The molecular pharmacology and in vivo activity of YS121 (2-(4-chloro-6-(2,3-dimethylphenylamino)pyrimidin-2-ylthio)octanoic acid), a dual inhibitor of microsomal prostaglandin E2 synthase-1 and 5-lipoxygenase*

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Running title: Inhibition of mPGES-1 by α-(n-hexyl)-pirinixic acid

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Abbreviations:
COX, cyclooxygenase; CV4151, (E)-7-phenyl-7-(3-pyridyl)-6-heptenoic acid; 12-HHT, 12(5)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid; MD52, 2-(2-chlorophenyl)-1H-phenanthro[9,10-d]-imidazole; mPGES, microsomal prostaglandin E2 synthase; MK-886 (3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid); NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin; pirinixic acid, (2-(4-chloro-6-(2,3-dimethylphenylamino)pyrimidin-2-ylthio)acetic acid; PPAR, peroxisome proliferator-activated receptor; SPR, surface plasmon resonance; Tx, thromboxane; YS121, 2-(4-chloro-6-(2,3-dimethylphenylamino)pyrimidin-2-ylthio)octanoic acid.

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Abstract

The microsomal prostaglandin E2 synthase (mPGES)-1 is one of the terminal isoenzymes of PGE2 biosynthesis. Pharmacological inhibitors of mPGES-1 are proposed as alternative to non-steroidal 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 lead compound. In cell-free assays, YS121 inhibited human mPGES-1 in a reversible and non-competitive manner (IC$_{50}$ = 3.4 µM), and surface plasmon resonance spectroscopy studies using purified in vitro-translated human mPGES-1 indicate a direct, reversible, and specific binding to mPGES-1 (K$_{d}$ = 10-14 µM). In lipopolysaccharide-stimulated human whole blood, PGE$_2$ formation was concentration-dependently inhibited (IC$_{50}$ = 2 µM), whereas the concomitant generation of the cyclooxygenase (COX)-2-derived thromboxane B$_2$ and 6-keto PGF$_{1\alpha}$ 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 PGE$_2$ and leukotriene B$_4$ but also of 6-keto PGF$_{1\alpha}$. Taken together, YS121 is a promising inhibitor of mPGES-1 with anti-inflammatory efficiency in human whole blood as well as in vivo.
Introduction

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 non-steroidal anti-inflammatory drugs (NSAIDs) 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 patients after long-term intake of coxibs was evident, apparently due to an imbalance of pro-thrombotic COX-1-derived thromboxane (Tx)A₂ and anti-thrombotic and vasodilatory COX-2-derived PGI₂ (McGettigan and Henry, 2006). Selective suppression of pro-inflammatory 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 endoplasmatic reticulum (Jegerschold et al., 2008), commonly acts in concert with COX-2 (Murakami et al., 2000) and is strongly upregulated by pro-inflammatory stimuli (e.g., interleukin-1β, 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 comparable efficiency to NSAIDs 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 afflicted with cardiovascular complications, i.e., hypertension or thrombogenesis at least in absence of risk factors like 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 (Koeberle et al., 2009b; Xu et al., 2008). 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). Also, 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-ythio)acetic acid, Fig. 1) led to α-substituted pirinixic acid derivatives such as YS121 (2-(4-chloro-6-(2,3-dimethylphenylamino)pyrimidin-2-ythio)octanoic acid, Fig. 1) that inhibits mPGES-1 (IC₅₀ = 3.4 µM (Koeberle et al., 2008b)) and 5-lipoxygenase (IC₅₀ = 4.1 and 6.5 µM 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 µM, 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.
Methods

