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Biological characterization of new mPGES-1 inhibitors in preclinical models of inflammation and vascular tone

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Running title: New mPGES-1 inhibitors for preclinical studies

Abstract

Background and Purpose

Microsomal prostaglandin E synthase-1 (mPGES-1), the inducible synthase that catalyses the terminal step in PGE_2 biosynthesis, is of high interest as therapeutic target to treat inflammation. Inhibition of mPGES-1 is suggested to be safer than traditional NSAIDs and recent data demonstrate anti-constrictive effects on vascular tone, indicating new therapeutic opportunities. However, there is a lack of potent mPGES-1 inhibitors lacking interspecies differences for conducting *in vivo* studies in relevant preclinical disease models.

Experimental Approach

Potency was determined based on the reduction of PGE_2 formation in recombinant enzyme assays, cellular assay, human whole blood assay, and air pouch mouse model. Anti-inflammatory properties were assessed by acute paw swelling in a paw oedema rat model. Effect on vascular tone was determined with human *ex vivo* wire-myography.

Key Results

We report five new mPGES-1 inhibitors (named 934, 117, 118, 322, and 323) that selectively inhibit recombinant human and rat mPGES-1 with IC_{50} values of 10-29 nM and 67-250 nM, respectively. The compounds inhibited PGE_2 production in a cellular assay (IC_{50} values 0.15-0.82 µM) and in a human whole blood assay (IC_{50} values 3.3-8.7 µM). Moreover, the compounds blocked PGE_2 formation in an air pouch mouse model and reduced acute paw swelling in a paw oedema rat model. Human *ex vivo* wire-myography analysis showed reduced adrenergic vasoconstriction after incubation with the compounds.

Conclusion and Implications

These mPGES-1 inhibitors can be used as refined tools in further investigations of the role of mPGES-1 in inflammation and microvascular disease.

Abbreviations

12-HHT	12-hydroxyheptadecatrienoic acid
AA	Arachidonic acid
CA	Carrageenan
CIII	Compound III
COX	Cyclooxygenase
Cxb	Celecoxib
EIA	Enzyme immunoassay
H-PGDS	Hematopoietic-type prostaglandin D synthase
KPSS	High potassium physiological solution
LC-MS/MS	Liquid chromatography tandem mass spectrometry
L-PGDS	Lipocalin-type prostaglandin D synthase
MDA	Malondialdehyde
mPGES-1	Microsomal prostaglandin E synthase-1
mPGES-2	Microsomal prostaglandin E synthase-2
NSAIDs	Nonsteroidal anti-inflammatory drugs
PG	Prostaglandin
PGIS	Prostacyclin synthase
PSS	Physiological salt solution
SPE	Solid-phase extraction
TBA	2-thiobarbituric acid

What is already known about this subject

- NSAIDs targeting COXs are valuables tools to treat inflammation but can cause severe side effects.
- Targeting downstream mPGES-1 constitutes a potentially safer therapeutic alternative to treat inflammation and cancer.

What this study adds

- Five new mPGES-1 inhibitors with cross-species activity.

Clinical significance

- Cross-species mPGES-1 inhibitors enable preclinical investigations of mPGES-1 as drug target necessary to drive clinical trials.

Introduction

Microsomal prostaglandin E synthase-1 (mPGES-1) is the key terminal enzyme in the production of prostaglandin E_2 (PGE_2) from arachidonic acid in the cyclooxygenase (COX) pathway. PGE_2 is a potent bioactive mediator involved in both physiological homeostatic functions e.g. regulation of blood flow (Kauffman, 1981), smooth muscle function (Ren *et al.*, 1995), and mucosal integrity (Takeuchi, 2012) as well as pathological processes in autoimmune diseases and cancer (Fattahi & Mirshafiey, 2012; Korotkova & Jakobsson, 2010; Wang & Dubois, 2010). Early upon inflammatory challenge, PGE_2 induces local vasodilation and vascular permeability, which promote leukocyte infiltration to the site of inflammation (Morimoto *et al.*, 2014). When the inflammatory stimuli are removed the inflammation can recede and resolve. To limit non-specific inflammation, PGE_2 also induces cytokines such as IL-10, leading to an immunosuppressive state that if persistent is associated with chronic inflammation and cancer (Nakanishi & Rosenberg, 2013; Stolina *et al.*, 2000). In a chronic inflammatory state, PGE_2 mediates pain, inflammatory angiogenesis, and tissue destruction (Kamei *et al.*, 2004; Robinson *et al.*, 1975). In cancer, PGE_2 is associated with increased proliferation and survival of tumour cells, increased angiogenesis, enhanced invasion, and metastasis (Buchanan *et al.*, 2003; Pai *et al.*, 2001; Sheng *et al.*, 1998; Sheng *et al.*, 2001).

