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Distinction of microsomal prostaglandin E synthase-1 (mPGES-1) inhibition from cyclooxygenase-2 inhibition in cells using a novel, selective mPGES-1 inhibitor

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ABSTRACT

Inflammation-induced microsomal prostaglandin E synthase-1 (mPGES-1) is the terminal enzyme that synthesizes prostaglandin E_2 (PGE_2) downstream of cyclooxygenase-2 (COX-2). The efficacy of nonsteroidal anti-inflammatory drugs and COX-2 inhibitors in the treatment of the signs and symptoms of osteoarthritis, rheumatoid arthritis and inflammatory pain, largely attributed to the inhibition of PGE_2 synthesis, provides a rationale for exploring mPGES-1 inhibition as a potential novel therapy for these diseases. Toward this aim, we identified PF-9184 as a novel mPGES-1 inhibitor. PF-9184 potently inhibited recombinant human (rh) mPGES-1 (IC_{50} = 16.5 ± 3.8 nM), and had no effect against rhCOX-1 and rhCOX-2 (>6500-fold selectivity). In inflammation and clinically relevant biological systems, mPGES-1 expression, like COX-2 expression was induced in cell context- and time-dependent manner, consistent with the kinetics of PGE_2 synthesis. In rationally designed cell systems ideal for determining direct effects of the inhibitors on mPGES-1 function, but not its expression, PF-9184 inhibited PGE_2 synthesis (IC_{50} in the range of 0.5–5 μM in serum-free cell and human whole blood cultures, respectively) while sparing the synthesis of 6-keto-$PGF_{1\alpha}$ ($PGF_{1\alpha}$) and $PGF_{2\alpha}$. In contrast, as expected, the selective COX-2 inhibitor, SC-236, inhibited PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ synthesis. This profile of mPGES-1 inhibition, distinct from COX-2 inhibition in cells, validates mPGES-1 as an attractive target for therapeutic intervention.

1. Introduction

Pain associated with human diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA) is treated with analgesic drugs, including nonsteroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase-2 (COX-2) inhibitors (COXibs). These broadly used drugs work by inhibiting PGE_2 synthesis through inhibition of the activity of COX-2 [1]. Consistent with the causative role of PGE_2 in the signs and symptoms of RA and OA, antagonistic molecules that prevent PGE_2 from binding to its receptor have shown efficacy in animal models of arthritis [2]. Furthermore, efficacy in preclinical models of inflammation and hyperalgesia was demonstrated with neutralizing PGE_2 antibodies [3]. Thus, it was reasonable to hypothesize that inhibition of microsomal prostaglandin E synthase-1 (mPGES-1), the enzyme that is responsible for PGE_2 synthesis in inflammation-associated pathologies, may be as efficacious as NSAIDs and COXibs.

mPGES-1 is the terminal enzyme one-step downstream of COX-2 that synthesizes prostaglandin E_2 (PGE_2) from PGH_2, the unstable peroxide intermediate derived from arachidonate catabolism. mPGES-1 expression, like COX-2 expression, is induced by various inflammatory stimuli in normal cells, and in cells and tissues derived from human patients and animal disease models [4]. These include for example, macrophages and synovial fibroblasts derived from RA patients (RASF), OA chondrocytes and several other cell types involved in inflammatory diseases and certain cancers [5,6]. The link between mPGES-1 and the production of PGE_2 in

Abbreviations: COX-2, cyclooxygenase-2; cPGES, cytosolic PGES; mPGES-1, microsomal prostaglandin E synthase-1; PGE_2, prostaglandin E_2; NSAIDs, nonsteroidal anti-inflammatory drugs; COXibs, COX-2 inhibitors; RA, rheumatoid arthritis; OA, osteoarthritis; TXB_2, thromboxane B_2; RASF, synovial fibroblasts derived from patients with rheumatoid arthritis; rh, recombinant human; FLAP, 5-lipoxygenase activating protein.

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inflammatory conditions was established first by data derived from genetic manipulation of mPGES-1 encoding gene. Indeed, mPGES-1-deleted cells produced significantly lower levels of PGE_2 in response to inflammatory stimuli [7,8], and consistently, mPGES-1 knockout (KO) mice were less sensitive to inflammatory and neuropathic pain and refractory to the development of joint pathology in rodent arthritis models [9–11]. These mice had no issues with thrombogenesis or blood pressure and renal function when placed on normal salt diet [12,13]. The findings that indomethacin-induced gastrointestinal (GI) tract lesions in animals can be alleviated by the administration of the prostacyclin agonist Beraprost [14], suggest that depletion of both PGE_2 and 6-keto-$PGF_{1\alpha}$ ($PGF_{1\alpha}$) may be required for the development of GI tract lesions. Consistent with this notion, mPGES-1 inhibition had no inhibitory effects on the synthesis of other prostanoids, including thromboxane B_2 (TXB_2) and $PGF_{1\alpha}$ and did not cause GI tract lesions in animals [15].

The exciting data generated with mPGES-1 KO mice triggered drug discovery efforts for the identification of pharmacological agents to inhibit this enzyme. In the process, compounds that modulate mPGES-1 expression or function have been identified [15–18]. Efficacy in animal models of inflammation has been shown with some of these tool compounds [15,16], though clinical benefits of these mPGES-1 inhibitors have yet to be demonstrated. Here, we described PF-9184 as a novel mPGES-1 inhibitor. PF-9184 is a potent inhibitor of mPGES-1 function, not its expression. In inflammation relevant cell systems, PF-9184 inhibited PGE_2 synthesis while sparing the synthesis of $PGF_{1\alpha}$, $PGF_{2\alpha}$ and TXB_2. Thus, this class of mPGES-1 inhibitors opens avenues for the development of novel inhibitors of PGE_2 synthesis and the treatment of signs and symptoms of OA and RA.

2. Materials and methods

2.1. Reagents

Nonidet P-40, BSA, *E. coli*-derived lipopolysaccharide (LPS), phosphatase inhibitor cocktail, stannous (tin) chloride ($SnCl_2$), pyridoxine hydrochloride, NaCl, Tween-20, diethyldithiocarbamate acid, phenylmethylsulfonyl fluoride, complete protease inhibitor mixture tablet, EDTA and dithiothreitol were obtained from Sigma (St. Louis, MO). Anti-GAPDH antibodies and Immobilon-P membranes were obtained from Millipore (Billerica, MA). Dulbecco's modified essential media (DMEM), Dulbecco's phosphate-buffered saline (DPBS), L-glutamine, HEPES, trypsin, penicillin–streptomycin, nitrocellulose membranes, Tris–glycine and acrylamide gels were purchased from Invitrogen (Carlsbad, CA). Recombinant human interleukin-1β (IL-1β) was purchased from R & D Systems (Minneapolis, MN), rabbit polyclonal anti-COX-2 antiserum (PG-27) from Oxford Biomedical Research (Burlington, Ontario, CA). ECL plus kit was obtained from GE Life Sciences (Piscataway, NJ), anti-mPGES-1 antibodies and ELSIA kits for the detection of PGH_2, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ were purchased from Cayman Chemical (Ann Arbor, MI). Sodium arachidonic acid was obtained from Nu-Chek Prep, Inc. (Elysian, MN). All other reagents used were of the highest grade commercially available.

