

The Molecular Pharmacology and In Vivo Activity of 2-(4-Chloro-6-(2,3-dimethylphenylamino)pyrimidin-2-ylthio)octanoic acid (YS121), a Dual Inhibitor of Microsomal Prostaglandin E₂ Synthase-1 and 5-Lipoxygenase

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ABSTRACT

The microsomal prostaglandin E₂ synthase (mPGES)-1 is one of the terminal isoenzymes of prostaglandin (PG) E₂ biosynthesis. Pharmacological inhibitors of mPGES-1 are proposed as an alternative to nonsteroidal anti-inflammatory drugs. We recently presented the design and synthesis of a series of pirinixic acid derivatives that dually inhibit mPGES-1 and 5-lipoxygenase. Here, we investigated the mechanism of mPGES-1 inhibition, the selectivity profile, and the in vivo activity of α -(*n*-hexyl)-substituted pirinixic acid [YS121; 2-(4-chloro-6-(2,3-dimethylphenylamino)pyrimidin-2-ylthio)octanoic acid] as a lead compound. In cell-free assays, YS121 inhibited human mPGES-1 in a reversible and noncompetitive manner (IC₅₀ = 3.4 μ M), and surface plasmon resonance spectroscopy studies using purified in vitro-translated human

mPGES-1 indicate direct, reversible, and specific binding to mPGES-1 (K_D = 10–14 μ M). In lipopolysaccharide-stimulated human whole blood, PGE₂ formation was concentration dependently inhibited (IC₅₀ = 2 μ M), whereas concomitant generation of the cyclooxygenase (COX)-2-derived thromboxane B₂ and 6-keto PGF_{1 α} and the COX-1-derived 12(S)-hydroxy-5-*cis*-8,10-*trans*-heptadecatrienoic acid was not significantly reduced. In carrageenan-induced rat pleurisy, YS121 (1.5 mg/kg i.p.) blocked exudate formation and leukocyte infiltration accompanied by reduced pleural levels of PGE₂ and leukotriene B₄ but also of 6-keto PGF_{1 α} . Taken together, these results indicate that YS121 is a promising inhibitor of mPGES-1 with anti-inflammatory efficiency in human whole blood as well as in vivo.

Prostaglandins (PGs) are potent lipid mediators that promote inflammatory reactions but also possess homeostatic functions (Funk, 2001). Their biosynthesis involves oxygenation of arachidonic acid by cyclooxygenase (COX)-1 or -2 to

PGH₂ and further conversion by PG synthases to the respective PGs (Funk, 2001). Inhibition of COX-1 and -2 by nonsteroidal anti-inflammatory drugs and selective suppression of COX-2 by coxibs are common and effective strategies for the therapy of inflammatory disorders, fever, and pain, but their long-term use is associated with severe side effects (Rainsford, 2007). Unselective COX-1/2 inhibitors may cause gastric toxicity, whereas an increased cardiovascular risk in

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ABBREVIATIONS: PG, prostaglandin; COX, cyclooxygenase; Tx, thromboxane; mPGES, microsomal prostaglandin E₂ synthase; pirinixic acid, 2-(4-chloro-6-(2,3-dimethylphenylamino)pyrimidin-2-ylthio)acetic acid; YS121, 2-(4-chloro-6-(2,3-dimethylphenylamino)pyrimidin-2-ylthio)octanoic acid; PPAR, peroxisome proliferator-activated receptor; MD52, 2-(2-chlorophenyl)-1*H*-phenanthro[9,10-*a*]-imidazole; DMSO, dimethyl sulfoxide; CV4151, (*E*)-7-phenyl-7-(3-pyridyl)-6-heptenoic acid; MK-886, 3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid; RP, reverse phase; HPLC, high-performance liquid chromatography; RM, reaction mixture; PBS, phosphate-buffered saline; SPR, surface plasmon resonance; 12-HHT, 12(S)-hydroxy-5-*cis*-8,10-*trans*-heptadecatrienoic acid; PAGE, polyacrylamide gel electrophoresis; LT, leukotriene; ANOVA, analysis of variance; HSD, honestly significant difference; ELISA, enzyme-linked immunosorbent assay.

patients after long-term intake of coxibs was evident, apparently due to an imbalance of prothrombotic COX-1-derived thromboxane (Tx) A₂ and antithrombotic and vasodilatory COX-2-derived PGI₂ (McGettigan and Henry, 2006). Selective suppression of proinflammatory PGE₂ biosynthesis by inhibition of microsomal prostaglandin E₂ synthase (mPGES)-1 without affecting the biosynthesis of homeostatic prostanoids (e.g., PGI₂) might overcome these limitations.

Among the three isomeric PGE₂ synthases, the inducible isoform mPGES-1 is essentially involved in massive PGE₂ formation, for example, during inflammation, fever, and pain (Samuelsson et al., 2007). mPGES-1, a homotrimeric membrane protein of the endoplasmic reticulum (Jegerschöld et al., 2008), commonly acts in concert with COX-2 (Murakami et al., 2000) and is strongly up-regulated by proinflammatory stimuli (e.g., interleukin-1 β and lipopolysaccharide) as well as under various pathological conditions (e.g., chronic inflammation, pain, fever, atherosclerosis, stroke, anorexia, and tumorigenesis) (Samuelsson et al., 2007). Genetic or pharmacological inhibition of mPGES-1 showed efficiency comparable with that of nonsteroidal anti-inflammatory drugs in animal models of inflammation, fever, and pain, while being essentially free of gastrointestinal adverse effects (Samuelsson et al., 2007; Xu et al., 2008). Deletion of mPGES-1 was not associated with cardiovascular complications, i.e., hypertension or thrombogenesis, at least in the absence of risk factors such as chronic salt loading (Wang et al., 2008).

Despite the increasing number of scientific reports and patents revealing novel mPGES-1 inhibitors (Friesen and Mancini, 2008; Koeberle and Werz, 2009), studies addressing the mechanistic basis and clinical relevance of mPGES-1 inhibition are rare (Xu et al., 2008; Koeberle et al., 2009b). Many mPGES-1 inhibitors with high efficiency in cell-free assays exhibit a strong loss of potency in cell-based assays, in particular in whole blood (Friesen and Mancini, 2008). In addition, these compounds fail to inhibit rodent mPGES-1 and thus are inactive in *in vivo* models based on mice or rats. We recently showed that target-oriented structural derivatization of pirinixic acid (2-(4-chloro-6-(2,3-dimethylphenylamino)pyrimidin-2-ylthio)acetic acid) (Fig. 1) led to α -substituted pirinixic acid derivatives such as YS121 (Fig. 1), which inhibits mPGES-1 (IC₅₀ = 3.4 μ M) (Koeberle et al., 2008b) and 5-lipoxygenase (IC₅₀ = 4.1 and 6.5 μ M in cell-based and cell-free assays, respectively) (Werz et al., 2008) and activates peroxisome proliferator-activated receptor (PPAR)- α and - γ (EC₅₀ = 1

and 3.6 μ M, respectively) (Rau et al., 2008). Here, we investigated the biochemical interaction of YS121 with mPGES-1. Moreover, we analyzed the interference of YS121 with prostanoid biosynthesis in human whole blood, and we assessed the anti-inflammatory efficacy in the carrageenan-induced rat pleurisy model.

