosolic PGES, also have been suggested to be involved in PGE2 production. mPGES-2 is expressed constitutively in several tissues along with COX-1 and is believed to play a housekeeping function (Murakami et al., 2003). Cytosolic PGES is present in the cytoplasm but its function in PGE2 production is poorly understood (Lovgren et al., 2007). Therefore, we have focused on identifying mPGES-1 inhibitors that are highly potent, selective, and orally active in relevant preclinical models.

Traditionally, NSAIDs and COX-2 selective inhibitors have been identified using in vitro enzyme activity, selectivity assays, human whole blood assays, and a variety of animal models that measure either pharmacodynamic end points such as PGE2 levels or behavioral response such as nociception or hyperalgesia. Because the mPGES-1 inhibitors identified here did not inhibit rat or mouse mPGES-1 enzymes (Table 1), we were unable to use traditional rodent animal models for efficacy studies. A meta-analysis of marketed NSAIDs and coxibs suggested that clinically efficacious doses of a variety of these
drugs effectively blocked PGE₂ production in LPS-stimulated human whole blood at their respective IC₅₀ concentrations (Huntjens et al., 2005). On this basis of observation, we initiated a biomarker-driven approach, which used IC₅₀ and IC₉₀ values from a human whole blood assay to determine compound efficacy (Werner et al., 2002). Our goal was to first identify molecules exhibiting high intrinsic potency in blocking PGE₂ production in LPS-stimulated human whole blood. Furthermore, a single oral dose of 200 mg celecoxib in humans provides blood levels of that drug, at Cmax, reach the in vitro human whole blood IC₅₀ value and exceed the IC₅₀ value for duration of 6–8 hours (Werner et al., 2002). Using these data, we sought compounds with high intrinsic potency that afforded exposure in rats approaching their IC₅₀ values in the human whole blood assay and remained above the human whole blood IC₅₀ for 6 hours or more after oral dosing (Schiffler et al., 2016).

The initial assessment of enzyme inhibitory activity was done by testing the ability of compounds to block mPGES-1 activity of microsomal preparation of HEK-293 cells transfected with a human mPGES-1 cDNA. The immunoblot analysis demonstrated the purity of the preparation and also showed that the enzyme was present in the microsomal fraction, with very little being present in the cytosolic fraction (Fig. 1). The Kᵳᵣᵣ value of the enzyme preparation (13.2 nM) is in the range of reported activity for a similar preparation (Ouellet et al., 2002). The compounds bound to the enzyme in a reversible manner (Fig. 4).

PGF₂α inhibition and selectivity was demonstrated in two cell-based assays: 1) in IL-1β treated human epithelial cell carcinoma cells (A549) and 2) in human whole blood treated with LPS. In both assays, the mPGES-1 inhibitors demonstrated selective inhibition of PGE₂. The A549 cell line is capable of synthesizing various prostanoids (PGE₂, PGF₂α, and TXA₂) in response to IL-1. Whereas NSAIDs and COX-2 inhibitors blocked all prostanoids, mPGES-1 inhibitors inhibited only the PGE₂ production. Actives from A549 cells were evaluated in human whole blood stimulated with LPS.

Both compounds 1 and 2 demonstrate shunting toward PGI₂. As shown previously, shunting toward other prostanoids is a mechanistic consequence of selective mPGES-1 inhibition (Trebbino et al., 2003). The biologic consequence of shunting to PGI₂ is unknown. Inhibition of PGI₂ production or function is associated with adverse cardiovascular function (Flavahan, 2007; Arehart et al., 2008). However, it is important to consider that PGI₂ shows paradoxical activities that include both cardiovascular protective function as well as proinflammatory activity in arthritic models and conditions (Stitham et al., 2011). Prostacyclin-deficient mice are resistant to an inflammatory and arthritic challenge and prostanoids is a mechanistic consequence of selective mPGES-1 inhibition. As shown previously, shunting toward other prostanoids is a mechanistic consequence of selective mPGES-1 inhibition. We do not know whether the therapeutic potential of mPGES-1 inhibitors as anti-inflammatory/analgesic drugs along with their potential safety features (cardiovascular and gastrointestinal) can be demonstrated in the clinic.

We do not know whether there is any PGI₂ that, at the tested doses, although we do not know whether there is any PGI₂ produced in this model.