2.2. Inhibitors

PF-9184 (N-(3′,4′-dichlorobiphenyl-4-yl)-4-hydroxy-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide) and SC-236 (COXib, [19]) were synthesized at Pfizer, Inc. (St. Louis, MO). PF-9184 was prepared according to the method described previously [20,21]. Briefly, commercially available methyl 4-hydroxy-2H-1,2-benzothiazine-3-carboxylate 1,1-dioxide and 3′,4′-dichloro-1,1′-biphenyl-4-amine in o-xylene, containing pyridine and 4A sieves was heated to 135 °C overnight. Analytical data was consistent with the desired product: ^{1}H NMR dH (DMSO, 400 MHz) 7.68–8.02 (m, 11H), 9.63 (s, 1H), 10.29 (s, 1H), 13.79 (s, 1H), LC/MS m/z = 461 (M+H), 483 (M+Na), M.P. = 285.5–289.3 °C. MK-886 (5-lipoxygenase activating protein (FLAP) and mPGES-1 inhibitor, [22]) and indomethacin (NSAID) were purchased from Sigma (St. Louis, MO). The inhibitors were dissolved in DMSO obtained from Sigma (St. Louis, MO), and were stored at −20 °C, as 10 mM aliquots.

2.3. mPGES-1 activity assay

PF-9184 was incubated with recombinant mPGES-1 (suspended in 100 mM K_3PO_4 at pH 6.2 buffer and 2.5 mM glutathione). The reaction was initiated by the addition of PGH_2 (2 μM, final concentration). The reaction was carried out for 41 s at room temperature. The reaction was terminated by the addition of 1:10 volumes of 2.5 mM $FeCl_2$. The reaction was immediately diluted 120× into ELISA buffer, and PGE_2 levels were measured by ELISA. The percentage control activity was calculated as the percentage difference between negative control (100% inhibited by a reference inhibitor) and mPGES-1 only control. The difference between enzymatic vs. non-enzymatic production of PGE_2 is typically 3–4-fold. IC_{50} were calculated by 4-parameter log fit of the % control data.

2.4. Cultures of human synovial fibroblasts derived from patients with rheumatoid arthritis (RASF)

Synovial fibroblasts derived from patients with rheumatoid arthritis (RASF), were isolated via enzymatic digestions from primary synovial tissues isolated after knee synovectomy. These experiments were performed in accordance with a protocol approved by the Ethics Committee of Pfizer. RASF were plated at 8×10^4 cells/cm^2 and cultured for 3 days in DMEM containing 10 μM L-glutamine, 25 μM HEPES, 10 units/ml penicillin, 10 μg/ml streptomycin, supplemented with 10% (v/v) fetal bovine serum (FBS), at 37 °C in 95% air, 5% CO_2 atmosphere. To test the effects of the inhibitors on prostaglandin (PG) production during 24 h incubation, the culture media were replaced with fresh DMEM containing 1% (v/v) FBS, and cells were treated for 24 h with 1 ng/ml IL-1β in the presence of vehicle (1% DMSO, final concentration) or the inhibitors (1% DMSO, final concentration). The conditioned media were then collected for PG analysis. To determine direct effects of the inhibitors on enzyme activity, cells were treated for 24 h with 1 ng/ml IL-1β as described above. The conditioned media were removed, and cells were washed twice with serum-free media. Cells were then treated with the media containing either the vehicle (1% DMSO) or the inhibitors (1% DMSO) for 50 min, and with 10 μM arachidonic acid for additional 10 min, at 37 °C in 95% air, 5% CO_2 atmosphere, and the conditioned media were collected for PG analysis. For $PGF_{2\alpha}$ studies, 2 mg/ml $SnCl_2$ was added to the cells 40 min after addition of the inhibitors (or 10 min before the addition of arachidonic acid). The levels of PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ in the conditioned media were measured by ELISA.

2.5. LPS-stimulated human whole blood assay (LPS/HWB)

Human whole blood was collected in 10 ml heparinized tubes (Vacutainer tubes, Becton Dickenson, Franklin Lakes, NJ) from healthy human donors who had not taken NSAIDS or COXibs for the preceding 5 days. Blood was stimulated with 20 μg/ml LPS for 24 h in the presence of vehicle (1% DMSO) or the inhibitors (1% DMSO), at 37 °C in 95% air, 5% CO_2 atmosphere. The assay plates were centrifuged at 930 × g for 10 min at room temperature and the plasma was removed for the quantitation of PGE_2 and TXB_2 levels by ELISA.

2.6. Modified human whole blood assays

Human whole blood obtained as described above, was pre-incubated at 37 °C in 95% air, 5% CO_2 atmosphere, with the inhibitors for 15 min then with 30 μM arachidonic acid for 10 min. The assay plates were centrifuged at 930 × *g* for 10 min at room temperature, and the plasma was removed for quantitation of PGE_2 and TXB_2 levels by ELISA. To demonstrate that mPGES-1 was the target of PF-9184 action, blood was mixed with human head and neck squamous cell carcinoma, 1483 cells. Before the day of the experiments, these cells were maintained in cultures at 37 °C in 95% air, 5% CO_2 atmosphere in DMEM/F-12 media containing 15 mM HEPES buffer, L-glutamine, pyridoxine hydrochloride and 10% FBS. On the day of the experiments, 1483 cells were trypsinized, centrifuged 930 × *g* for 10 min, and the pellets were resuspended with human whole blood. The cell mixtures were treated with the inhibitors and 30 μM arachidonic acid, and the samples processed for PG analysis by ELISA.

2.7. Carrageenan-induced prostaglandin production in the rat air pouch model

Male Lewis rats (175–200 g) obtained from Charles River Laboratories (Wilmington, MA) were used in the study. The Pfizer Institutional Animal Care and Use Committee reviewed and approved the animal use in these studies. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Air pouches were produced by subcutaneous injection of 20 ml of sterile air into the intrascapular area of the back as described previously [23]. After 24 h of initial air injection, 2 ml of a 1% suspension of carrageenan (FMC BioPolyer, Philadelphia, PA) dissolved in saline was injected directly into the pouch. At time 0, then 1, 2, 4 and 24 h post-carrageenan injection, 1 ml of DPBS was injected into the pouch, mixed and the fluid was collected. The fluid was centrifuged at 800 × *g* for 10 min at 4 °C, and the supernatants were collected for the analysis of PGE_2 and $PGF_{1\alpha}$ levels by ELISA.

2.8. RNA isolation and purification

RNA from cells or frozen pulverized pouch lining tissues was extracted using the Qiagen RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA pellets were DNAse treated (Qiagen) and quantified by OD_{260}. Purity and integrity were confirmed by RNA Nano 6000 analysis (Agilent Technologies, Santa Clara, CA).