Non-steroidal anti-inflammatory drugs (NSAIDs) that reduce PGE_2 production via COX inhibition are widely used drugs for inflammation and pain management. Since COX inhibition also blocks the production of the other prostanoids, i.e. PGD_2, $PGF_{2\alpha}$, prostacyclin (PGI_2), and thromboxane (TXA_2), which are important for normal cellular functions and homeostasis, NSAIDs are associated with severe side effects. COX-1 selective inhibitors are associated with bleeding and gastrointestinal side effects (Bombardier *et al.*, 2000) whereas COX-2 selective inhibitors increase the risk of cardiovascular adverse effects (Baron *et al.*, 2008; Bresalier *et al.*, 2005), which has led to caution in the use of these drugs. Recent studies have also indicated an increased risk of cardiovascular adverse effects even for non-selective COX inhibitors (Nissen *et al.*, 2016; Sondergaard *et al.*, 2017). In contrast, selective inhibition of downstream mPGES-1 has been suggested as a potential safer alternative to NSAIDs (Samuelsson *et al.*, 2007).

Despite promising results with genetic knock-out of mPGES-1 in numerous mouse models of inflammation (Kojima *et al.*, 2008; Trebino *et al.*, 2003) and cancer (Howe *et al.*, 2013; Nakanishi *et al.*, 2011; Nakanishi *et al.*, 2008), there are no mPGES-1 inhibitors in the clinic

today. The first phase I trials with an mPGES-1 inhibitor, LY3023703, in healthy volunteers showed inhibition of LPS-induced PGE_2 production in *ex vivo* blood and increased levels of systemic prostacyclin, as measured by stable urine metabolite PGIM. LY3023703, as well as an additional mPGES-1 inhibitor from the same program, were discontinued due liver toxicity (Jin *et al.*, 2018; Jin *et al.*, 2016), and the toxicity could subsequently be attributed to a reactive metabolite of 2-aminoimidazole, which was a common feature to both compounds (Norman *et al.*, 2018). Aside from such compound-specific set-backs, there are at least two likely reasons for the lack of mPGES-1 inhibitors on the market. First, the inhibitors developed by pharmaceutical companies have been screened towards the human enzyme. There are differences in amino acid sequences between human and murine mPGES-1 in the active site rendering the murine catalytic cleft less accessible to compounds, thus published inhibitors developed towards human mPGES-1 are generally ineffective against murine mPGES-1 (Pawelzik *et al.*, 2010; Sjögren *et al.*, 2013). This has limited preclinical investigations in commonly used animal models of several diseases and thus hampered the exploration of novel mPGES-1 inhibitors for use in indications beyond those for which they were initially developed, typically inflammatory pain.

We have previously characterized mPGES-1 inhibitors lacking interspecies differences in murine models of inflammation (Leclerc *et al.*, 2013a; Leclerc *et al.*, 2013b) as well as in cancer models (Kock *et al.*, 2018; Olesch *et al.*, 2015) and recently Ding and co-workers described new cross-species inhibitors (Ding *et al.*, 2018), but there is still a need for further improved mPGES-1 inhibitors for preclinical investigations. New mPGES-1 inhibitors are required to have superior affinity and improved bioavailability. Second, COX-2 inhibitors are associated with cardiovascular side effects, and since mPGES-1 is functionally coupled to COX-2, there has been a fear that also mPGES-1 inhibitors will present cardiovascular adverse effects. However, recent studies have indicated that mPGES-1 depletion will not only evade cardiovascular concerns associated with COX-2 inhibition (Raouf *et al.*, 2016a; Raouf *et al.*, 2016b), but also that the mPGES-1 inhibitor Compound III (CIII) increases levels of vasoprotective prostacyclin (Leclerc *et al.*, 2013a) and reduces vasoconstriction in large blood vessels, measured by wire-myography (Ozen *et al.*, 2017). Collectively, the observed anti-inflammatory (through reduction of PGE_2) and vasoprotective (through increase of prostacyclin) properties of mPGES-1 inhibitors suggests further studies in models of resistance-sized arteries.

In this study, we have characterized new inhibitors of human and rodent mPGES-1, demonstrating improved pharmacological properties, and tested them *in vitro, in vivo,* and *ex vivo* models of inflammation and vascular tone in human small arteries.

METHODS

Compounds

For experimental descriptions of the synthesis of compounds 934, 117, 118, 322, and 323 used in this study (Figure 1) see Supporting Information. COX-1 inhibitor SC-560, COX-2 inhibitor celecoxib, mPGES-1 inhibitor MK-886, and dual COX-1/2 inhibitor diclofenac were purchased from Sigma-Aldrich. COX-2 inhibitor NS-398 was purchased from Biomol, Germany. Hematopoietic-type prostaglandin D synthase (H-PGDS) inhibitor HQL-79 and prostacyclin synthase (PGIS) inhibitor U-51605 were purchased from Cayman Chemicals, Ann Arbor, MI, USA. Selective mPGES-1 inhibitor CIII was produced by NovaSaid AB, Stockholm, Sweden. All compounds used were diluted from DMSO stock solutions if not stated otherwise.