Materials

YS121 was synthesized as previously reported (Koeberle et al., 2008b; Werz et al., 2008). Pirinixic acid was purchased from Sigma. The mPGES-1 inhibitor 2-(2-chlorophenyl)-1H-phenanthro[9,10-d]-imidazole (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 achieving a final DMSO concentration of 4%. Thromboxane synthase inhibitor (E)-7-phenyl-7-(3-pyridyl)-6-heptenoic acid (CV4151, Kato et al., 1985) was kindly provided by Dr. S. Laufer (University of Tuebingen, 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 (Deisenhofen, Germany). Materials used: DMEM High Glucose (4.5 g/l) medium, penicillin, streptomycin, trypsin/EDTA solution, PAA (Coelbe, Germany); PGH₂, Larodan (Malmö, Sweden); 11β-PGE₂, MK-886, PGB₁, and enzyme immunoassay, Cayman Chemical (Ann Arbor, MI). N-formyl-methionyl-leucyl-phenylalanine (fMLP), Alexis (Lörrach, Germany); 1-lauroyl-2-hydroxy-sn-glycero-3-phosphocholine (Anatrace, Maumee, OH); λ-Carrageenan type IV isolated from Gigartina aciculaire and Gigartina pistillata, and indomethacin were purchased from Sigma-Aldrich (Milan, Italy). [³H]-PGE₂ was from PerkinElmer Life Sciences (Milan, Italy) and PGE₂ antibody from Sigma-Aldrich (Milan, Italy). 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 DMEM/High glucose (4.5 g/l) medium supplemented with heat-inactivated fetal calf serum (10%, v/v), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37 °C in a 5% CO₂
incubator. After 3 days, confluent A549 cells were detached using 1 × trypsin/EDTA solution and reseeded at 2 × 10^6 cells in 20 ml medium in 175 cm^2 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 E.C. 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-1β for 48 h at 37 °C and 5% CO₂. After sonification, the homogenate was subjected to differential centrifugation at 10,000×g for 10 min and 174,000×g for 1 h at 4 °C. The pellet (microsomal fraction) was resuspended in 1 ml homogenization buffer (0.1 M potassium phosphate buffer pH 7.4, 1 mM phenylmethanesulphonyl fluoride, 60 µg/ml soybean trypsin inhibitor, 1 µg/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 µl total volume) was initiated by addition of PGH₂ (20 µM, final concentration). After 1 min at 4 °C, the reaction was terminated using stop solution (100 µl; 40 mM FeCl₂, 80 mM citric acid, and 10 µM of 11β-PGE₂ as internal standard). PGE₂ was separated by solid phase extraction and analyzed by RP-HPLC as described (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 *E. 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, NJ). The RM is dialysed against the feeding mixture (FM) that supplies amino acids, energy equivalents acetyl phosphate and phosphoenol pyruvate as well as 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 per ml of 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, USA) for 2 h at 30 °C, and insoluble parts were removed by centrifugation (10,000×g 10 min, 10 °C).

Determination of the activity of in vitro-translated mPGES-1

After re-solubilisation, the in vitro-translated mPGES-1 was incorporated into liposomes, which were prepared by sonification of phosphatidylethanolamine (0.15 mM in 0.1 M potassium phosphate buffer 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 which forms 1 µmol PGE2 per minute under standard assay conditions, i.e., 20 µM PGH2, 2.5 mM glutathione, 4°C, pH 7.4) that corresponds to 0.5 µmol PGE2 formed per mg protein per min.
**Determination of PGE$_2$ 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 (10$^6$/ml) and pre-incubated with the indicated compounds at 37 °C for 10 min. PGE$_2$ 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 PGE$_2$, samples were extracted, fractionated by HPLC and then quantified using a PGE$_2$ High Sensitivity EIA Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's protocol.

**Surface plasmon resonance (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) 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/mm$^2$ corresponding to 4700 resonance units) or cathepsin G (112 fmol/mm$^2$ 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 Freiburg, Germany) at 25 °C and a flow rate of 30 µl/min. Following 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 non-specific bulk refractive index effects (BIAevaluation Version 3.1 software).
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: 

\[ R_{eq} = \frac{R_{max}[\text{compound}]}{K_D + [\text{compound}]} \]