2.9. RT-PCR analysis

mRNA was quantitated with One-step TaqMan or Syber-green RT-PCR (Applied Biosystems, Foster City, CA). The TaqMan probes were labeled at the 5′-end with 6-carboxyfluorescein (FAM) reporter dye and at the 3′-end with a minor groove binder/nonfluorescent quencher (MGB/NFQ) (Applied Biosystems, Foster City, CA). Standard curves using 5-fold dilutions of total RNA (from 200 pg to 320 pg) were performed to determine the efficiency of each primer set prior to the analysis of the experimental samples. Duplicate samples were assayed with no enzyme as control for fluorescent contaminants. Dissociation curves were run after the Syber-green assays to ensure the absence of non-specific amplification. All results were normalized to the housekeeping gene, cyclophilin. Total RNA (50–100 ng), 500 nM each primer and 100 nM each probe were used for each reaction run in duplicate. The following protocol was applied to all reactions: 30 min at 48 °C (reverse transcription), 10 min at 95 °C (inactivation of reverse transcriptase), 40 cycles of 15 s at 95 °C and 1 min at 60 °C (polymerization). Data analysis was performed using the Sequence Detection System software from Applied Biosystems. Sequences of the primers and probes used in the reactions were as follows: rat mPGES-1: forward (F) = ACCCTCTCATCGCCTGGATAC, reverse (R) = CAGGTAGGCCACGGTGTGTA, MGB = TTCCTCGTGGTCCTCACA; rat COX-2, F = TCAAAGACACTCAGGTAGA CATGATCT, R = CGGCAC-CAGACCAAAGACTT, MGB = CCCTCCCCACGTCC; human mPGES-1, F = CCTGGGCTTCGTCTACTCCTTT, R = CAGGTAGGCCACGG TGTGT; human COX-2, F = TGGCTGAGGGAACACAACAG, R = TAGCCTGCTTG TCTGGAACAACT; human mPGES-2, F = CCTCTATGAGGCTGCTGACA, R = CCATACACCGCCAAATCAG; human cPGES, F = GCCTGCTTCTGC-AAAGTGG TA, R = CACTTCCTCCGAGACAACTGAA; human COX-1, F = GCCCAGCCCC TCTTTCA, R = GGCATTGACAAACTCCCAGAA.

2.10. Immunoblot analysis

Cells (human embryonic kidney (HEK) cells, 1483 cells or human fibroblasts stimulated with IL-1β) or frozen pouch lining tissues, which were ground under liquid nitrogen, were lyzed in buffer containing 100 mM Tris-buffered saline (pH 8), 150 mM NaCl, 1% Tween-20, 50 mM diethyldithiocarbamate acid, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor mixture tablet with 1 mM EDTA. Lysates were sonicated for 20 s on ice and centrifuged at 10,000 × *g* for 10 min. The proteins were quantified in the supernatants using the Bradford Protein Assay (Bio-Rad, Hercules, CA). Proteins (50 μg/lane) were loaded and separated by SDS-PAGE. SDS-PAGE was performed under reducing conditions on 10% polyacrylamide Tris/glycine gels. The resolved proteins were transferred onto Immobilon-P membrane using an XCell II Blot Module (Invitrogen, Carlsbad, CA). The membrane was blocked and then probed with primary antibodies. Antigen–antibody complexes were visualized by incubation of the blots in a dilution of horseradish peroxidase-conjugated secondary antibodies and the ECL detection system (Pierce, Rockford, IL).

2.11. Statistical analysis

Samples were always run in triplicates and values are expressed as means ± standard error of the mean (S.E.M.). Independent experiments were performed at least twice. Statistical significance was assessed using unpaired *T*-test (two groups) and one-way ANOVA (more than two groups).

3. Results

3.1. PF-9184 is a novel, potent and selective inhibitor of mPGES-1

PF-9184 (N-(3′,4′-dichlorobiphenyl-4-yl)-4-hydroxy-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide) (Fig. 1) was identified from structure activity relationship of an oxicam series. It is a novel, potent inhibitor of recombinant human (rh) mPGES-1 (IC_{50} = 16.5 ± 3.8 nM, n = 8), and had no effect against rhCOX-1 and rhCOX-2 with IC_{50} of 118 and 263 μM, respectively.

3.2. Inflammation induces mPGES-1 expression in vivo in rat air pouch: effects of PF-9184

To investigate mPGES-1 expression *in vivo* in inflammatory conditions, air pouches generated in rats by subcutaneous injection of sterile air into the intrascapular area of the back were injected with carrageenan for various times, and the fluid and lining pouch tissues were collected as described previously [23]. Carrageenan-induced COX-2 mRNA (Fig. 2A) and protein (Fig. 2B) expression occurred as early as 1 h, with maximal expression achieved around 4 h before declining (relative to GAPDH levels)

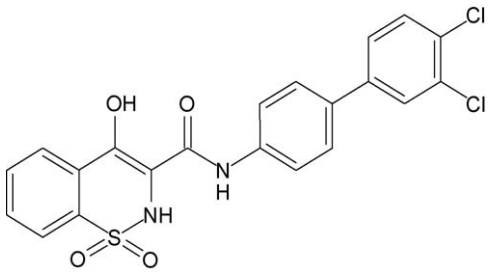

Fig. 1. Chemical structure of PF-9184.

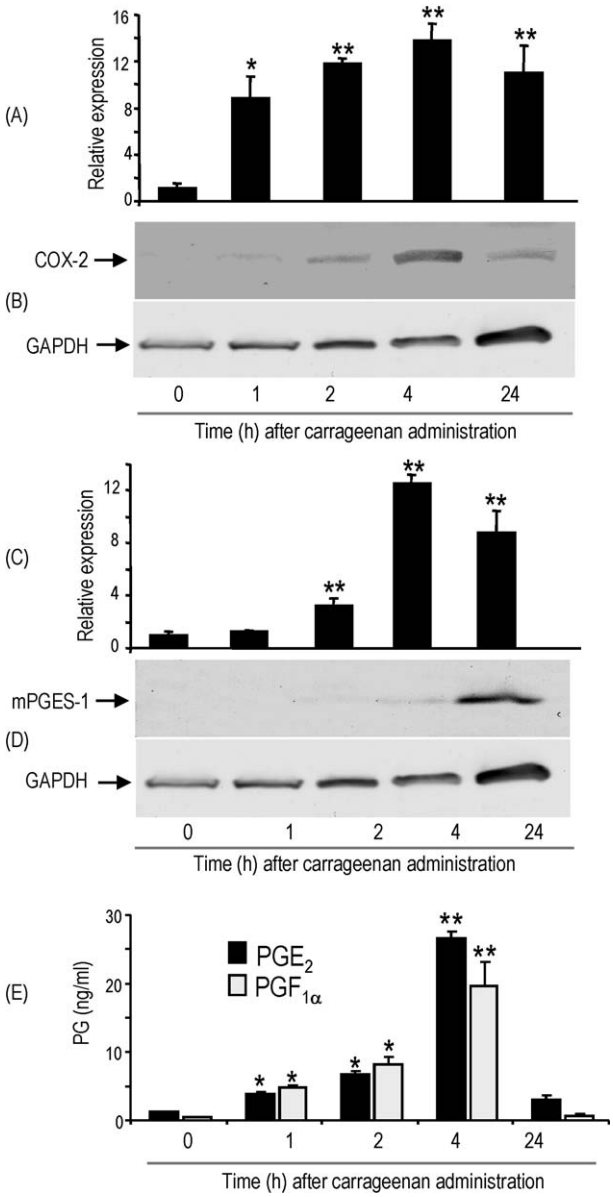

Fig. 2. mPGES-1 and COX-2 expression is induced in vivo in rat air pouches by carrageenan. Air pouches generated in rats by subcutaneous injection of sterile air into the intrascapular area of the back were injected with carrageenan for the indicated times. The lining pouch tissues were collected for expression analysis of COX-2 mRNA (A), COX-2 protein (B), mPGES-1 mRNA (C) or mPGES-1 protein (D). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as control for protein loading. (E) Pouch fluid was collected for the determination of PGE_2 (black columns) and $PGF_{1\alpha}$ (gray columns) levels. mRNA and PG data represent the mean ± S.E.M. of 4 individual rats from 1 representative experiment. For Western blot analysis, samples from 4 rats from each group were pooled prior to the analysis. $^{*}p < 0.05$; $^{**}p < 0.005$, significance differences compared with time 0.