Enzyme inhibition assay

In order to determine the ability of the test compounds to inhibit mPGES-1 enzyme activity, PGH_2 (Lipidox, Sweden) was incubated with recombinant mPGES-1 and remaining PGH_2 was indirectly assessed by measuring its degradation product malondialdehyde (MDA) as described previously (Basevich *et al.*, 1983). In brief, recombinant human (30 µg/ml) and rat (1 mg/ml) mPGES-1 membrane fraction produced in *Escherichia coli* was pre-incubated with the test compounds at 4°C at concentrations ranging from 0.1 nM to 3.3 µM for human and 5 nM to 37 µM for rat, in duplicates. After 30 min, the substrate PGH_2 (10 µM final concentration) was added to the enzyme-compound mixture and incubated for 90 s at room temperature. An excess of $FeCl_2$ in the presence of citric acid, pH 3, stopped the reaction by converting any remaining PGH_2 into MDA and 12-hydroxyheptadecatrienoic acid (12-HHT). Subsequently, 2-thiobarbituric acid (TBA, Sigma-Aldrich) was added and the samples were heated at 80°C for 30 min. Any formed MDA-TBA conjugate was measured using absorbance at 530 nm (and subtracting absorbance at 560 nm) or using fluorescence at excitation 485 nm/emission 545 nm. Inhibition of mPGES-1 by the test compounds was expressed as the percentage relative to the inhibition of mPGES-1 by a reference mPGES-1 inhibitor MK-886 or CIII, to reduce inter-assay variability, and calculated as follows:

$$Inhibition = (Test\ Compound - Positive) \div (Reference - Positive) \times 100$$

Inhibition is the percent inhibitory activity, *Positive* is the signal obtained after incubation of PGH_2 with mPGES-1, *Test compound* is the signal obtained after incubation of PGH_2 with mPGES-1 in the presence of test compound, and *Reference* is the signal obtained after incubation of PGH_2 with mPGES-1 in the presence of 10 μM MK-886 or 10 μM CIII. The assay was performed without mPGES-1 enzyme or with denatured mPGES-1 enzyme (boiled for 5 min) as negative controls. The inhibition assay was repeated three times for 118 and four times for 934, 322 and 323.

The test compounds were also assayed for inhibition of microsomal prostaglandin E synthase-2 (mPGES-2), PGDS, and PGIS activity using the same approach as the mPGES-1 inhibition assay. Recombinant simian mPGES-2 membrane fraction was used, and the compounds were assayed in duplicates at 10 concentrations between 2.5 nM and 50 μM or 100 μM. The inhibition assay was performed four times for 934, 117, and 118 and twice for 322 and 323. For PGDS, human recombinant lipocalin-type PGDS (L-PGDS, #10006788) and human recombinant hematopoietic-type PGDS (H-PGDS, #10006593, both from Cayman Chemical, Ann Arbor, MI, USA) were used. Compounds were assayed in duplicates with 9 concentrations between 7.5 nM to 50 μM for L-PGDS and 10 concentrations between 5 nM to 100 μM for H-PGDS. The L-PGDS inhibition assay was performed twice for all compounds. The H-PGDS inhibition assay was performed twice for 934, 117, and 188 and once for 322 and 323. The inhibitory activity of a test compound was calculated as % inhibition using the same formula as for mPGES-1, with L-PGDS inhibition relative to denatured protein and H-PGDS inhibition relative to the H-PGDS inhibitor HQL-79.

Human recombinant PGIS (membrane fraction) was used to assess the test compounds inhibition on PGIS activity. Inhibition of PGIS was reported as relative to inhibition by PGIS inhibitor U-51605 at 10 μM. All compounds were tested once in duplicates between 2.5 nM and 50 μM.

COX inhibition assay

To screen for cross-reactivity against COX-1 and COX-2, the compounds were tested in a COX inhibitor screening assay (#560131, Cayman Chemical) according to manufacturer's recommendations with a minor modification, and formed $PGF_{2\alpha}$ was measured by EIA (Cayman Chemical). In brief, compounds 934, 117, 118, 322 and 323 were assayed in triplicates at 10 μM and compared to reference compounds SC-560 and NS-398. Compounds

were incubated with ovine COX-1 and human recombinant COX-2 for 10 min at 37 °C prior to addition of arachidonic acid. After 3 min, the reaction was stopped with HCl, stannous chloride was added, and the reaction mixture was incubated at room temperature for 1 h in order to allow formed PGH_2 to convert into $PGF_{2\alpha}$. The reaction volumes were reduced to one fifth of the recommended volumes.

mPGES-1 inhibition in intact cells

A549 human lung carcinoma cells (ATCC, Cat# CCL-185, RRID:CVCL_0023) were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptavidin and L-glutamine (all from Invitrogen AB, Sweden) at 37°C in a humidified atmosphere containing 5% CO_2. A549 cells were seeded in 96-well plates at a density of 25.000 cells/well and incubated for 20 h in RPMI-1640 culture medium supplemented with 2% FBS. After 20 h, cells were treated with 10 ng/ml interleukin-1β (IL-1β, # I9401, Sigma-Aldrich) and various concentrations of test compounds or vehicle control (1% DMSO) in culture medium supplemented with 2% FBS and incubated for 24 h. NS-398 at 10 µM was used as positive control. The reaction was stopped by aspiring the supernatants and cell viability was assessed by MTT assay (Sigma-Aldrich) according to manufacturer's protocol. PGE_2 concentration was determined by enzyme immunoassay (EIA, Cayman chemicals) in cell culture supernatants according to manufacturer's instructions.