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 employing the 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 \( K_D \) (Karlsson and Falt, 1997; Roden and Myszka, 1996). The quality of the fit was determined by the \( \chi^2 \) values as well as 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 two weeks under informed consent, was obtained by venepuncture and collected in syringes containing heparin (20 U/ml). For determination of PGE₂ and 6-keto PGF₁α, 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 pre-incubation 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 (2300×g, 10 min, 4 °C), and 6-keto PGF₁α and TxB₂ were quantified in the supernatant using High Sensitivity EIA Kits (Assay Designs, Ann Arbor, MI) for 6-keto PGF₁α and TxB₂, respectively, according to the manufacturer's protocols. PGE₂ was determined as described (Koeberle et al., 2009b). In brief, the
supernatant was acidified with citric acid (30 µl, 2 M), and after centrifugation (2300×g, 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 co-elution 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 (Assay Designs, Ann Arbor, MI) 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 pre-incubated 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 (600×g/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 (600×g/15 min/4 °C). The supernatants were collected and diluted with 2.5 ml PBS, and 75 µl HCl 1 N was added. Formed 12-HHT was extracted and analyzed by HPLC as described (Albert et al., 2002).

**SDS-PAGE and Western blot**

Cells (4 × 10⁶ cells) were resuspended in 50 µl 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 glycerol/0.1% bromophenol blue (1:1, v/v), and proteins were separated by SDS-PAGE. After electroblotting to nitrocellulose membrane (GE Healthcare, Munich, Germany) and blocking with 5% BSA 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) were given i.p. 30 min before carrageenan. A group of male rats received the vehicle (DMSO, 4%, i.p.) 30 min before carrageenan. Rats were anaesthetized with enflurane 4% mixed with O₂, 0.5 l/min, N₂O 0.5 l/min and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected, and saline (0.2 ml) or λ-carrageenan type IV 1% (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 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 Burker’s chamber after vital trypan blue staining.

The amounts of PGE₂, LTB₄ and 6-keto PGF₁α in the supernatant of centrifuged exudate (800×g for 10 min) were assayed by radioimmunoassay (PGE₂) and enzyme immunoassay (LTB₄, 6-keto PGF₁α), respectively (Cayman Chemical (Ann Arbor, MI), according to manufacturer’s protocol. The results are expressed as ng per rat and represent the mean ± S.E. of 10 rats.

**Statistics**
Data are expressed as mean ± S.E. Concentration response curves were fitted to a one side binding competition equation, and 50% inhibitory concentration (IC$_{50}$) 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. A $P$ value <0.05 (*) was considered significant.
Results

**YS121 is a reversible and non-competitive inhibitor of mPGES-1**

In agreement with previous studies (Koeberle et al., 2008b), YS121 concentration-dependently inhibited the mPGES-1-mediated conversion of PGH$_2$ to PGE$_2$ (IC$_{50}$ of 3.4 µM, essentially complete inhibition at 30 µM) in microsomal preparations of IL-1β-stimulated human A549 cells (Fig. 2A). Suppression of other PGs such as PGD$_2$ could not be observed (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 (~ 30% inhibition at 30 µM), and MD52 (2 µM, reference mPGES-1 inhibitor) did not significantly inhibit PGE$_2$ formation (not shown). In order to investigate whether YS121 inhibits human mPGES-1 activity in a reversible manner, wash-out experiments were performed. Thus, microsomes from IL-1β-stimulated A549 cells were pre-incubated with YS121 at 1 or 10 µM for 15 min. The sample containing 10 µM YS121 was split and one aliquot was diluted 10-fold to obtain a final inhibitor concentration of 1 µM. Then, PGE$_2$ formation was initiated by addition of PGH$_2$. At a concentration of 1 µM, YS121 did not significantly reduce PGE$_2$ formation (16.9±4.8% inhibition, P>0.05), whereas 10 µM YS121 significantly inhibited PGE$_2$ synthesis by 60.6±5% (P<0.001, Fig. 2B). Dilution of the sample containing 10 µM YS121 restored mPGES-1 activity (Fig. 2B) suggesting a reversible mode of inhibition. Although the diluted sample still contained 1 µM YS121, PGE$_2$ formation was entirely reversed for unknown reasons.