around 24 h. mPGES-1 expression followed COX-2 expression with noticeable induction 2 h post-carrageenan injection of both mRNA (Fig. 2C) and protein (Fig. 2D). Consistent with the kinetics of COX-2 and mPGES-1 expression, PGE_2 levels in the fluid derived from pouch lavages increased progressively from 1 to 4 h before declining around 24 h post-carrageenan injection (Fig. 2E). $PGE_{1\alpha}$ synthesis also followed the kinetics of COX-2 expression. Besides reflecting the profile of COX-2 and mPGES-1 expression, the pronounced decline in PG synthesis at 24 h may also be due to a decrease in the release of arachidonic acid as we found that adding exogenous arachidonic acid to the pouches resulted in an increase of PG synthesis (data not shown).

In light of mPGES-1 expression in pouch tissues, we evaluated PF-9184 efficacy in this model. Several attempts to demonstrate the inhibitory effects of PF-9184 on PGE_2 synthesis in this model following oral administration or even local delivery of PF-9184 to the air pouches were unsuccessful (data not shown). These unexpected results led us to determine the potency of PF-9184 on recombinant rat mPGES-1. Surprisingly, we found that PF-9184 was a poor inhibitor of recombinant rat mPGES-1 ($IC_{50} = 1080 \pm 398$ nM) as compared to rhmPGES-1 ($IC_{50} = 16.5 \pm 3.8$ nM). Collectively, the data shows that in response to inflammatory stimuli, the induction of COX-2 expression occurred earlier than mPGES-1 expression in the lining tissues, findings that are in agreement with other models [24]. The low potency of PF-9184 against rat mPGES-1 re-directed the focus of these studies to human cell-based assays.

3.3. IL-1β induces mPGES-1 expression in vitro in synovial fibroblasts derived from patients with rheumatoid arthritis (RASF)

The expression of eicosanoid enzymes was investigated in synovial fibroblasts derived from patients with rheumatoid arthritis (RASF). We found that IL-1β induced both COX-2 and mPGES-1 mRNA expression (Fig. 3A). In agreement with the data from the inflamed rat air pouches, COX-2 mRNA induction, which started 1 h post-IL-1β stimulation, preceded mPGES-1 mRNA expression, which was noticeable at least 6 h post-IL-1β stimulation. IL-1β treatment had no effect on the expression of COX-1, mPGES-2 or cytosolic PGES (cPGES). The levels of PGE_2 were 48.16 ± 10.05 pg/μg proteins vs. 2.58 ± 0.49 pg/μg proteins, in the 24 h-conditioned media derived from cells stimulated with IL-1β or from unstimulated cells, respectively. The levels of 6-keto$PGF_{1\alpha}$ ($PGF_{1\alpha}$) were 7.08 ± 1.87 pg/μg proteins vs. 0.15 ± 0.04 pg/μg proteins, in the 24 h-conditioned media derived from cells stimulated with IL-1β or from unstimulated cells, respectively. Thus, IL-1β is a potent stimulator of mPGES-1 and COX-2 expression as well as PGE_2 synthesis in RASF.

3.4. PF-9184 inhibits IL-1β-stimulated PGE_2 synthesis in RASF

The inducible nature of mPGES-1 and COX-2 expression in RASF made this a potentially suitable cell system to screen for selective mPGES-1 inhibitors. To validate and ensure the relevance of this cell system we tested the efficacy of indomethacin, a nonselective COX-1 and COX-2 inhibitor, and SC-236, a selective COX-2 inhibitor [19]. Indomethacin (Fig. 3B) or SC-236 (Fig. 3C), incubated concomitantly with IL-1β for 24 h inhibited the synthesis of PGE_2 and $PGF_{1\alpha}$ with equal potency yielding IC_{50} in the range of 50–80 nM. These findings were consistent with the notion that COX-2 is the primary enzyme providing the substrate PGH_2 to be converted to PGE_2 by mPGES-1. The data also validated this cell system for further pharmacology studies with mPGES-1 inhibitors. Toward this goal, we found that the mPGES-1 inhibitor, MK-886 (Fig. 3D and [22]), and our novel, selective mPGES-1 inhibitor, PF-9184 (Fig. 3E) decreased PGE_2 synthesis. PF-9184 had no apparent cytotoxic effects up to 100 μM (data not shown). The