Whole blood assay and prostanoid profiling using LC-MS/MS

Compounds, reference compounds (diclofenac and NS-398), and vehicle controls (DMSO) in 25 µl of PBS were prepared in a 96-well plate. 200 µl of freshly drawn heparin blood (Ethical approval Dnr 02-196, Karolinska Institutet) was added to each well and the plate was incubated at 37°C for 30 min. After incubation, 25 µl of 0.1 mg/ml LPS (Sigma-Aldrich) in PBS was added (final concentration 10 µg/ml of LPS) followed by pipetting up and down 3 times. The plate was incubated at 37°C for 24 h and then centrifuged at 3000 g for 10 min at 4°C. Working on ice, 120 µl of plasma was recovered to a new plate that was sealed with aluminium foil and stored at -80°C. For prostanoid profiling using LC-MS/MS, plasma samples were thawed on ice and then transferred to a collection plate prepared with 50 µl of deuterated internal standard mix containing 6-keto-$PGF_{1\alpha}$-d4, $PGF_{2\alpha}$-d4, PGE_2-d4, PGD_2-d4, TxB_2-d4, and 15-deoxy-Δ12,14PGJ_2-d4 (Cayman Chemical, Ann Arbor, MI, USA) in 100% MeOH. Proteins were precipitated by addition of 800 µl 100% MeOH, pipetting up and down 10 times, and the plate was incubated on ice for 20 min. The plate was then centrifuged at

3000 g for 10 min at 4°C. The supernatants were transferred to a new plate and evaporated under vacuum for 4 h. The evaporated samples (about 200 µl) were diluted with 1 ml of 0.05% formic acid in water and then loaded onto Oasis HLB 1cc 30 mg plate (Waters, Ireland) that had been pre-conditioned with 1 ml of 100% MeOH and 1 ml of 0.05% formic acid in water. The plate was washed with 10% MeOH, 0.05% formic acid in water, and analytes were eluted with 100% MeOH. The eluates were evaporated under vacuum to complete dryness and then stored at -20°C until reconstituted in 50 µl of 20% acetonitrile in water prior to LC-MS/MS analysis. Analytes were quantified in negative mode with multiple reaction monitoring method, using an Acquity triple quadrupole detector mass spectrometer equipped with an Acquity H-class UPLC (Waters, MA, USA). Separation was performed on a 50 x 2.1 mm Acquity UPLC BEH C18 column 1.7 µm (Waters, Ireland) with a 12 min stepwise linear gradient (20-95%) at a flowrate of 0.6 ml/min with 0.05% formic acid in acetonitrile as mobile phase B and 0.05% formic acid in water as mobile phase A. Data were analysed using MassLynx software, version 4.1, with internal standard calibration.

Air pouch model

The air pouch model is an established model for preclinical anti-inflammatory drug efficiency studies. The air pouch mimics the synovial cavity and when challenged with carrageenan, provides a localized sterile inflammatory environment suitable to study prostaglandins (Duarte *et al.*, 2012). Male C57BL/6JBomTac mice (Taconic, Denmark), weighing approximately 20 g, were used for the air pouch experiments. The mice were housed in groups of 4 to 8 animals in cages containing bedding and environmental enrichment. Husbandry and care practices were based on veterinary guidance. The animals had access to food and water *ad libitum* and were inspected at least twice a day so that any health issues were immediately apparent and veterinary guidance could be obtained. Ethical approval for this study was granted by the regional ethical committee of Stockholm, Sweden (N86/13).

To form the air pouches, 3 ml of sterile air were injected into the interscapular area of the back of the mice under isoflurane anaesthesia (Univentor 400, 3%). To provide stable air pouches, they were re-inflated with 1.5 ml sterile air after 5 days under light anaesthesia. 24 h after the second air injection, animals were randomized into receiving 30 mg/kg or 100 mg/kg of mPGES-1 inhibitor (934, 117, 118, 322 or 323), 50 mg/kg celecoxib, or vehicle control (1% Tween 80 and 0.5% carboxymethyl cellulose in MilliQ water) administered through oral gavage (*p.o.*). One hour after administration of compounds or vehicle, 1 ml 1%