Materials and Methods

Materials. YS121 was synthesized as reported previously (Koeberle et al., 2008b; Werz et al., 2008). Pirinixic acid was purchased from Sigma-Aldrich (Deisenhofen, Germany). The mPGES-1 inhibitor MD52 was synthesized according to Côté et al. (2007). The compounds were dissolved in DMSO and kept in the dark at -20°C, and freezing/thawing cycles were kept to a minimum. For animal studies, the compounds were dissolved in DMSO and diluted with saline to achieve a final DMSO concentration of 4%. The thromboxane synthase inhibitor CV4151 (Kato et al., 1985) was kindly provided by Dr. S. Laufer (University of Tübingen, Tübingen, Germany). The antibody against human mPGES-1 was from Cayman Chemical (Ann Arbor, MI). Antibodies against COX-2 and β -actin were obtained from Sigma-Aldrich. Materials used were as follows: Dulbecco's modified Eagle's high glucose (4.5 g/l) medium, penicillin, streptomycin, and trypsin/EDTA solution from PAA Laboratories GmbH (Coelbe, Germany); PGH₂ from Larodan (Malmö, Sweden); 11 β -PGE₂, MK-886, PGB₁, and enzyme immunoassay from Cayman Chemical; *N*-formyl-methionyl-leucyl-phenylalanine from Alexis Corporation (Lörrach, Germany); 1-lauroyl-2-hydroxy-*sn*-glycero-3-phosphocholine from Anatrace (Maumee, OH); λ -carrageenan type IV isolated from *Gigartina aciculata* and *Gigartina pistillata* and indomethacin was from Sigma-Aldrich (Milan, Italy); [³H]PGE₂ was from PerkinElmer Life Sciences (Milan, Italy); and PGE₂ antibody was from Sigma-Aldrich. All other chemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany) unless stated otherwise.

Cells. Human lung carcinoma A549 cells and murine RAW 264.7 cells were cultured in Dulbecco's modified Eagle's high glucose (4.5 g/l) medium supplemented with heat-inactivated fetal calf serum (10%, v/v), penicillin (100 U/ml), and streptomycin (100 μ g/ml) at 37°C in a 5% CO₂ incubator. After 3 days, confluent A549 cells were detached using 1 \times trypsin/EDTA solution and reseeded at 2 \times 10⁶ cells in 20 ml of medium in 175-cm² flasks.

Animals. Male adult Wistar Han rats (200–220 g; Harlan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care complied with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116192) as well as with the European Economic Community regulations (Official Journal of European Community L 358/1 12/18/1986).

Preparation of Crude mPGES-1 in Microsomes of A549 Cells and Determination of PGE₂ Synthase Activity.

Preparation of A549 cells and determination of mPGES-1 activity was performed as described previously (Koeberle et al., 2008a). In brief, cells were treated with 1 ng/ml interleukin-1 β for 48 h at 37°C and 5% CO₂. After sonification, the homogenate was subjected to differential centrifugation at 10,000g for 10 min and 174,000g for 1 h at 4°C. The pellet (microsomal fraction) was resuspended in 1 ml of homogenization buffer (0.1 M potassium phosphate buffer, pH 7.4, 1 mM phenylmethanesulfonyl fluoride, 60 μ g/ml soybean trypsin inhibitor, 1 μ g/ml leupeptin, 2.5 mM glutathione, and 250 mM sucrose), and the total protein concentration was determined. Microsomal membranes were diluted in potassium phosphate buffer (0.1 M, pH 7.4) containing 2.5 mM glutathione. Test compounds or vehicle were added, and after 15 min at 4°C, the reaction (100 μ l total volume) was initiated by addition of PGH₂ (20 μ M, final concentration). After 1 min at 4°C, the reaction was terminated using stop solution (100 μ l; 40 mM FeCl₂, 80 mM citric acid, and 10 μ M 11 β -PGE₂ as internal

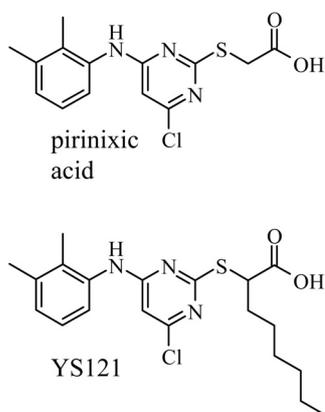


Fig. 1. Chemical structures of pirinixic acid and YS121.

standard). PGE₂ was separated by solid-phase extraction and analyzed by RP-HPLC as described previously (Koeberle et al., 2008a).

Cell-Free Expression of Human mPGES-1. Human mPGES-1 was obtained by the continuous-exchange cell-free expression system according to Schwarz et al. (2007). This system comprises a reaction mixture (RM) that contains the *Escherichia coli* S30 extract (derived from the A19 strain), T7 polymerase, tRNAs, pyruvate kinase, and the template DNA for human mPGES-1 (cloned in the pBH4 vector derived from pET19b; Novagen, Madison, WI). The RM is dialyzed against the feeding mixture that supplies amino acids, energy equivalents acetyl phosphate and phosphoenol pyruvate, and nucleotides. Reactions were incubated at 30°C for up to 20 h. Protein synthesis takes place in the RM and up to 1.5 mg of mPGES-1/ml RM can be obtained. mPGES-1 was resuspended in 50 mM potassium phosphate buffer, pH 7.4, 1 mM glutathione, 10% glycerol, and 2% (w/v) 1-lauroyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LysoFos12 choline; Anatrace, Maumee, OH) for 2 h at 30°C, and insoluble parts were removed by centrifugation (10,000g 10 min, 10°C).

Determination of the Activity of In Vitro-Translated mPGES-1. After resolubilization, the in vitro-translated mPGES-1 was incorporated into liposomes, which were prepared by sonification of phosphatidylethanolamine [0.15 mM in potassium phosphate buffer (0.1 M), pH 7.4, containing 2.5 mM glutathione] for 1 min on ice. The PGE₂ synthase activity of these liposomal preparations was determined as described for microsomal preparations of interleukin-1β-stimulated A549 cells. The activity of the in vitro-translated mPGES-1 was 0.5 U/mg (where 1 U is defined as the amount of enzyme that forms 1 μmol of PGE₂/min under standard assay conditions, i.e., 20 μM PGH₂, 2.5 mM glutathione, 4°C, pH 7.4) that corresponds to 0.5 μmol of PGE₂ formed/mg of protein/min.

Determination of PGE₂ Formation in Intact RAW 264.7 Cells. Expression of mPGES-1 in RAW 264.7 cells was induced by incubation with lipopolysaccharide (1 μg/ml) for 20 h. Cells were washed twice with PBS, resuspended in PBS (10⁶/ml), and preincubated with the indicated compounds at 37°C for 10 min. PGE₂ formation was started by the addition of arachidonic acid (1 μM). The reaction was stopped after 15 min at 37°C, and the samples were put on ice. For quantification of PGE₂, samples were extracted, fractionated by HPLC, and then quantified using a PGE₂ High Sensitivity EIA Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's protocol.