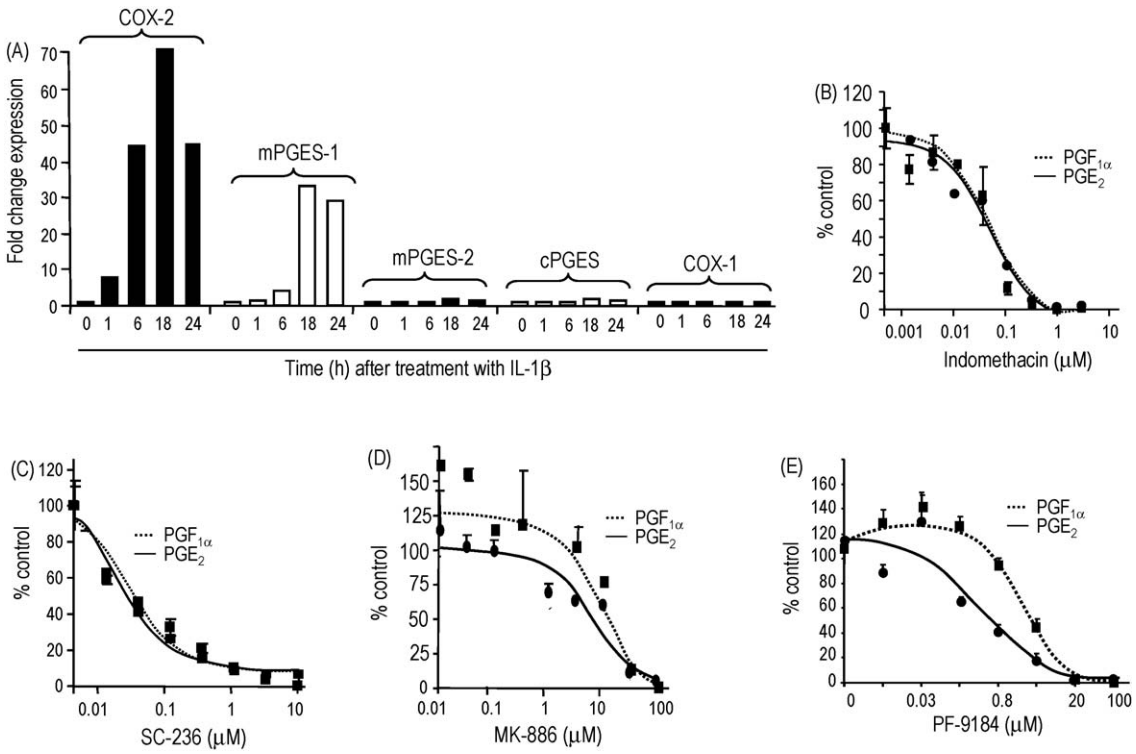

Fig. 3. Effects of the inhibitors on IL-1β-stimulated PGE_2 synthesis on synovial fibroblasts isolated from patients with rheumatoid arthritis (RASF). (A) RASF were stimulated with 1 ng/ml IL-1β for 24 h, and RNA was isolated for gene expression analysis by TaqMan. Conditioned media from IL-1β-stimulated RASF for 24 h in the absence or presence of indomethacin (B), SC-236 (C), MK-886 (D) or PF-9184 (E) were used to measure the levels of PGE_2 (solid lines) or $PGF_{1\alpha}$ (dotted lines). The data represent the mean ± S.E.M. of at least three independent determinations.

unexpected inhibition of $PGF_{1\alpha}$ synthesis, though at higher concentrations of mPGES-1 inhibitors, led us to explore assay conditions where the incubation time with the inhibitors was as short as possible. Such conditions were ideal for determining direct effects of inhibitors on the function of mPGES-1 while minimizing any feedback mechanisms on mPGES-1 or COX-2 expression that might occur in a 24 h time frame.

3.5. IL-1β-stimulated RASF synthesize prostanoids de novo in the presence of added exogenous arachidonic acid

When cells were first stimulated to induce the expression of the enzymes involved in prostanoid metabolism, including mPGES-1 and COX-2, then supplied with exogenous arachidonic acid or PGH_2, they synthesize prostanoids de novo, achieving maximal synthesis within a time frame as short as 10 min [5,7]. Therefore, RASF were stimulated with IL-1β for 24 h, washed to discard accumulated PGE_2 then incubated for 10 min with 10 μM arachidonic acid. The data shows that the addition of arachidonic acid to IL-1β-stimulated cells resulted in an increase in de novo synthesis of PGE_2 (Fig. 4A) and $PGF_{1\alpha}$ (Fig. 4B) by approximately 10-fold and 3-fold compared to unstimulated cells and IL-1β-stimulated cells, respectively.

As shown in Fig. 4A and B, the levels of $PGF_{1\alpha}$ represent approximately 10% of the PGE_2 levels in RASF. Due to a concern for misinterpretation of $PGF_{1\alpha}$ levels attributable to a 1% cross reactivity of the $PGF_{1\alpha}$ antibodies to PGE_2, we developed another cell-based efficacy and selectivity assay. This was based on the notion that endogenous PGH_2 may be converted to $PGF_{2\alpha}$ non-enzymatically by $SnCl_2$ (Fig. 4C and [25]). If this premise holds true, then a COX-2 inhibitor is expected to block the production of PGH_2 and consequently neither PGE_2 nor $PGF_{2\alpha}$ is synthesized whereas PGE_2 not $PGF_{2\alpha}$ production should be inhibited by an mPGES-1 inhibitor. As predicted, the addition of $SnCl_2$ to IL-1β-stimulated RASF treated with arachidonic acid resulted in a 5-fold increase of $PGF_{2\alpha}$ levels (Fig. 4D) and a slight decrease of PGE_2 levels (Fig. 4E). Thus, the efficacy of PF-9184 on mPGES-1 (PGE_2) and selectivity against COX-2 ($PGF_{2\alpha}$) and prostacyclin synthase ($PGF_{1\alpha}$) can be evaluated in RASF.

3.6. mPGES-1 inhibition by PF-9184 is distinctive from COX-2 inhibition in cells

To determine the effects of the inhibitors on mPGES-1 function, IL-1β-stimulated RASF were pre-treated with the inhibitors, then incubated with or without $SnCl_2$ before adding arachidonic acid. Indomethacin (Fig. 5A and E) or SC-236 (Fig. 5B and F), inhibited PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ with the same potency with IC_{50} in the 80 nM range in the absence (Fig. 5A and B) or presence of $SnCl_2$ (Fig. 5E and F). MK-886 (Fig. 5C and G) or PF-9184 (Fig. 5D and H) also inhibited PGE_2 synthesis. Maximal inhibition of PGE_2 synthesis by mPGES-1 inhibitors was around 80%, suggesting that there is an additional source for the remaining 10–20% PGE_2 synthesis in this system. Interestingly, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ levels increased in an inhibitor concentration-dependent manner in the absence (Fig. 5C and D) or presence of $SnCl_2$ (Fig. 5G and H), respectively. Collectively, these data indicate the shunting of PGH_2 synthesis to $PGF_{1\alpha}$ or $PGF_{2\alpha}$ in the presence of PF-9184, and are consistent with the notion that this inhibitor is mPGES-1 selective, with no apparent inhibitory effects on COX-2 and prostacyclin synthase.

3.7. LPS induces mPGES-1 expression in human whole blood whereas mPGES-1 is constitutively expressed in 1483 cells

As described above, we were unable to demonstrate PF-9184 efficacy in *in vivo* rodent models of inflammation. However, since there is a strong correlation between analgesic effects of NSAIDs