carrageenan (CA) was injected into the pouch under light isoflurane anaesthesia. Sterile inflammation was allowed to develop for 6 h before sacrifice by an overdose of isoflurane combined with cervical dislocation, and exudate was collected. Exudates were immediately centrifuged at 1500 g for 3 min and then stored at -20°C until further analysis. PGE_2 and 6-keto-$PGF_{1\alpha}$ concentrations were measured by EIA according to manufacturer's protocol (#514010, #515211, Cayman Chemical). Four of the unstimulated control samples and three of the celecoxib treated mice were below the detection limit of the EIA analysis (15 pg/ml). Therefore, these samples were assigned a value of 20 pg/pouch for the subsequent data analysis. Due to non-successful pouch formation, 7 out of 48 mice were excluded from the study. The remaining 41 mice were randomized among the different groups, ensuring a minimum number of mice (n=3) for the saline control and equal distribution for each treatment group with the aim of 10 per group based on earlier experiments. This resulted in two treatment groups with a sample size of 9 (30 mg/kg and celecoxib) and two treatment groups with 10 mice each (CA control and 100 mg/kg). For the following four experiments the same number of mice was used in each experiment.

Carrageenan-induced paw oedema model

The CA-induced paw oedema model is a widely used model to study the anti-inflammatory response of NSAIDs *in vivo* (Morris, 2003). Anthem Biosciences was assigned to perform experiments to evaluate the anti-inflammatory effect of the compounds in the CA-induced paw oedema model in rats. All experiments were performed according to protocols approved by the Institutional Animal Ethics Committee (IAEC) under the supervision of the Committee for the Purpose of Control and Supervision on Experiment on Animals (CPCSEA), Ministry of Environment, Forest and Climate Change, Government of India. Male Wistar rats, 6 weeks old and weighing approximately 150 g, kept on regular chow diet were used in the experiment. Seven rats were randomized into treatment groups based on weight. The identity of compounds was unknown to Anthem Biosciences. Sample size was based on earlier experience and existing literature (Morris, 2003). Animals were fasted overnight before the day of experiment. Compounds were administered at 1, 3, 10, 30 and 100 mg/kg and the reference compound, celecoxib was administered at 10 mg/kg in a suspension containing a final concentration of 1% Tween 80 and 0.5% carboxymethyl cellulose by oral gavage. One hour later, inflammation was induced with 0.1 ml of 1% CA solution that was injected into the subplantar region of the hind paw of the rats. Swelling of the paw was monitored using a

plethysmometer (Ugo Basile, Italy) before (baseline) and at 1, 2, 3, and 4 h after CA injection.

In vivo pharmacokinetic studies in rat

Eurofins Cerep Laboratories was assigned to investigate the pharmacokinetic properties of the new mPGES-1 inhibitors. The pharmacokinetic study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). Each value represents the mean of three animals (Supporting Information Table S1). Intravenous (*i.v.*) dose was 2 mg/kg and oral dose was 10 mg/kg. Blood was drawn 3,10, 30, 60, 120, 240, 360, and 1440 min after *i.v.* dosing and 10, 30, 60, 120, 240, 360, 480, and 1440 min after *p.o.* dosing. The pharmacokinetic properties of the inhibitors were assessed in male CD IGS rats from Charles River Laboratories, weighing between 180-250 g.

Vascular reactivity studies in resistance size arteries

Ethical approval for this study was granted by the Ethics Committee at Karolinska University Hospital, Huddinge (273/94). Full informed consent according to the Declaration of Helsinki was obtained from all subjects. Arterial segmentation and vascular reactivity studies were carried out as previously described (Arefin *et al.*, 2014). Briefly, the subcutaneous fat biopsies were obtained from the lower abdomen from healthy donors and placed in cold physiological salt solution (PSS: NaCl 119 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.17 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1.18 mM, EDTA 0.026 mM, and glucose 5.5 mM). In ice-cold PSS, resistance size arteries (Ø100-500µm) were dissected from subcutaneous fat biopsies and cleaned from surrounding non-vascular tissue using a stereomicroscope. Vessel tension was measured using a Mulvany's type 4-channel Multi Myograph system (Danish Myotechnology, Model 610), and isometric force was registered using Lab chart 8 software (AD Instruments, New Zealand). Each organ bath contained warmed (37°C) PSS that was continuously bubbled with 5% CO_2 /95% O_2. Every 30 min all solutions, including the incubation solutions were refreshed. Viability and endothelial function was assessed by an initial stretching protocol, followed by specific smooth muscle activation that included a first stimulation with a mixture of high potassium physiological salt solution (KPSS, equimolar substitution of 125 mM Na+ with K+). The arteries were then washed with PSS to return to resting basal tone followed by treatment with norepinephrine at 10 µM. The arteries were washed with PSS, and then relaxation was tested with 1 µM acetylcholine or 1 µM

bradykinin after preconstruction with 1 μM norepinephrine. Arteries that did not fulfil viability criteria of >50% relaxation to acetylcholine or bradykinin were excluded.