SPR Spectroscopy. In vitro-translated mPGES-1 (100 μg/ml) in 10 mM sodium acetate buffer (pH 6.0) was coupled to a carboxymethylated dextran surface (CM-5 chip; GE Healthcare, Munich, Germany) using a standard amine coupling procedure according to the manufacturer's instructions. Flow cell 1 on the chip was not altered (reference), whereas on flow cell 2, mPGES-1 (236 fmol/mm² corresponding to 4700 resonance units) or cathepsin G (112 fmol/mm² corresponding to 2500 resonance units, used as negative control) was immobilized. Then the chip surface was equilibrated by a continuous flow of assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% surfactant P20, and 1% DMSO, pH 7.4), the stock solutions of test compounds in DMSO were diluted into assay buffer, and measurements were performed on a BIAcore X device (GE Healthcare) at 25°C and a flow rate of 30 μl/min. After the association of the test compounds to flow cell 2, the mobile phase was replaced by assay buffer, and the dissociation was monitored. The binding profiles were obtained after subtracting the response signals of the untreated reference cell 1, and the resulting sensograms were processed by using automatic correction for nonspecific bulk refractive index effects (BIAevaluation software, version 3.1).

To obtain the dissociation constants from the equilibrium binding data, two different fitting models were adopted. First, the change in the equilibrium amount of compound bound as a function of the concentration of compound was fitted to the equation for a simple 1:1 binding model [$R_{eq} = (R_{max} \times [\text{compound}]) / (K_D + [\text{compound}])$], where R_{eq} is the equilibrium response, R_{max} is the maximum response, and K_D is the dissociation constant. Alternatively, Scatchard

plot analysis was used to estimate K_D values. Kinetic analysis were performed using BIAevaluation software (version 3.1) by fitting an integrated rate equation describing a 1:1 Langmuir interaction simultaneously to the entire concentration range for YS121. This fit yielded the association rate k_a , the dissociation rate k_d , and the dissociation constant K_D (Roden and Myszk, 1996; Karlsson and Fält, 1997). The quality of the fit was determined by the χ^2 values as well as by the magnitude and distribution of the residuals.

Determination of Prostanoid Formation in Human Whole Blood. Peripheral blood from healthy adult volunteers, who had not received any medication for at least 2 weeks under informed consent, was obtained by venipuncture and collected in syringes containing heparin (20 U/ml). For determination of PGE₂ and 6-keto PGF_{1α}, aliquots of whole blood (0.8 ml) were mixed with CV4151 (1 μM) and with aspirin (50 μM). For determination of TxB₂, aliquots of whole blood (0.5 ml) were used without addition of CV4151. A total volume of 1 ml was adjusted with sample buffer (10 mM potassium phosphate buffer, pH 7.4, 3 mM KCl, 140 mM NaCl, and 6 mM *d*-glucose). After preincubation with the indicated compounds for 5 min at room temperature, the samples were stimulated with lipopolysaccharide (10 μg/ml) for 5 h at 37°C. Prostanoid formation was stopped on ice, the samples were centrifuged (2300g, 10 min, 4°C), and 6-keto PGF_{1α} and TxB₂ were quantified in the supernatant using High Sensitivity EIA Kits for 6-keto PGF_{1α} and TxB₂, respectively, according to the manufacturer's protocols. PGE₂ was determined as described previously (Koeberle et al., 2009b). In brief, the supernatant was acidified with citric acid (30 μl, 2 M), and after centrifugation (2300g, 10 min, 4°C), solid-phase extraction, and RP-HPLC, analysis of PGE₂ was performed to isolate PGE₂. The PGE₂ peak (3 ml), identified by coelution with the authentic standard, was collected, and acetonitrile was removed under a nitrogen stream. The pH was adjusted to 7.2 by addition of 10× PBS buffer, pH 7.2 (230 μl) before PGE₂ contents were quantified using a PGE₂ High Sensitivity EIA Kit according to the manufacturer's protocol.

For determination of 12(*S*)-hydroxy-5-*cis*-8,10-*trans*-heptadecatrienoic acid (12-HHT), human whole blood (2 ml) was preincubated with the indicated compounds at 37°C for 5 min, and formation of 12-HHT was initiated by addition of 30 μM Ca²⁺-ionophore A23187 and 100 μM arachidonic acid. After 10 min at 37°C, the reaction was stopped on ice, and the samples were centrifuged (600g, 10 min, 4°C). Aliquots of the resulting plasma (500 μl) were then mixed with 2 ml of methanol, and 200 ng of PGB₁ was added as internal standard. The samples were placed at -20°C for 2 h and centrifuged again (600g, 15 min, 4°C). The supernatants were collected and diluted with 2.5 ml of PBS, and 75 μl of 1 N HCl was added. Formed 12-HHT was extracted and analyzed by HPLC as described previously (Albert et al., 2002).

SDS-PAGE and Western Blot. Cells (4 × 10⁶ cells) were resuspended in 50 μl of PBS buffer (pH 7.2), mixed with the same volume of 2× SDS/PAGE sample loading buffer [20 mM Tris-HCl, pH 8, 2 mM EDTA, 5% (m/v) SDS, and 10% (v/v) β-mercaptoethanol], and boiled for 5 min at 95°C. Aliquots (20 μl) corresponding to equivalents of 0.8 × 10⁶ cells were mixed with 4 μl of glycerol-0.1% bromophenol blue (1:1, v/v), and proteins were separated by SDS-PAGE. After electroblotting to nitrocellulose membrane (GE Healthcare) and blocking with 5% bovine serum albumin for 1 h at room temperature, membranes were washed and incubated with primary antibodies overnight at 4°C. The membranes were washed and incubated with a 1:1000 dilution of alkaline phosphatase-conjugated immunoglobulin G for 3 h at room temperature. After washing, proteins were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Carrageenan-Induced Pleurisy in Rats. YS121 (1.5 mg/kg) or indomethacin (5 mg/kg) was given intraperitoneally 30 min before carrageenan. A group of male rats received the vehicle (DMSO, 4%, intraperitoneally) 30 min before carrageenan. Rats were anesthetized with enflurane 4% mixed with 0.5 l/min O₂ and 0.5 l/min N₂O and submitted to a skin incision at the level of the left sixth inter-

costal space. The underlying muscle was dissected, and saline (0.2 ml) or 1% λ -carrageenan type IV (w/v, 0.2 ml) was injected into the pleural cavity. The skin incision was closed with a suture, and the animals were allowed to recover. At 4 h after the injection of carrageenan, the animals were killed by inhalation of CO₂. The chest was carefully opened, and the pleural cavity was rinsed with 2 ml of saline solution containing heparin (5 U/ml). The exudate and washing solution were removed by aspiration, and the total volume was measured. Any exudate that was contaminated with blood was discarded. The amount of exudates was calculated by subtracting the volume injected (2 ml) from the total volume recovered. Leukocytes in the exudates were resuspended in PBS and counted with an optical light microscope in a Burkner's chamber after vital trypan blue staining.