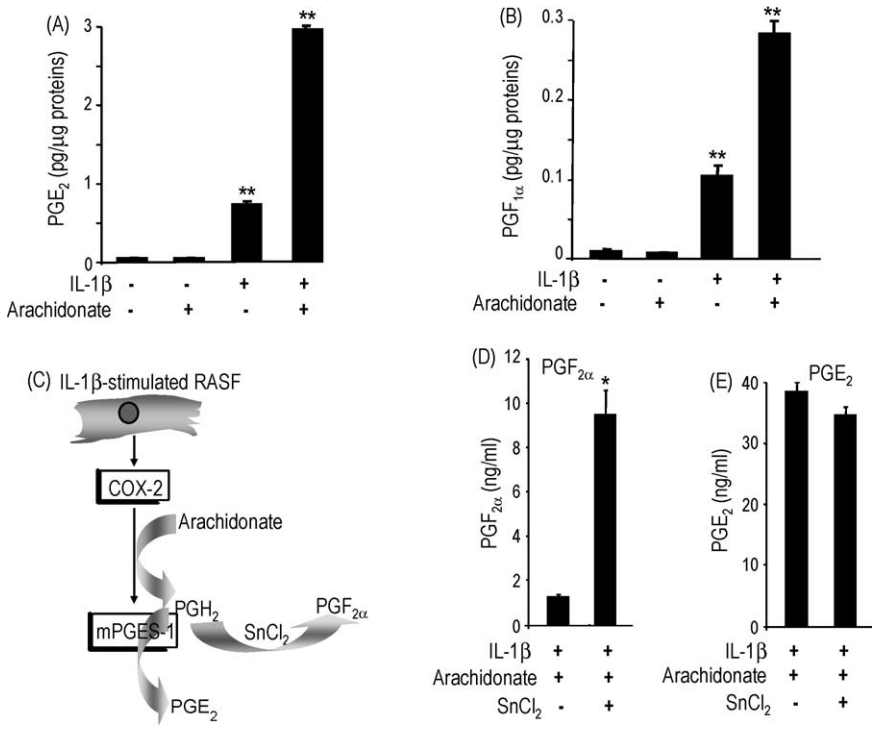

Fig. 4. IL-1β-stimulated RASF synthesize prostanoids de novo in the presence of added exogenous arachidonic acid. RASF were stimulated with 1 ng/ml IL-1β for 24 h, washed to discard accumulated PGE_2 then incubated without (A and B) or with 2 mg/ml $SnCl_2$ (D and E) for 10 min before adding 10 μM arachidonic acid for an additional 10 min. The levels of PGE_2 (A and E), $PGF_{1\alpha}$ (B) or $PGF_{2\alpha}$ (D) were measured by ELISA. (C) Schematic representation of PG synthesis in the presence of $SnCl_2$. The data represent the mean ± S.E.M. of at least three independent determinations. $^*p < 0.05$; $^{**}p < 0.005$, significance differences compared with unstimulated cells without adding exogenous arachidonic acid (A and B) or cells without $SnCl_2$ (D and E).

and COXibs in the clinic and their potency to inhibit PGE_2 synthesis in human whole blood assays [26], we wanted to determine PF-9184 potency in these blood assays. We first assessed the expression of mPGES-1 and COX-2 in human blood stimulated or not with LPS. LPS induced COX-2 (Fig. 6A) and mPGES-1 (Fig. 6B) mRNA expression. mPGES-1 expression was delayed, and reflected our results with RASF. Also consistent with the RASF data, PGE_2 synthesis followed the expression profile of these enzymes (Fig. 6C), while LPS treatment had no effect on expression of mPGES-2, cPGES and COX-1 (data not shown).

To create a condition of acute synthesis of high levels of PGE_2, we modified the human blood assay. Specifically, human head and neck squamous cell carcinoma, 1483 cells, which are known to express COX-2 constitutively [27] were mixed with human whole blood and exposed to arachidonic acid for 10 min. First, we evaluated the expression of mPGES-1 by 1483 cells, and found that they also constitutively expressed mPGES-1 (Fig. 6D) as well as mPGES-2, cPGES and COX-1 mRNA (data not shown). When exogenous arachidonic acid was added to 1483 cell cultures, they produced PGE_2 levels that were comparable to those produced in the 24 h-LPS/human whole blood assay (Fig. 6C), and were about 10-fold higher than those produced by unstimulated human whole blood incubated with arachidonic acid (Fig. 6E). It is worth mentioning that high levels of TXB_2 were also produced in this mixed blood assay (10.49 ± 1.10 ng/ml, $n = 6$). The comparable levels of PGE_2 in 1483 cells vs. 1483 cells mixed with blood (Fig. 6E) strongly indicate that 1483 cells were the main source of PGE_2 in these experimental conditions.

3.8. mPGES-1 inhibition by PF-9184 is distinctive from COX-2 inhibition in human whole blood and modified blood assays

The selectivity and efficacy profiles of these inhibitors were evaluated in human whole blood assay. Since PGE_2 synthesis in blood was maximally induced >8 h after stimulation with LPS (Fig. 6C), pharmacology studies were therefore carried out for 24 h. Indomethacin inhibited PGE_2 and TXB_2 synthesis with equal potency (Fig. 7A). In contrast, while SC-236 was as potent as indomethacin in inhibiting PGE_2 synthesis, it only inhibited TXB_2 synthesis significantly at higher concentrations known to cross-over to thromboxane A synthase (TXAS) (Fig. 7B and [19]). Although PF-9184 inhibited PGE_2 weakly, it had no effect on TXB_2 synthesis except at 100 μM (Fig. 7C). MK-886 failed to inhibit PGE_2 synthesis in the LPS-stimulated blood (data not shown), and was therefore dropped from further testing.

Next, the effects of the inhibitors were investigated in the unstimulated human whole blood assay, where PG synthesis is dependent on exogenously added arachidonic acid and is mainly driven by COX-1 since COX-2 and mPGES-1 are expressed at very low levels. Indomethacin inhibited PGE_2 and TXB_2 synthesis with the same potency as in the LPS-stimulated blood assay (Fig. 7D). In contrast, SC-236 inhibited PGE_2 and TXB_2 (Fig. 7E) with an IC_{50} that was 100-fold higher compared to its potency in inhibiting LPS-stimulated PGE_2 synthesis in blood (COX-2 driven PGE_2 synthesis). The equal potency of SC-236 in inhibiting PGE_2 and TXB_2 synthesis in the unstimulated blood clearly indicates that these inhibitory effects were due to crossover of the COX-2 inhibitor to COX-1 at concentrations >3 μM. In this system, PF-9184 was ineffective at all concentrations (Fig. 7F), data that were consistent with mPGES-1 mRNA levels, which were barely detectable by quantitative RT-PCR (data not shown).

Finally, we evaluated PF-9184 in the modified human blood assay where 1483 cells were added to the unstimulated whole blood. We found that Indomethacin potently inhibited PGE_2 synthesis as expected (Fig. 7G). Interestingly, the addition of 1483 cells to blood restored the ability of SC-236 (Fig. 7H) or PF-9184 (Fig. 7I) to inhibit PGE_2 synthesis. The acute high levels of PGE_2 produced in this system may explain the right shift in IC_{50} for