To determine any effect of mPGES-1 inhibitors in respect to contractility, arteries were first contracted with increasing concentrations of norepinephrine (0.001-3 μM) in a cumulative manner until a stable plateau was reached. The arteries were then washed with PSS to return to a resting basal tone followed by a 30 min treatment with CIII (10 μM), 934 (3 μM), 118 (3 μM), or vehicle control (DMSO). After the 30 min incubation, a second norepinephrine (0.001-3 μM) concentration-response curve was recorded, and concentration response curves were expressed as % of initial high potassium contraction. Thereafter, arterial viability was assessed by treatment with norepinephrine at 1 μM. Due to paired analysis, randomization was not needed.

Data and statistical analyses

Data are presented as mean ± SD or median with inter-quartile range (25th-75th percentile) and individual data points. All IC_{50} values were calculated using nonlinear regression and sigmoidal concentration-response curve fit. Statistical significance in the air pouch model and in the paw swelling assay was calculated using one-way ANOVA (normally distributed data) followed by Dunnett's multiple comparison test, with a single pooled variance (the mean of each treatment group was compared with the mean of the vehicle control group). Post-hoc tests were only run if F achieved $P<0.05$. Statistical analysis of constriction of arteries was performed by paired t-test of individual EC_{50} values (before and after inhibitor incubation). Significance was set to $P<0.05$ (denoted by * in figures). Calculations and graphs were prepared using GraphPad Prism 7.0e. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2018).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a; Alexander *et al.*, 2017b).

RESULTS

Compound potency and selectivity towards mPGES-1

The compounds profiled herein are the result of an optimization effort that started from a screen of a compound library towards human mPGES-1. One of the original hit series consisted of 1-(benzothiazol-2-yl)piperidine-4-carboxamides. This hit series evolved into a series of 1-(1H-benzoimidazol-2-yl)piperidine-4-carboxamides with various substitutions on the benzimidazole and amide parts with CIII (Leclerc *et al.*, 2013a) as an early example. Further optimizations focused on properties such as potency, aqueous solubility, and *in vitro* metabolic stability. The improved compounds combined with the knowledge that earlier bezimidazole compounds could reach submicromolar IC_{50} values in rat recombinant mPGES-1 (e.g. CIII) provided the basis for further *in vitro* and *in vivo* profiling of these potentially cross species human/murine mPGES-1 inhibitors.

The five compounds 934, 117, 118, 322, and 323 (Figure 1) were found to be potent inhibitors of recombinant human and rat mPGES-1 (Figure 2, Supporting Information Figure S1). The IC_{50} values were 10-29 nM and 67-250 nM towards human or rat enzyme, respectively. The selectivity towards mPGES-1 was determined by screening other enzymes in the prostanoid synthesis pathway. The compounds showed no inhibitory capacities towards COX-1, PGIS, L-PGDS or H-PGDS at concentrations up to 10 µM (Table 1). All compounds showed weak to moderate inhibition of mPGES-2 at 10 µM. Compound 323 showed weak inhibition of COX-2.

Inhibition of PGE_2 production in intact cells

Human lung cancer cell line A549 in culture medium supplemented with 2% FBS was used to determine the potency of the compounds to inhibit PGE_2 production in a cell assay. All compounds inhibited the IL-1β induced PGE_2 production in a concentration dependent manner, showing IC_{50} values in the range of 0.15-0.82 µM (Figure 3). The COX-2 inhibitor NS-398, used as positive control, completely reduced PGE_2 production at 10 µM. The tested compounds did not affect cell viability when used below 100 µM (Supporting Information Figure S2).

Inhibition of PGE_2 production in human whole blood

Human whole blood assay was used to assess the compounds' capacity to inhibit LPS-induced PGE_2 production in a complex biological matrix. The compounds targeting mPGES-

1 displayed similar potency to block PGE_2 production, with IC_{50} values in the range of 3.3-8.7 µM (Figure 4). The positive control, diclofenac, fully inhibited PGE_2 production at 10 µM, while the tested compounds showed 10-15% residual PGE_2 production at the highest tested concentration (20 µM). Reference compound NS-398 showed weak inhibition at 0.1 µM. Diclofenac fully inhibited the thromboxane production (as measured by the stable metabolite TXB_2), while NS-398 and the compounds targeting mPGES-1 showed no consistent effect on TXB_2 formation (Supporting Information Figure S3).

***In vivo* pharmacokinetic study in rats**

All the compounds displayed similar properties, although 117 reached a higher maximum concentration and displayed a higher bioavailability than the other compounds (Supporting Information Table S1). The obtained exposure of the compounds after oral administration supports an *in vivo* effect with the doses used in the animal studies.