Next, we attempted to define whether YS121 competes with the substrate (PGH$_2$) to inhibit mPGES-1 activity. In the mPGES-1 assay using microsomes of IL-1β-stimulated A549 cells (total protein = 87 µg/ml), the substrate concentration was varied over a concentration range of 10 to 50 µM PGH$_2$, and the rate of PGE$_2$ formation was determined. Fitting of the data for the non-inhibited reaction to a Michaelis Menten kinetic model (Fig. 2C) and reciprocal linear regression analysis (Lineweaver-Burk plot, Fig. 2C) yielded K$_m$ values of 54 and 30 µM and
v_{\text{max}} \text{ values of 2.6 and 1.9 nmol/min, respectively. Variation of the PGH}_2 \text{ concentration (10 – 50 µM) and subsequent Lineweaver-Burk analysis indicate a non-competitive type of inhibition by YS121 (Fig. 2C). These results rather preclude a preferential binding of YS121 to the PGH}_2 \text{ binding pocket of mPGES-1.}

**Characterisation of the binding of YS121 to mPGES-1 by SPR spectroscopy**

The direct (physical) interaction of YS121 with human mPGES-1 was analysed using SPR spectroscopy. Purified *in vitro*-translated mPGES-1 (236 fmol mPGES-1/mm$^2$) with a specific activity of 0.5 U/mg (when tested in a cell-free assay where 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. Fig. 3A shows the corrected sensograms for different concentrations of YS121 (upper left panel). The maximal concentration of YS121 was limited to 10 µM since unspecific (super-stoichiometric) binding to mPGES-1 and to the reference surface was observed at concentrations > 10 µM. The unspecific binding at high analyte concentration could be minimized by inclusion of 0.01% detergent (P20) in the running buffer, at least at concentrations of YS121 < 10 µM.

In order to discriminate unspecific binding behaviour of YS121, the structurally related lead compound pirinixic acid, which did not inhibit mPGES-1 activity (Koeberle et al., 2008b), was analysed. Pirinixic acid failed to interfere with mPGES-1 up to 40 µM (Fig. 3B). Further, YS121 (up to 20 µM) failed to bind to an immobilized reference protein, i.e., cathepsin G (Fig. 3C, previously used in SPR studies, unpublished data), which is also not functionally affected by YS121 (not shown). Taken together, 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 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 \( \mu \)M were calculated from the nonlinear fit (upper panel) and for the Scatchard plot (lower 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 3.1 (Rich et al., 2001). The dissociation constant \( K_d \) was calculated from the rate constants \( (k_d/k_a = 0.14 \text{ s}^{-1} / 13900 \text{ M}^{-1}\text{s}^{-1} = K_d = 10 \mu \text{M}) \) and is consistent to the \( K_d \) determined by equilibrium measurements (Fig. 3A). The actual \( K_d \) values are rather similar to the \( IC_{50} \) value of 3.4 \( \mu \)M 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 PGE2 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 pre-incubated with the thromboxane synthase inhibitor CV4151 (1 \( \mu \)M) and the test compounds, stimulated with lipopolysaccharide (10 \( \mu \)g/ml) for 5 h, and then, formed PGE2 was separated by RP-HPLC and quantified by ELISA (Koeberle et al., 2009b). In agreement with previous studies, MD52 (that inhibits human mPGES-1 with an \( IC_{50} = 87 \text{ nM} \) in cell-free assays (Côté et al., 2007)) maximally suppressed PGE2 synthesis in whole blood by 48.3±7.7% at high concentrations (2 - 6 \( \mu \)M), whereas celecoxib (20 \( \mu \)M) efficiently inhibited PGE2 formation under these conditions (78.3±8.4%; \( IC_{50} = 0.87 \mu \text{M} \) (Riendeau et al., 2001)). YS121 concentration-dependently inhibited PGE2 formation with an \( IC_{50} \) value of 3 \( \mu \)M (Fig. 4A),
and in analogy to MD52, a 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 PGF$_{1α}$ (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 (IC$_{50}$ ~ 5 µM, 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 pre-incubation 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-1β-mediated COX-2/mPGES-1 expression**