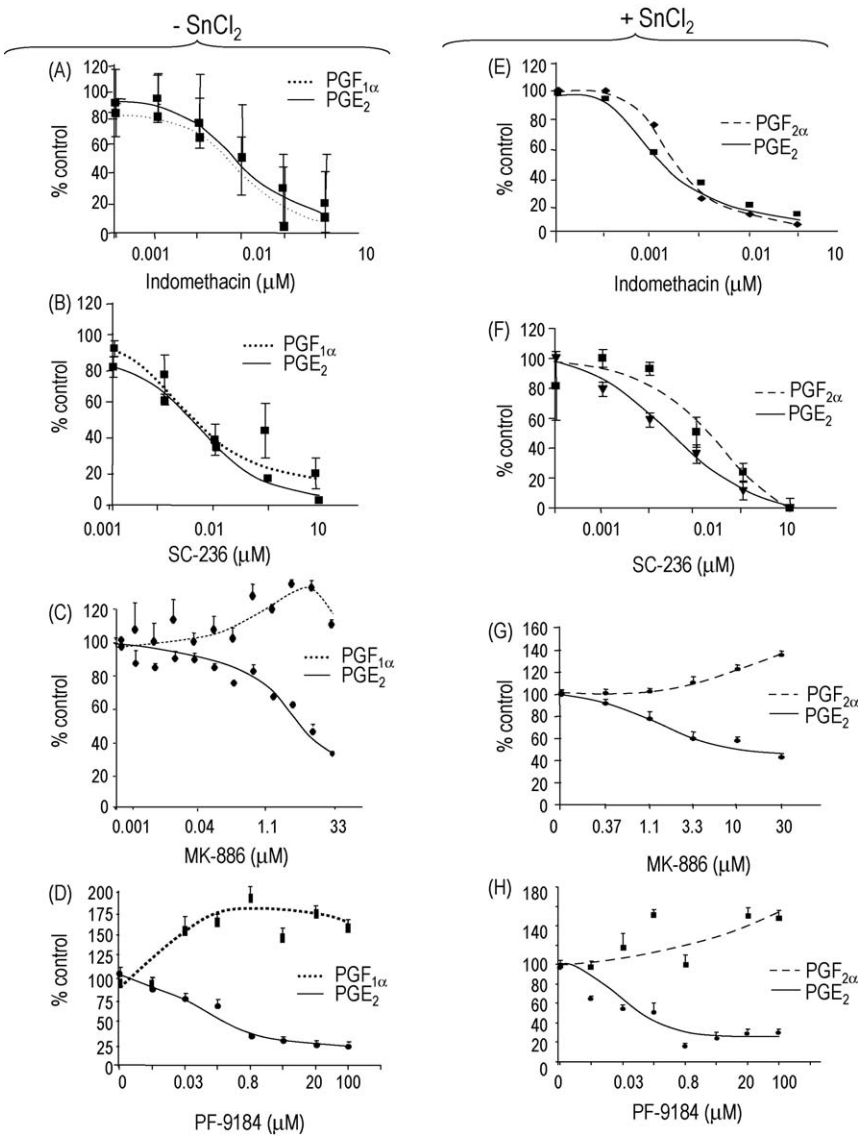

Fig. 5. mPGES-1 inhibition by PF-9184 is distinctive from COX-2 inhibition in RASF. IL-1β-stimulated RASF were pre-treated with indomethacin (A and E), SC-236 (B and F), MK-886 (C and G) or PF-9184 (D and H), then incubated with 10 μM arachidonic acid, except that 2 mg/ml $SnCl_2$ was added to the cultures before the addition of arachidonic acid (E–H). PGE_2 (solid lines), $PGF_{1\alpha}$ (dotted lines) and $PGF_{2\alpha}$ (dashed lines) levels were measured by ELISA. The data represent the mean ± S.E.M. of at least three independent determinations.

PF-9184 (competitive, reversible inhibitor), not SC-236 (a pseudo-irreversible COX-2 inhibitor) as compared to their potency in the LPS-stimulated blood assay (Fig. 7B and C). Together, the data indicates that in clinically relevant human blood assays, PF-9184 selectively inhibited PGE_2 synthesis by mPGES-1 while sparing TXB_2 synthesis by the COX-1/TXAS axis.

4. Discussion

We identified PF-9184 from an oxicam series as a potent and highly selective inhibitor of human mPGES-1. The selectivity of PF-9184 was further demonstrated in a variety of biological systems optimally set up for determining direct effects of the inhibitors on mPGES-1 function, not its expression. Indeed, we showed that in cell systems, PF-9184 potently blocked mPGES-1 ability to synthesize PGE_2 from PGH_2, and consequently, increased the levels of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ via a process known as shunting of the eicosanoids [7]. Furthermore, in the unstimulated human whole blood assays where mPGES-1 is expressed at very low levels, PF-9184 had no effect on PGE_2 and TXB_2 synthesis. Remarkably, the addition to the unstimulated blood of 1483 cells, which express mPGES-1 and COX-2 constitutively [27], restored the ability of PF-9184 to inhibit PGE_2 synthesis while still sparing TXB_2 synthesis. We also found that PF-9184 had no effect on 5-hydroxyeicosatetraenoic acid (5-HETE) synthesis by 5-lipoxygenase in cell and blood assays (data not shown). Since 1483 cells also express mPGES-2 and cPGES, to rule them out as potential targets of PF-9184 action, we could have used 1483 cells rendered unresponsive to PF-9184 effects by siRNA-mediated depletion of mPGES-1. However, the constitutive expression of mPGES-2 and cPGES, the lack of homology between these three proteins combined with the unclear functions of mPGES-2 and cPGES as genuine PGE_2 synthases [28,29] made this possibility unlikely. Collectively, our data demonstrates that PF-9184 is a selective mPGES-1 inhibitor.

Unexpectedly, we found that longer exposures (>16 h) of cultures to PF-9184 resulted in the inhibition of PGE_2 and $PGF_{1\alpha}$ synthesis, an observation that necessitated developing cell systems with shorter incubation times (<1 h). Although this was observed only at high inhibitor concentrations, it was

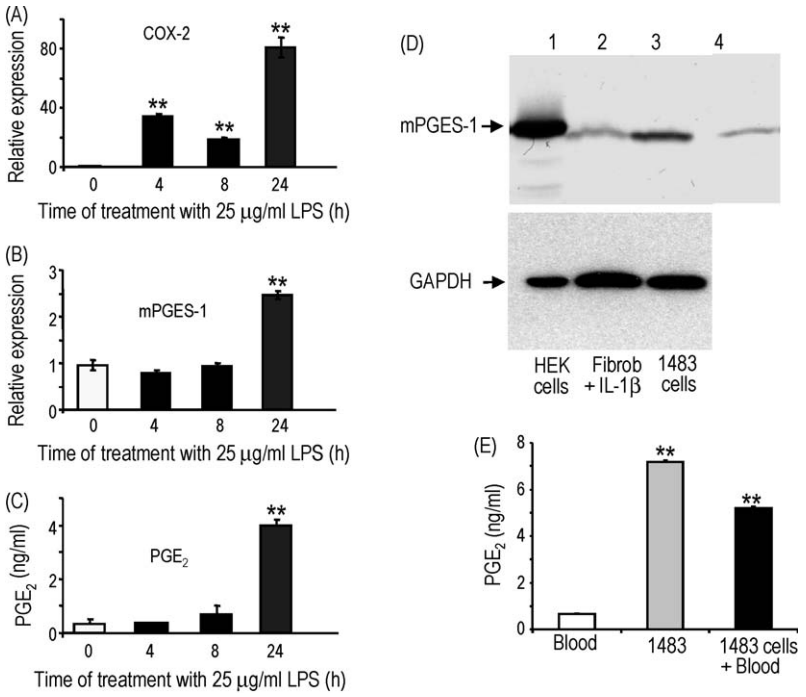

Fig. 6. Expression of mPGES-1 and COX-2 in LPS-stimulated human whole blood and cell lines. Human whole blood was stimulated with 20 μg/ml LPS for 24 h, and COX-2 (A) and mPGES-1 (B) mRNA expression was analyzed by TaqMan. (C) PGE_2 plasma levels from LPS-stimulated blood. (D) mPGES-1 protein expression by HEK cells (lane 1), IL-β-stimulated fibroblasts (lane 2) or 1483 cells (lane 3). Lane 4, recombinant human mPGES-1. GAPDH was used as control for protein loading. (E), PGE_2 synthesis in unstimulated blood (white columns), 1483 cells (gray columns) or mixtures of blood with 1483 cells (black columns), in the presence of added exogenous arachidonic acid. The data represent the mean ± S.E.M. of at least three independent determinations. $^{**}p < 0.005$, significance differences compared with time 0 (A–C) or blood (E).