Inhibition of PGE_2 production in air pouch model

To assess the compounds' ability to reduce PGE_2 *in vivo,* the CA air pouch model was used to induce inflammation and prostaglandin production in mice (Leclerc *et al.*, 2013a). Compounds or vehicle were administered via *p.o.* one hour before injection of CA in the pouch. CA successfully induced PGE_2 production in the air pouches, and celecoxib, used as a positive control in all experiments, significantly reduced the formation of PGE_2. All test compounds reduced PGE_2 production in the pouch exudates (Figure 5). Compound 322 and 323 significantly reduced PGE_2 production at 30 mg/kg versus 100 mg/kg for the other compounds. In contrast to celecoxib, the mPGES-1 inhibitors did not reduce prostacyclin levels. For Compound 117 there was even an increase in prostacyclin formation (Supporting Information Figure S4).

Efficacy on acute inflammation *in vivo*

To further investigate the compounds' efficacy *in vivo,* a carrageenan-induced paw oedema model in rats was used. Celecoxib was used as a reference compound and significantly reduced paw swelling in all the individual experiments with 50% (one experiment per compound). A significant reduction in swelling 1 h post CA induction was seen for all compounds compared to vehicle control, with a maximum reduction in paw swelling of 45-65%. 934, 322 and 323 reduced paw swelling at all tested doses (1-100 mg/kg) with a clear trend to dose-dependency (Figure 6). A significant reduction in paw swelling was also seen for 117 and 118 1 h post CA, but a dose-response trend was not observed for 117, and only a

weak dose-response was observed for 118 (Figure 6). At 4 h post CA induction, paw swelling reached its maximum (Figure S5). When comparing the area under the curve (AUC) of the different inhibitors to the vehicle treated CA induced control, a significant decrease in paw swelling over 4 h was observed for 934 (10, 30 and 100 mg/kg), 117 (1, 3, 10 and 30 mg/kg), 118 (10 mg/kg), 322 (3 and 100 mg/kg), and 323 (100 mg/kg). Celecoxib (10 mg/kg) significantly reduced paw swelling in all experiments.

Effect of mPGES-1 inhibition on vasoconstriction in small arteries

To study the effects of mPGES-1 inhibition on human peripheral resistance vasculature, we assessed norepinephrine-induced vasoconstriction using wire-myography. The reference mPGES-1 inhibitor CIII (Leclerc *et al.*, 2013a; Ozen *et al.*, 2017) reduced constriction at 10 μM; however, without reaching statistical significance. Based on structural differences and performance *in vitro* and *in vivo*, 934 and 118 were selected for the wire-myography experiments. Compound 118 showed better efficacy than CIII at 3-fold lower concentration (3 μM), indicating an increased potency (Figure 7). Compound 934 showed a high variability, possibly due to poor solubility in the assay buffer; however, a trend towards reduced constriction was seen.

DISCUSSION

Inhibition of mPGES-1 was initially proposed as a promising alternative to traditional COX inhibitors to manage pain and inflammation. Selective mPGES-1 inhibitors are envisioned to present less side effects than COX inhibitors, as they target only inducible PGE_2 production and spare the production of other prostanoids that are important for physiological functions. However, as recent data suggests that mPGES-1 inhibition is not only safe, but also may elicit beneficial cardiovascular effects, we set out to first develop improved human/rodent mPGES-1 inhibitors that can be used to study such effects in multiple preclinical disease models and then to demonstrate the ability of such inhibitors to attenuate norepinephrine-induced vasoconstriction in resistance-size human arteries.

The compounds 934, 117, 118, 322, and 323 selectively inhibited recombinant human and rat mPGES-1 *in vitro* with IC_{50} values in the low nanomolar range. The compounds displayed potent inhibition of PGE_2 production in IL-1β treated human intact cells. The compounds were further tested in an LPS-treated human whole blood assay (24 h), where the compounds blocked PGE_2 production with IC_{50} values in the low micromolar range without affecting

thromboxane synthesis. A summary of the compounds performance in *in vitro* assays is given in Table 2.

To establish the compounds' efficacy in rodent models *in vivo*, we used the CA-induced air pouch mouse model (Leclerc *et al.*, 2013a) and a CA-induced paw oedema rat model. All five compounds dose-dependently reduced the concentration of PGE_2 in the pouch exudates. While the COX-inhibitor celecoxib almost completely blocked PGE_2 formation, full inhibition of PGE_2 production was not reached with the mPGES-1 inhibitors. This difference could be due to non-enzymatic degradation of PGH_2 or because PGH_2 is converted to PGE_2 by the other PGE_2 synthases mPGES-2 and cPGES (Murakami *et al.*, 2003; Tanioka *et al.*, 2000). Inhibition of mPGES-1 in the air pouch did not affect prostacyclin concentration, while celecoxib inhibited both PGE_2 and prostacyclin production. Moreover, the five compounds significantly reduced acute paw swelling.