The amounts of PGE₂, LTB₄, and 6-keto PGF_{1 α} in the supernatant of centrifuged exudate (800g for 10 min) were assayed by radioimmunoassay (PGE₂) and enzyme immunoassay (LTB₄ and 6-keto PGF_{1 α}), respectively (Cayman Chemical), according to the manufacturer's protocol. The results are expressed as nanograms per rat and represent the mean \pm S.E. of 10 rats.

Statistics. Data are expressed as mean \pm S.E. Concentration-response curves were fitted to a one-side binding competition equation, and IC₅₀ values were determined using SigmaPlot 9.0 (Systat Software Inc., San Jose, CA). The program GraphPad Instat (GraphPad Software Inc., San Diego, CA) was used for statistical comparisons. Statistical evaluation of the data was performed by one-way or two-way ANOVAs for independent or correlated samples followed by Tukey HSD post hoc tests. $P < 0.05$ was considered significant.

Results

YS121 Is a Reversible and Noncompetitive Inhibitor of mPGES-1. In agreement with previous studies (Koeberle et al., 2008b), YS121 concentration-dependently inhibited the mPGES-1-mediated conversion of PGH₂ to PGE₂ (IC₅₀ of

3.4 μ M, essentially complete inhibition at 30 μ M) in microsomal preparations of interleukin-1 β -stimulated human A549 cells (Fig. 2A). Suppression of other PGs such as PGD₂ could not be observed (data not shown). As observed for other mPGES-1 inhibitors that fail to inhibit the rodent enzyme (Friesen and Mancini, 2008), YS121 hardly affected the activity of mPGES-1 in murine RAW264.7 macrophages (\sim 30% inhibition at 30 μ M) and MD52 (2 μ M, reference mPGES-1 inhibitor) did not significantly inhibit PGE₂ formation (data not shown). To investigate whether YS121 inhibits human mPGES-1 activity in a reversible manner, washout experiments were performed. Thus, microsomes from IL-1 β -stimulated A549 cells were preincubated with YS121 at 1 or 10 μ M for 15 min. The sample containing 10 μ M YS121 was split, and one aliquot was diluted 10-fold to obtain a final inhibitor concentration of 1 μ M. Then, PGE₂ formation was initiated by addition of PGH₂. At a concentration of 1 μ M, YS121 did not significantly reduce PGE₂ formation (16.9 \pm 4.8% inhibition, $P > 0.05$), whereas 10 μ M YS121 significantly inhibited PGE₂ synthesis by 60.6 \pm 5% ($P < 0.001$) (Fig. 2B). Dilution of the sample containing 10 μ M YS121 restored mPGES-1 activity (Fig. 2B), suggesting a reversible mode of inhibition. Although the diluted sample still contained 1 μ M YS121, PGE₂ formation was entirely reversed for unknown reasons.

Next, we attempted to define whether YS121 competes with the substrate (PGH₂) to inhibit mPGES-1 activity. In the mPGES-1 assay using microsomes of IL-1 β -stimulated A549 cells (total protein = 87 μ g/ml), the substrate concentration was varied over a concentration range of 10 to 50 μ M PGH₂, and the rate of PGE₂ formation was determined. Fitting of the data for the noninhibited reaction to a Michaelis-Menten kinetic model (Fig. 2C) and reciprocal linear regres-

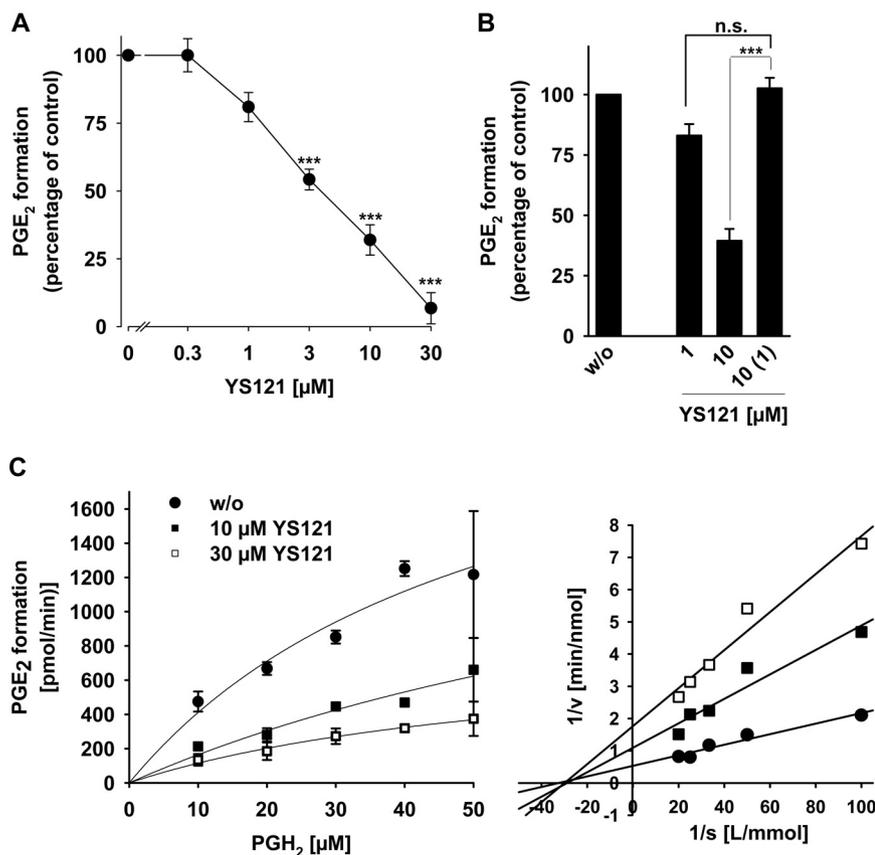


Fig. 2. YS121 inhibits mPGES-1 in a reversible and noncompetitive manner. A, microsomal preparations of interleukin-1 β -stimulated A549 cells were preincubated with vehicle (DMSO, w/o) or the test compounds at the indicated concentrations for 15 min at 4°C, and the reaction was started with 20 μ M PGH₂. After 1 min at 4°C, the reaction was terminated using a stop solution containing FeCl₂ and 11 β -PGE₂ (1 nmol) as internal standard. The 100% values in the individual experiments are in the range of 3 to 4 μ g/ml PGE₂ and correspond to enzyme activities of 6 to 18 μ mol of PGE₂/mg of protein/min. MK-886, used as control, inhibited PGE₂ formation with an IC₅₀ of 2.2 μ M. Data are given as means \pm S.E. ($n = 3-4$). ***, $P < 0.001$ versus vehicle (0.1% DMSO) control, ANOVA + Tukey HSD post hoc tests. B, reversibility of mPGES-1 inhibition by YS121. Microsomal preparations of interleukin-1 β -stimulated A549 cells were preincubated with 10 μ M YS121 for 15 min at 4°C. An aliquot was diluted 10-fold to obtain an inhibitor concentration of 1 μ M. Then 20 μ M PGH₂ was added (no dilution), all samples were incubated for 1 min on ice, and PGE₂ formation was analyzed by RP-HPLC as described. Data are given as means \pm S.E. ($n = 3-4$). ***, $P < 0.001$. C, the activity of mPGES-1 was determined at different PGH₂ and YS121 concentrations as indicated (left), and Lineweaver-Burk analysis (right) was performed. Data are given as means \pm S.E. ($n = 2-4$).

sion analysis (Lineweaver-Burk plot) (Fig. 2C) yielded K_m values of 54 and 30 μM and V_{max} values of 2.6 and 1.9 nmol/min, respectively. Variation of the PGH_2 concentration (10–50 μM) and subsequent Lineweaver-Burk analysis indicate a noncompetitive type of inhibition by YS121 (Fig. 2C). These results preclude preferential binding of YS121 to the PGH_2 binding pocket of mPGES-1.