α-Alkyl substituted pirinixic acid derivatives such as YS121 are dual agonists of PPARα and γ (Rau et al., 2008). As PPARα/γ agonists were described to down-regulate the expression of COX-2 (Grau et al., 2006; Inoue et al., 2000) 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-1β-treated A549 cells. Stimulation with interleukin-1β (1 ng/ml) led to an increased expression of mPGES-1 and of COX-2 protein within 24 h compared to unstimulated cells which were not affected by YS121 (10 µM, Fig. 5). Incubation in the presence of YS121 (10 µM) led to a decrease of 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 hours, characterized by the accumulation of fluid that contained large numbers of inflammatory cells (Table 1). Based on previously performed in vivo studies (Rossi et al., 2009; Feisst et al., 2009) with compounds of similar pharmacological profile 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 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 PGF1α (94%) levels as expected. On the other hand, indomethacin failed to significantly reduce LTB4 levels which were lowered by YS121 (48% inhibition), seemingly due to direct inhibition of 5-lipoxygenase (Werz et al., 2008). Surprisingly, YS121 also reduced the levels of 6-keto PGF1α (45% inhibition).
Discussion

We showed before that α-substituted pirinixic acid derivates (that activate PPARα/γ) dually inhibit mPGES-1 and 5-lipoxygenase, but only moderately affect COX enzymes (Werz et al., 2008; Koeberle et al., 2008b). Here, we have characterized the biochemical interaction of the lead YS121 with human mPGES-1, and we assessed its efficacy and the 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, non-competitive manner. In human whole blood, YS121 inhibits PGE2 formation without affecting the synthesis of COX-2-derived 6-keto PGF1α, 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, Ca2+ signaling) or to modulation of preceding substrate supply (i.e., inhibition of PLA2s or COX enzymes). However, YS121 hardly inhibited isolated COX-1 and -2 (Koeberle et al., 2008b) and the activity of isolated cPLA2 is not affected up to 10 µM (unpublished data). Also, the failure of YS121 to block the expression of mPGES-1 in A549 cells rather excludes an 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 to indomethacin and celecoxib) solely inhibited PGE2 formation in human whole blood but failed to block the formation of COX-2-derived 6-keto PGF1α, 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 PGH$_2$, but instead relates to inhibition of PGE$_2$ formation from PGH$_2$ due to interference with mPGES-1. Direct interference of YS121 with mPGES-1 is demonstrated in two ways: (i) by suppression of mPGES-1-mediated transformation of PGH$_2$ to PGE$_2$ in the cell-free assay, and (ii) 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 Myszka, 2008). In fact, at concentrations >10 µM, YS121 also binds to the reference dextrane surface and causes aggregation or micelle formation with super-stoichiometric binding pattern (Giannetti et al., 2008). Hence, binding experiments were performed at concentrations <10 µM YS121 and were validated by using cathepsin G as 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 the interference of YS121 with other PG synthases (e.g., cPGES-1 and mPGES-2) are currently limited on the basis of the availability of such purified proteins and will be subject of future studies.

Because binding studies cannot discriminate between a functional (i.e., enzyme inhibition) and a non-functional interference, we performed kinetic studies for cell-free mPGES-1 by varying the PGH$_2$ and inhibitor concentrations. The calculated $K_m$ value for PGH$_2$ ($K_m = 30$-$50$ µM) is consistent with results from previous studies using purified recombinant mPGES-1 ($K_m = 14$–$160$ µM, Ouellet et al., 2002; Thoren et al., 2003). Our data indicate a non-competitive inhibitory mechanism for YS121 as observed for myrtucommulone (Koeberle et al., 2009b) or the dual FLAP/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-1H-indol-2-yl)-2,2-dimethylpropanoic acid (AbdulHameed et al., 2008) or to the consensus-sequence ERXXXAXXNXXD/E to which MK-886 binds FLAP and eventually also other members of the family of membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG), 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 (IC$_{50} = 3$ nM) but loose efficacy in presence of 50% fetal calf serum and fail to suppress PGE$_2$ formation in human whole blood (Riendeau et al., 2005). In contrast, YS121 suppressed COX-2/mPGES-1-derived PGE$_2$ formation in human whole blood equally well as in the cell-free assay. However, compared to indomethacin and celecoxib, YS121 as well as MD52 reduced PGE$_2$ formation in lipopolysaccharide-stimulated whole blood only partially with maximum inhibition of 61 and 48%, respectively, even at high concentrations. Such partial suppression of PGE$_2$ formation was reported also for other mPGES-1 inhibitors (Koeberle et al., 2009a; Koeberle et al., 2009b; Koeberle et al., 2008a), and was ascribed to remaining PGE$_2$ synthesis by constitutively expressed PGE$_2$ synthases (i.e., cPGES-1 and mPGES-2). Along these lines, PGE$_2$ levels in mPGES-1 knockout mice after injection of a noxious agent were only reduced by 52% (Trebin et al., 2003).