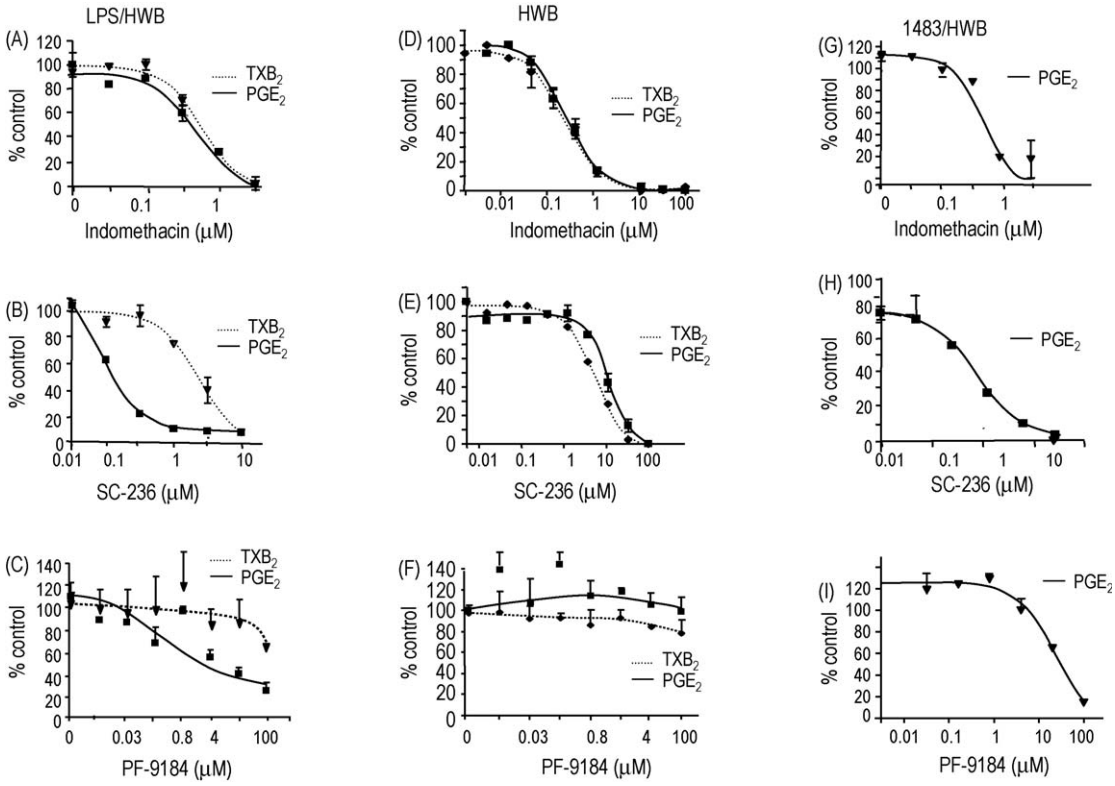

Fig. 7. mPGES-1 inhibition by PF-9184 is distinctive from COX-2 inhibition in human whole blood and modified blood assays. (A–C) Whole blood was stimulated with 20 μg/ml LPS for 24 h in the presence of the inhibitors. (D–F) Unstimulated whole blood was pre-incubated with the inhibitors for 50 min then treated with 30 μM arachidonic acid for 10 min. (G–I) Unstimulated whole blood mixed with 1483 cells was pre-incubated with the inhibitors before adding arachidonic acid. PGE_2 (solid lines) and TXB_2 (dotted lines) levels were measured by ELISA. The data represent the mean ± S.E.M. of at least three independent determinations.

however, in contrast to the described pharmacology profiles of other selective mPGES-1 inhibitors [15] as well as genetic data from some mPGES-1 knockout cells [7,9]. The reasons for this discrepancy are not clear but may be at least partially attributed to the difference in the cell models employed as we used RASF in this study instead of macrophages or the other cell types as reported [7,9,15]. In fact, differential profiles of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ synthesis was found between thioglycollate elicited macrophages and resident macrophages from mPGES-1 KO mice [8]. It may be that positive feedback mechanisms where RASF-produced PGE_2 contributes to sustained expression of mPGES-1 or COX-2 takes place. In this case, lower expression of COX-2 due to suppressed levels of PGE_2 in the presence of mPGES-1 inhibitors lead to decreased levels of PGH_2 needed for $PGF_{1\alpha}$ synthesis. This scenario would resemble an *in vivo* situation where the resolution of inflammation by PGE_2 blockers leads indirectly to decreased expression of inflammatory mediators, including mPGES-1 and COX-2, at the site of inflammation. Although PGE_2 was shown to up-regulate COX-2 expression in lung fibroblasts in synergy with IL-1β [30], this hypothesis was not tested in our study, and is beyond the scope of this manuscript.

Although we demonstrated that mPGES-1 expression was induced in the rat air pouch inflammatory model, we were not able to evaluate the efficacy of PF-9184 in this or other rodent inflammation models. Our findings that the mPGES-1 inhibitor, PF-9184, as well as several analogs initially identified using human mPGES-1, failed to inhibit mouse or rat enzyme made the studies unfeasible in rodent models. These observations are consistent with the recently published data where mPGES-1 inhibitors from distinct chemical series also failed to efficiently inhibit mouse or rat enzyme [15]. Interestingly, guinea pigs or engineered chimeric mice bearing human mPGES-1 had to be used for pharmacology studies with mPGES-1 inhibitors [15]. In our study, the efficacy of PF-9184 in the LPS/human whole blood as well as in the modified human whole blood assay supplemented with 1483 cells, where high levels of PGE_2 are produced acutely when exogenous arachidonic acid was added, is consistent with the notion that PF-9184 potently inhibits PGE_2 synthesis in clinically relevant inflammatory settings.

Despite the efficacy of NSAIDs and COXibs in relieving pain, some adverse events associated with the use of some of these drugs have been reported [31,32]. In agreement with these observations, indomethacin causes gastrointestinal (GI) tract lesions when administered to animals [15]. Thus, there remains an unmet need for analgesic agents with better safety profiles. The findings that indomethacin-induced GI tract lesions in animals can be alleviated by the administration of the prostacyclin agonist Beraprost [14], suggest that depletion of both PGE_2 and $PGF_{1\alpha}$ may be required for the development of GI tract lesions. Consistent with this notion, genetic or pharmacological mPGES-1 inhibition had no inhibitory effects on the synthesis of other prostanoids and did not cause GI tract lesions in animals [15]. Thus, the desirable profile of PF-9184 on eicosanoid metabolism is an incentive for future efforts aim at optimizing the proprieties of this class of mPGES-1 inhibitors. Ultimately, the determination whether the efficacy of these inhibitors as demonstrated in pre-clinical models translates to efficacy in human inflammatory diseases, is the main goal of this endeavor.

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