Characterization of the Binding of YS121 to mPGES-1 by SPR Spectroscopy. The direct (physical) interaction of YS121 with human mPGES-1 was analyzed using SPR spectroscopy. Purified *in vitro*-translated mPGES-1 (236 fmol of mPGES-1/ mm^2) with a specific activity of 0.5 U/mg (when tested in a cell-free assay in which mPGES-1 was first embedded in liposomes and then supplied with PGH_2 as substrate) was immobilized to one of the two flow cells of the CM5 sensor chip. YS121 was diluted into assay buffer (1.25–10 μM) and sequentially injected over the chip surface. For calculating the binding responses, the response of the untreated reference cell was subtracted from the response of the mPGES-1 surface. Figure 3A shows the corrected sensograms for different concentrations of YS121 (top left panel). The maximal concentration of YS121 was limited to 10 μM because unspecific (superstoichiometric) binding to mPGES-1 and to the reference surface was observed at concentrations >10 μM . The unspecific binding at high analyte concentrations could be minimized by inclusion of 0.01% detergent (P20) in the running buffer, at least at concentrations of YS121 <10 μM .

To discriminate unspecific binding behavior of YS121, the structurally related lead compound pirinixic acid, which did not inhibit mPGES-1 activity (Koeberle et al., 2008b), was analyzed. Pirinixic acid failed to interfere with mPGES-1 up to 40 μM (Fig. 3B). Furthermore, YS121 (up to 20 μM) failed to bind to an immobilized reference protein, i.e., cathepsin G (Fig. 3C) (previously used in SPR studies; U. Siemoneit and O. Werz, unpublished data), which is also not functionally affected by YS121 (data not shown). Taken together, these results indicate that YS121 binds directly and selectively to mPGES-1.

For determination of the equilibrium binding constants of YS121, we fitted the equilibrium response (R_{eq}) as a function of the analyte concentration to a 1:1 interaction model, and the fit was superimposed over each data set (Fig. 3D). Dissociation constants (K_D) of 14 and 11 μM were calculated from the nonlinear fit (top panel) and for the Scatchard plot (bottom panel), respectively. The kinetic parameters (rate constants) of the association and dissociation phases were obtained by fitting the data sets to a 1:1 Langmuir binding model using BIAevaluation software (version 3.1) (Rich et al., 2001). The dissociation constant K_D was calculated from the rate constants ($k_d/k_a = 0.14 \text{ s}^{-1}/13,900 \text{ M}^{-1} \text{ s}^{-1} = K_D = 10 \mu\text{M}$) and is consistent with the K_D determined by equilibrium measurements (Fig. 3A). The actual K_D values are similar to the IC_{50} value of 3.4 μM obtained in the cell-free mPGES-1 activity assay, supporting a functional correlation between mPGES-1 binding and inhibition.

Effects of YS121 on Eicosanoid Formation in Human Whole Blood. Many potent mPGES-1 inhibitors constitute highly lipophilic molecules with strong albumin-binding features and this abolishes their efficiency in biological assays (Friesen and Mancini, 2008). The effectiveness of YS121 to inhibit cellular COX-2-derived PGE_2 biosynthesis was investigated in a human whole blood assay. The COX-2 selective

inhibitor celecoxib and the mPGES-1 inhibitor MD52 were used as controls. First, heparinized blood was preincubated with the thromboxane synthase inhibitor CV4151 (1 μM) and the test compounds and stimulated with lipopolysaccharide (10 $\mu\text{g}/\text{ml}$) for 5 h, and then formed PGE_2 was separated by RP-HPLC and quantified by ELISA (Koeberle et al., 2009b). In agreement with previous studies, MD52 (which inhibits human mPGES-1 with an $\text{IC}_{50} = 87 \text{ nM}$ in cell-free assays) (Côté et al., 2007) maximally suppressed PGE_2 synthesis in whole blood by $48.3 \pm 7.7\%$ at high concentrations (2–6 μM), whereas celecoxib (20 μM) efficiently inhibited PGE_2 formation under these conditions ($78.3 \pm 8.4\%$; $\text{IC}_{50} = 0.87 \mu\text{M}$) (Riendeau et al., 2001). YS121 concentration-dependently inhibited PGE_2 formation with an IC_{50} value of 3 μM (Fig. 4A), and, in analogy with MD52, complete inhibition of PGE_2 synthesis could not be achieved even at 30 μM . Of interest, the COX-2/ PGI_2 synthase-derived 6-keto $\text{PGF}_{1\alpha}$ (a stable metabolite of PGI_2) was not significantly reduced (Fig. 4A). In the absence of CV4151, PGE_2 synthesis was only moderate but still was suppressed by YS121 ($\text{IC}_{50} \sim 5 \mu\text{M}$; data not shown), whereas the concomitant, strong generation of COX-2-derived TxB_2 was not inhibited (Fig. 4A). These data suggest that YS121 does not suppress PGH_2 formation (i.e., by COX inhibition) but instead inhibits transformation of PGH_2 to PGE_2 . Furthermore, we investigated the effects of YS121 in another human whole-blood assay under stimulation conditions, designed to analyze COX-1 activity. After preincubation with YS121, Ca^{2+} -ionophore plus arachidonic acid (100 μM) was added to induce 12-HHT formation (predominantly produced by constitutively expressed COX-1 under these experimental conditions). YS121 failed to significantly suppress 12-HHT formation up to 30 μM (Fig. 4B).

Effects of YS121 on Interleukin-1 β -Mediated COX-2/mPGES-1 Expression. α -Alkyl-substituted pirinixic acid derivatives such as YS121 are dual agonists of PPAR α and γ (Rau et al., 2008). Because PPAR α/γ agonists were described to down-regulate the expression of COX-2 (Inoue et al., 2000; Grau et al., 2006) and mPGES-1 (Cheng et al., 2004), we analyzed the effects of YS121 on the expression of COX-2 and mPGES-1 protein in IL-1 β -treated A549 cells. Stimulation with interleukin-1 β (1 ng/ml) led to increased expression of mPGES-1 and of COX-2 protein within 24 h compared with that in unstimulated cells, which were not affected by YS121 (10 μM) (Fig. 5). Incubation in the presence of YS121 (10 μM) led to a decrease in COX-2 expression after 24 to 48 h, whereas expression of mPGES-1 was not affected within 48 h.