Another problem of 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, also YS121 was less active on rodent mPGES-1 in murine RAW264.7 cells as compared to human mPGES-1. Nevertheless, in comparison to indomethacin, YS121 was equally efficient to reduce exudate formation in the pleurisy model, although the efficacy with regard to PGE$_2$ repression was impaired. It should be noted that YS121 also decreased formation of 6-keto PGF$_{1\alpha}$ and (in contrast to
indomethacin) the pleural levels of LTB₄ to a similar extent as 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). Unfortunately, results from experiments with mice deficient in both mPGES-1 and 5-lipoxygenase are not available yet.

Taken together, 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 anti-inflammatory efficacy of YS121 in an animal model of inflammation though the exact underlying mechanisms are unclear, and the failure of YS121 (as of other mPGES-1 inhibitors) to efficiently inhibit rodent mPGES-1 impede to assess whether mPGES-1 inhibition contributes to the in vivo efficacy. Based on encouraging results from mPGES-1 knockout studies (Trebin 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, due to 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 pre-clinical studies addressing these issues as well as the potency and safety of YS121 will give further insights into its therapeutic potential.
Acknowledgments

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References


Koeberle A, Siemoneit U, Buehring U, Northoff H, Laufer S, Albrecht W and Werz O (2008a) Licofelone suppresses prostaglandin E2 formation by interference with the


*Footnotes*

C.P. received a Carl-Zeiss stipend.

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Legends for Figures

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

Fig. 2. YS121 inhibits mPGES-1 in a reversible and non-competitive manner. A, Microsomal preparations of interleukin-1β-stimulated A549 cells were pre-incubated 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 µM PGH₂. After 1 min at 4 °C, the reaction was terminated using a stop solution containing FeCl₂ and 11β-PGE₂ (1 nmol) as internal standard. The 100% values in the individual experiments are in the range of 3-4 µg/ml PGE₂ and correspond to enzyme activities of 6 - 18 µmol PGE₂ per mg protein per min. MK-886, used as control, inhibited PGE₂ formation with an IC₅₀ of 2.2 µM. Data are given as mean ± S.E., n = 3-4, ***p < 0.001 vs. vehicle (0.1% DMSO) control, ANOVA + Tukey HSD post-hoc tests. B, Reversibility of mPGES-1 inhibition by YS121. Microsomal preparations of interleukin-1β-stimulated A549 cells were pre-incubated with 10 µM YS121 for 15 min at 4 °C. An aliquot was diluted 10-fold to obtain an inhibitor concentration of 1 µM. Then, 20 µM PGH₂ was added (no dilution), all samples were incubated for 1 min on ice, and PGE₂ formation was analyzed as described by RP-HPLC. Data are given as mean ± S.E., n = 3-4, ***p < 0.001; n.s., not significant. C, The activity of mPGES-1 was determined at different PGH₂ and YS121 concentrations as indicated (left panel), and Lineweaver Burk analysis (right panel) was performed. Data are given as mean +/- S.E., n = 2-4.

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 µl/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 was fitted to a 1:1 Langmuir binding model, and the results are shown in solid lines (upper panel). The lower panel shows the distribution of the residuals in order 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 to mPGES-1. C, Binding of YS121 to cathepsin G. Sensograms are representatives of three independent experiments. D, The equilibrium responses $R_{eq}$ were plotted against the indicated concentrations of YS121 (upper panel), and the corresponding Scatchard plots are given (lower panel). The solid lines represent the nonlinear fit to a simple 1:1 interaction model. Data are given as mean +/- S.E., n = 3.