YS121 Suppresses Carrageenan-Induced Pleurisy in Rats. The anti-inflammatory effectiveness of YS121 was assessed *in vivo* using carrageenan-induced pleurisy in rats. Injection of carrageenan into the pleural cavity of rats (DMSO 4% group) elicited an inflammatory response within 4 h, characterized by the accumulation of fluid that contained large numbers of inflammatory cells (Table 1). Based on *in vivo* studies performed previously (Feisst et al., 2009; Rossi et al., 2009) with compounds having similar pharmacological profiles *in vitro*, the dose of YS121 was chosen at 1.5 mg/kg *i.p.*, 30 min before carrageenan. YS121 significantly inhibited the inflammatory response as demonstrated by the significant attenuation of exudate formation (62%) and cell infiltration (40%). Indomethacin (5 mg/kg) also reduced exudate formation and cell infiltration (75 and 65%, respectively) (Table 1). In comparison with the corresponding exudates

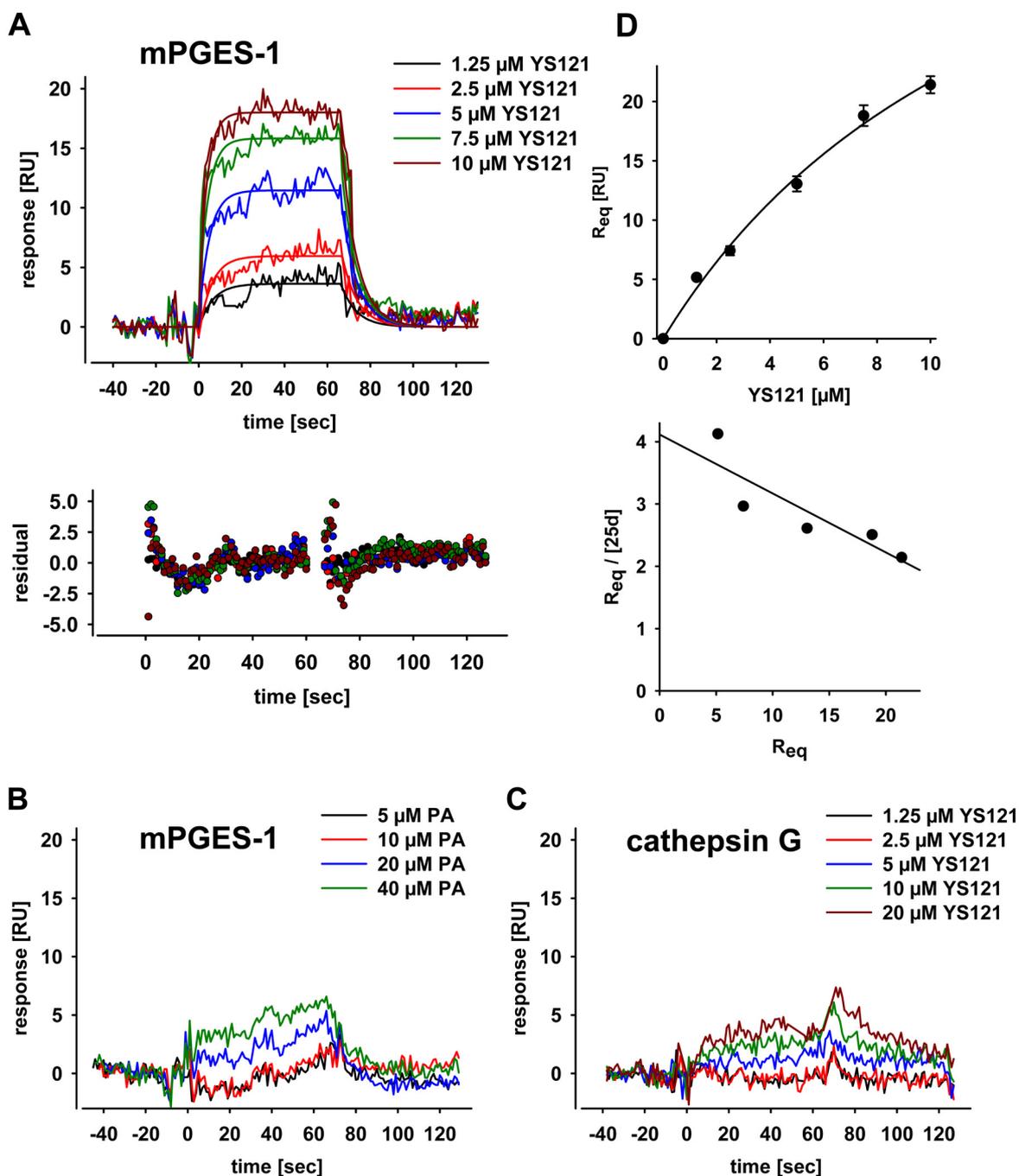


Fig. 3. Analysis of the interaction of YS121 with mPGES-1 by SPR spectroscopy. The test compounds at the indicated concentrations were injected sequentially over the mPGES-1 (236 fmol/mm²) or the cathepsin G (112 fmol/mm²) surface as well as over the untreated reference surface at a continuous flow of 30 μ l/min. Sensograms were obtained by subtracting the response of the reference cell from the cell with the immobilized enzyme. A, binding of YS121 to mPGES-1. For kinetic evaluation of inhibitor binding, the data were fitted to a 1:1 Langmuir binding model, and the results are shown by solid lines (top). The bottom panel shows the distribution of the residuals to allow evaluation of the quality of the fitting model. The residuals are the difference between the response data (observed value) and the estimated value, derived from the fitting model adopted to represent the response. B, binding of pirinixic acid (PA) to mPGES-1. C, binding of YS121 to cathepsin G. Sensograms are representative of three independent experiments. D, the equilibrium responses R_{eq} were plotted against the indicated concentrations of YS121 (top), and the corresponding Scatchard plots are given (bottom). The solid lines represent the nonlinear fit to a simple 1:1 interaction model. Data are given as means \pm S.E. ($n = 3$). RU, resonance units.

from DMSO-treated rats, exudates of YS121-treated animals exhibited decreased PGE₂ levels (36% inhibition), whereas indomethacin almost completely suppressed PGE₂ (88%) as well as 6-keto PGF_{1 α} (94%) levels as expected. On the other hand, indomethacin failed to significantly reduce LTB₄ levels, which were lowered by YS121 (48% inhibition), seemingly because of direct inhibition of 5-lipoxygenase (Werz et

al., 2008). We were surprised to find that YS121 also reduced the levels of 6-keto PGF_{1 α} (45% inhibition).

Discussion

We showed before that α -substituted pirinixic acid derivatives (that activate PPAR α/γ) dually inhibit mPGES-1 and

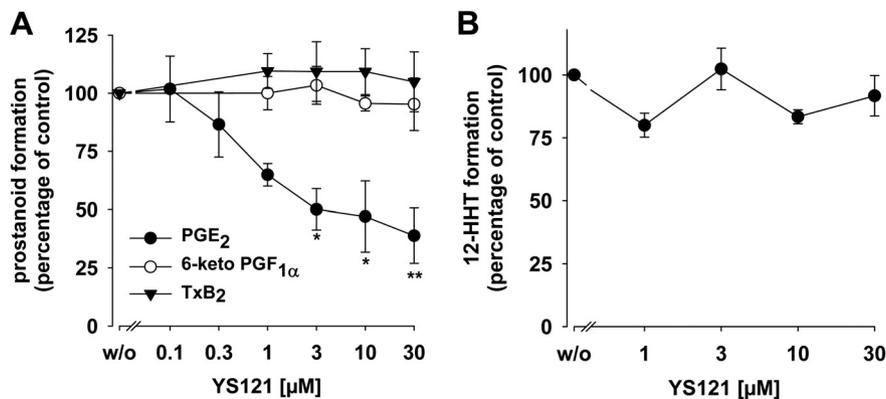


Fig. 4. Effects of YS121 on prostanoid formation in human whole blood. A, PGE₂, TxB₂, and 6-keto PGF_{1α} formation. Heparinized human whole blood, treated without (for determination of TxB₂) or with 1 μM thromboxane synthase inhibitor and 50 μM aspirin (for determination of PGE₂ and 6-keto PGF_{1α}) was preincubated with the indicated concentrations of YS121 or vehicle (DMSO, w/o) for 5 min at room temperature, and then prostanoid formation was induced by addition of 10 μg/ml lipopolysaccharide. After 5 h at 37°C, PGE₂ was extracted from plasma by RP-18 solid-phase extraction, separated by RP-HPLC, and quantified by ELISA as described. The 100% value corresponds to 10 to 22 ng/ml PGE₂ in the individual experiments, respectively; the PGE₂ levels in unstimulated blood were 1 to 3 ng/ml. MD52 (2 μM) and celecoxib (20 μM) were used as controls and inhibited PGE₂ formation by 48 ± 8 and 78 ± 8%, respectively. 6-Keto PGF_{1α} was directly determined in blood plasma by ELISA. The 100% value corresponds to 4 to 5 ng/ml 6-keto PGF_{1α} (unstimulated ≤ 1 ng/ml) in the individual experiments, respectively. Celecoxib (20 μM) inhibited 6-keto PGF_{1α} formation by 85 ± 4%. Indomethacin (20 μM) completely inhibited TxB₂ synthesis. B, 12-HHT formation. Heparinized whole blood was preincubated with YS121 or vehicle (DMSO, w/o) at the indicated concentrations for 5 min, and arachidonic acid (100 μM) and Ca²⁺-ionophore (30 μM) were added to induce COX product formation. After 10 min at 37°C, 12-HHT was extracted and analyzed by RP-HPLC as described. The 100% value corresponds to 1 to 2 μg/ml 12-HHT in the individual experiments, respectively. Indomethacin (20 μM) was used as control and inhibited 12-HHT formation by 90 ± 2%. Data are given as mean ± S.E. (*n* = 3–4). *, *P* < 0.05; **, *P* < 0.01, versus vehicle (0.1% DMSO) control, ANOVA + Tukey HSD post hoc tests.

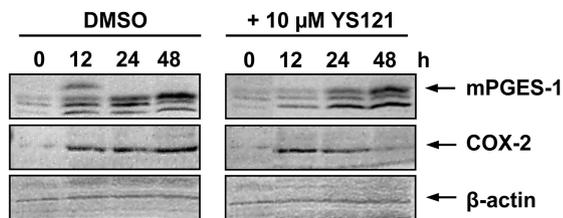


Fig. 5. Effects of YS121 on the expression of COX-2 and mPGES-1. A549 cells, 60% confluent, were incubated with 1 ng/ml interleukin-1β together with vehicle (DMSO) or with YS121 (10 μM) in cell culture medium containing 2% (v/v) fetal calf serum. Cells were harvested after the indicated periods, and total cell lysates were prepared and analyzed for induction of COX-2 (72 kDa) and mPGES-1 (16 kDa) using SDS-PAGE and Western blotting. β-Actin (45 kDa) was used as loading control. Data are representative of two independent experiments.

5-lipoxygenase but only moderately affect COX enzymes (Koeberle et al., 2008b; Werz et al., 2008). Here, we have characterized the biochemical interaction of the lead compound YS121 with human mPGES-1, and we assessed its efficacy and pharmacological profile in human whole blood and in an animal model of inflammation. Our data demonstrate that YS121 selectively and reversibly binds mPGES-1 as demonstrated by SPR spectroscopy and at comparable concentrations inhibits mPGES-1 activity in a concentration-dependent, reversible, noncompetitive manner. In human whole blood, YS121 inhibits PGE₂ formation without affecting the synthesis of COX-2-derived 6-keto PGF_{1α} and TxB₂ or COX-1-derived 12-HHT. Finally, the anti-inflammatory efficacy of YS121 *in vivo* is demonstrated.

YS121 suppressed PGE₂ formation in intact A549 cells (Koeberle et al., 2008b) and as shown here also in human whole blood. Cell-based test systems often fail to unequivocally specify molecular targets of a given inhibitor and to provide concrete insights into the molecular inhibitory mechanisms. Thus, reduced formation of PGE₂ in the cell-based assays might also be due to interference with upstream signaling events (e.g., protein kinases or Ca²⁺ signaling) or to

modulation of the preceding substrate supply (i.e., inhibition of phospholipase A₂ or COX enzymes). However, YS121 hardly inhibited isolated COX-1 and -2 (Koeberle et al., 2008b), and the activity of isolated cytosolic phospholipase A₂ is not affected up to 10 μM (M. Verhoff, C. Greiner, and O. Werz, unpublished data). In addition, the failure of YS121 to block the expression of mPGES-1 in A549 cells excludes interference at the transcriptional level. Instead, YS121 directly suppressed mPGES-1 activity in a cell-free assay (microsomal preparations of IL-1β-stimulated human A549 cells). Accordingly, YS121 (in contrast with indomethacin and celecoxib) solely inhibited PGE₂ formation in human whole blood but failed to block the formation of COX-2-derived 6-keto PGF_{1α} and TxB₂ or COX-1-derived 12-HHT. Suppression of PGE₂ formation (in cell-based models of human origin) is consequently not the result of an interruption of the release and transformation of arachidonic acid to PGH₂ but instead relates to inhibition of PGE₂ formation from PGH₂ because of interference with mPGES-1. Direct interference of YS121 with mPGES-1 is demonstrated in two ways: 1) by suppression of mPGES-1-mediated transformation of PGH₂ to PGE₂ in the cell-free assay and 2) by physical interaction of YS121 with mPGES-1 using SPR spectroscopy, yielding concrete data on rate constants of the association and dissociation phase, the equilibrium constants, and the stoichiometry. Reliable controls are obligatory for binding studies to exclude unspecific interactions between analyte, immobilized macromolecule, or surrounding matrix (Rich and Myszk, 2008). In fact, at concentrations >10 μM, YS121 also binds to the reference dextran surface and causes aggregation or micelle formation with superstoichiometric binding pattern (Giannetti et al., 2008). Hence, binding experiments were performed at concentrations <10 μM YS121 and were validated by using cathepsin G as an indifferent control protein or by use of the inactive pirinixic acid as analyte. A marked binding response was only evident between mPGES-1 and YS121, supporting a specific interaction. Investigation of

TABLE 1

Effect of YS121 on carrageenan-induced pleurisy in rats

Thirty minutes before intrapleural injection of carrageenan, male rats were treated intraperitoneally with YS121 (1.5 mg/kg), indomethacin (5 mg/kg), or vehicle (DMSO). Exudate volume, PGE₂, 6-keto PGF_{1 α} , LTB₄, and inflammatory cell accumulation in the pleural cavity were assessed 4 h after carrageenan injection. PGE₂, 6-keto PGF_{1 α} , and LTB₄ levels are expressed as nanograms per rat, which is the overall content of the prostanoid in the exudates. Data are given as mean \pm S.E. (%); *n* = 10.