Fig. 4. Effects of YS121 on prostanoid formation in human whole blood. A, PGE$_2$, TxB$_2$, and 6-keto PGF$_{1\alpha}$ formation. Heparinized human whole blood, treated without (for determination of TxB$_2$) or with 1 μM thromboxane synthase inhibitor and 50 μM aspirin (for determination of PGE$_2$ and 6-keto PGF$_{1\alpha}$) was pre-incubated 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, PGE$_2$ 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 10-22 ng/ml PGE$_2$ in the individual experiments, respectively; the PGE$_2$ levels in unstimulated blood were 1-3 ng/ml. MD52 (2 μM) and celecoxib (20 μM) were used as controls and inhibited PGE$_2$ formation by 48 ± 8% and 78 ± 8%, respectively. 6-keto PGF$_{1\alpha}$ was directly determined in blood plasma by ELISA. The 100% value corresponds to 4-5 ng/ml 6-keto PGF$_{1\alpha}$ (unstimulated ≤1 ng/ml) in the individual experiments, respectively. Celecoxib (20 μM) inhibited 6-keto PGF$_{1\alpha}$ formation by 85 ± 4%. Indomethacin (20 μM) completely inhibited TxB$_2$ synthesis. B, 12-HHT formation. Heparinized whole blood was pre-incubated 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 1-2 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 or **p < 0.01 vs. 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, 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 representatives of 2 independent experiments.
Table 1 Effect of YS121 on carrageenan-induced pleurisy in rats. Thirty min before intrapleural injection of carrageenan, male rats were treated i.p. with YS121 (1.5 mg/kg), indomethacin (5 mg/kg), or vehicle (DMSO). Exudate volume, PGE$_2$, 6-keto PGF$_{1\alpha}$, LTB$_4$, and inflammatory cell accumulation in the pleural cavity were assessed 4 h after carrageenan injection. PGE$_2$, 6-keto PGF$_{1\alpha}$, and LTB$_4$ levels are expressed as ng/rat, which is the overall content of the prostanoid in the exudates. Data are given as mean +/- S.E., n = 10, *p<0.05, **p<0.01, or ***p<0.001 vs. vehicle (4% DMSO) control, ANOVA + Tukey HSD post-hoc tests.

<table>
<thead>
<tr>
<th>treatment</th>
<th>exudate volume (ml)</th>
<th>inflammatory cells × 10$^6$</th>
<th>LTB$_4$ (ng/rat)</th>
<th>PGE$_2$ (ng/rat)</th>
<th>6-keto PGF$_{1\alpha}$ (ng/rat)</th>
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</thead>
<tbody>
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<td>vehicle</td>
<td>0.40±0.03</td>
<td>45.2±5.2</td>
<td>0.99±0.12</td>
<td>2.48±0.28</td>
<td>5.54±0.55</td>
</tr>
<tr>
<td>YS121 (1.5 mg/kg)</td>
<td>0.15±0.026***</td>
<td>27.2±1.9**</td>
<td>0.51±0.07**</td>
<td>1.58±0.19*</td>
<td>3.07±0.44**</td>
</tr>
<tr>
<td></td>
<td>62%</td>
<td>40%</td>
<td>48%</td>
<td>36%</td>
<td>45%</td>
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<tr>
<td>indomethacin</td>
<td>0.1±0.04***</td>
<td>15.8±2.21***</td>
<td>0.86±0.16</td>
<td>0.30±0.036***</td>
<td>0.33±0.09***</td>
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<tr>
<td>(5 mg/kg)</td>
<td>75%</td>
<td>65%</td>
<td>13%</td>
<td>88%</td>
<td>94%</td>
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Fig. 1

pirinixic acid

YS121
Fig. 4

(A) Prostanoid formation (percentage of control) versus YS121 [μM].
- PGE₂
- 6-keto PGF₁₂α
- TxB₂

(B) 12-HHT formation (percentage of control) versus YS121 [μM].
Fig. 5

![Western blot analysis of mPGES-1, COX-2, and β-actin expression in DMSO and + 10 μM YS121 treated cells at 0, 12, 24, and 48 hours.](image-url)