Treatment	Exudate Volume <i>ml</i>	Inflammatory Cells $\times 10^6$	LTB ₄	PGE ₂ <i>ng/rat</i>	6-Keto PGF _{1α}
Vehicle	0.40 \pm 0.03	45.2 \pm 5.2	0.99 \pm 0.12	2.48 \pm 0.28	5.54 \pm 0.55
YS121 (1.5 mg/kg)	0.15 \pm 0.026 (62)***	27.2 \pm 1.9 (40)**	0.51 \pm 0.07 (48)***	1.58 \pm 0.19 (36)*	3.07 \pm 0.44 (45)**
Indomethacin (5 mg/kg)	0.1 \pm 0.04 (75)***	15.8 \pm 2.21 (65)***	0.86 \pm 0.16 (13)	0.30 \pm 0.036 (88)***	0.33 \pm 0.09 (94)***

* *P* < 0.05, vs. vehicle (4% DMSO) control. ANOVA + Tukey HSD post hoc tests.** *P* < 0.01.*** *P* < 0.001.

the interference of YS121 with other PG synthases (e.g., cPGES-1 and mPGES-2) is currently limited on the basis of the availability of such purified proteins and will be the subject of future reports.

Because binding studies cannot discriminate between a functional (i.e., enzyme inhibition) and a nonfunctional interference, we performed kinetic studies for cell-free mPGES-1 by varying the PGH₂ and inhibitor concentrations. The calculated *K_m* value for PGH₂ (*K_m* = 30–50 μ M) is consistent with results from previous studies using purified recombinant mPGES-1 (*K_m* = 14–160 μ M) (Ouellet et al., 2002; Thorén et al., 2003). Our data indicate a noncompetitive inhibitory mechanism for YS121 as observed for myrtocommulone (Koeberle et al., 2009b) or the dual 5-lipoxygenase-activating protein/mPGES-1 inhibitor MK-886 (Koeberle et al., 2008a). Because of its structural feature (carboxylic acid substituted with a large lipophilic residue), one may speculate that YS121 targets a postulated fatty acid-binding site of mPGES-1 (Mancini et al., 2001; Quraishi et al., 2002). This site might be identical to the suggested binding site for the MK-886 derivative 3-(1-(4-chlorobenzyl)-5-(2-fluoro-2'-methylbiphenyl-4-yl)-3-methyl-1*H*-indol-2-yl)-2,2-dimethylpropanoic acid (AbdulHameed et al., 2008) or to the consensus sequence ERXXXAXNXD/E to which MK-886 binds 5-lipoxygenase-activating protein and eventually also other members of the family of membrane-associated proteins in eicosanoid and glutathione metabolism, including mPGES-1 (Mancini et al., 2001).

One major problem of many synthetic mPGES-1 inhibitors relates to their strong loss of potency in whole blood assays because of their strong plasma protein-binding tendency (Friesen and Mancini, 2008). For example, biphenyl derivatives of MK-886 are extremely potent in cell-free assays (IC₅₀ = 3 nM) but lose efficacy in presence of 50% fetal calf serum and fail to suppress PGE₂ formation in human whole blood (Riendeau et al., 2005). In contrast, YS121 suppressed COX-2/mPGES-1-derived PGE₂ formation in human whole blood as well as it did in the cell-free assay. However, compared with indomethacin and celecoxib, YS121 and MD52 reduced PGE₂ formation in lipopolysaccharide-stimulated whole blood only partially, with maximum inhibition of 61 and 48%, respectively, even at high concentrations. Such partial suppression of PGE₂ formation was reported also for other mPGES-1 inhibitors (Koeberle et al., 2008a, 2009a,b) and was ascribed to the remaining PGE₂ synthesis by constitutively expressed PGE₂ synthases (i.e., cPGES-1 and mPGES-2). Along these lines, PGE₂ levels in mPGES-1 knockout mice after injection of a noxious agent were only reduced by 52% (Trebino et al., 2003).

Another problem with mPGES-1 inhibitors is their failure

to inhibit mPGES-1 from rodents, excluding assessment of their in vivo efficacy in commonly used rodent models of inflammation and pain (Friesen and Mancini, 2008). In fact, YS121 was also less active on rodent mPGES-1 in murine RAW264.7 cells than on human mPGES-1. Nevertheless, in comparison with indomethacin, YS121 was as efficient in reducing exudate formation in the pleurisy model, although the efficacy with regard to PGE₂ repression was impaired. It should be noted that YS121 also decreased formation of 6-keto PGF_{1 α} and (in contrast with indomethacin) the pleural levels of LTB₄ to an extent similar to PGE₂, implying that the compound affects additional pathways of eicosanoid formation, such as 5-lipoxygenase (Werz et al., 2008) or may act via PPARs (Rau et al., 2008) and thus far unknown targets. Reduced levels of LTB₄ might contribute to the overall anti-inflammatory effectiveness of YS121, and dual inhibition of the PG and the leukotriene synthetic pathway might synergize in terms of higher anti-inflammatory efficacy and also in terms of lower side effects (Leone et al., 2007). It is unfortunate that results from experiments with mice deficient in both mPGES-1 and 5-lipoxygenase are not available yet.

Taken together, these results indicate that binding and kinetic approaches provided the molecular basis for the inhibition of human mPGES-1 by YS121 and functional studies demonstrated suppression of PGE₂ formation under inflammatory conditions in whole blood. Moreover, we showed the anti-inflammatory efficacy of YS121 in an animal model of inflammation although the exact underlying mechanisms are unclear and the failure of YS121 (as of other mPGES-1 inhibitors) to efficiently inhibit rodent mPGES-1 impede assessment of whether mPGES-1 inhibition contributes to the in vivo efficacy. Based on encouraging results from mPGES-1 knockout studies (Trebino et al., 2003) and pharmacological analysis (Xu et al., 2008), mPGES-1 inhibitors possess a high therapeutic potential as anti-inflammatory, analgesic, and antipyretic drugs. YS121 represents, in this respect, a promising drug candidate, because of multiple interference with proinflammatory targets. Neither YS121 nor any other α -substituted pirinixic acid derivative was analyzed in vivo before, and, thus, experiences regarding dosing and pharmacokinetics (e.g., half-life, metabolism) and effectiveness in humans are still missing. Ongoing preclinical studies addressing these issues as well as the potency and safety of YS121 will give further insights into its therapeutic potential.

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