In contrast to COX inhibitors, mPGES-1 inhibitors are likely to have cardioprotective properties based on several studies in mPGES-1 knockout mice (Cheng *et al.*, 2006; Leclerc *et al.*, 2013a; Tang *et al.*, 2016; Wang *et al.*, 2011; Wang *et al.*, 2006). Also, it was recently reported that treatment with mPGES-1 inhibitor CIII results in reduced contraction of larger human blood vessels *ex vivo* (Ozen *et al.*, 2017). We set out to prove that mPGES-1 inhibition results in reduced contraction in human small resistance size arteries. We conclude that the new compounds replicate the effects of CIII regarding reduction of vasoconstriction. However, further studies are warranted to elucidate the underlying mechanisms of mPGES-1 inhibition and cardiovascular protection. The established dogma behind the cardiovascular side effects imposed by NSAIDs is a reduction in vasodilating prostacyclin, while platelet derived thromboxane (platelet activator and vasoconstrictor) biosynthesis remains (Grosser *et al.*, 2006). The proposed cardioprotective effect of mPGES-1 inhibition is mediated by shunting of PGH_2 from PGE_2 to prostacyclin (Cheng *et al.*, 2006; Jin *et al.*, 2016; Ozen *et al.*, 2017; Tang *et al.*, 2016).

Recently, the results from the first clinical phase I trial with an mPGES-1 inhibitor were published (Jin *et al.*, 2016). The Eli Lilly compound LY3023703 showed very potent inhibition of PGE_2, and in contrast to a COX-2 inhibitor celecoxib, there was an increased release of urinary prostacyclin metabolite, suggesting a systemic increase of cardioprotective prostacyclin during mPGES-1 inhibition in man. Mechanistic data in mice showed that mPGES-1 derived PGE_2 drives vascular remodelling, stiffness, and endothelial dysfunction in

hypertension (Avendano *et al.*, 2018). This potentially protective side effect of mPGES-1 inhibition opens up the possibility to tackle the production of pro-inflammatory and immunosuppressive PGE_2, while increasing the production of cardioprotective prostacyclin.

In the present study, we have characterized five new cross-species mPGES-1 inhibitors suitable for oral delivery with improved potency and selectivity compared to published inhibitors lacking interspecies differences (Ding *et al.*, 2018; Leclerc *et al.*, 2013a; Leclerc *et al.*, 2013b). All five compounds presented comparable selectivity and potency. Our results indicate a class effect of mPGES-1 inhibition in reduction of inflammation and protection against cardiovascular events. We envision that these compounds will be valuable tools in preclinical research to evaluate mPGES-1 as a therapeutic target in inflammation, cancer, and microvascular disease.

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CONFLICTS OF INTEREST

R.M., P.S., and P-J.J. are engaged in Gesynta Pharma AB, a company that develops mPGES-1 inhibitors. All other authors have no conflicts of interests to declare.

AUTHOR CONTRIBUTIONS

K.L., J.S., F.B., L.S., J.W., R.M., P.S., K.K., M.K, and P-J.J. contributed to study conception and design. J.S., F.B., S.A., L.S., J.W., and P.S. performed experiments. K.L., J.S., F.B., S.A., L.S., J.W., and P.S. analysed data. K.L., J.S., F.B, and J.W. drafted the manuscript. R.M, P.S., K.K., M.K, and P-J.J. critically revised the manuscript. S-C.P. contributed with new reagents. All authors read and approved the final manuscript.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Table 1. Selectivity of mPGES-1 inhibitors
Biochemical *in vitro* inhibition of enzymes involved in prostaglandin biosynthesis by mPGES-1 inhibitors: 934, 117, 118, 322, and 323. No inhibition (No inh.) signifies less than 10% inhibition at 10 μM compound concentration.

Enzyme	% Inhibition by compound (10 μM)				
	934	117	118	322	323
COX-1	No inh.	No inh.	No inh.	No inh.	12
COX-2	No inh.	No inh.	No inh.	11	25
mPGES-2	25	39	43	40	52
L-PGDS	13	No inh.	No inh.	No inh.	No inh.
H-PGDS	No inh.	No inh.	No inh.	No inh.	No inh.
PGIS	No inh.	No inh.	No inh.	12	No inh.

Table 2. *In vitro* performance of mPGES-1 inhibitors
Biochemical and cellular *in vitro* performance of mPGES-1 inhibitors 934, 117, 118, 322, and 323 presented as mean IC_{50} value±SD.

In vitro	Compound IC_{50} [μM]				
	934	117	118	322	323
Biochemical assays					
mPGES-1 human	0.024±0.0071 n=4	0.017±0.0094 n=4	0.023±0.0059 n=3	0.037±0.011 n=4	0.031±0.0031 n=4
mPGES-1 rat	0.17±0.098 n=4	0.055±0.033 n=4	0.078±0.049 n=4	0.27±0.21 n=4	0.14±0.085 n=3
Cellular assays					
A549 cells	0.15 n=1	0.26 n=1	0.15 n=1	0.82 n=1	0.66 n=1
whole blood	3.7±0.28 n=2	3.4±0.14 n=2	2.5±1.4 n=2	6.4±3.6 n=2	4.9±3.7 n=2

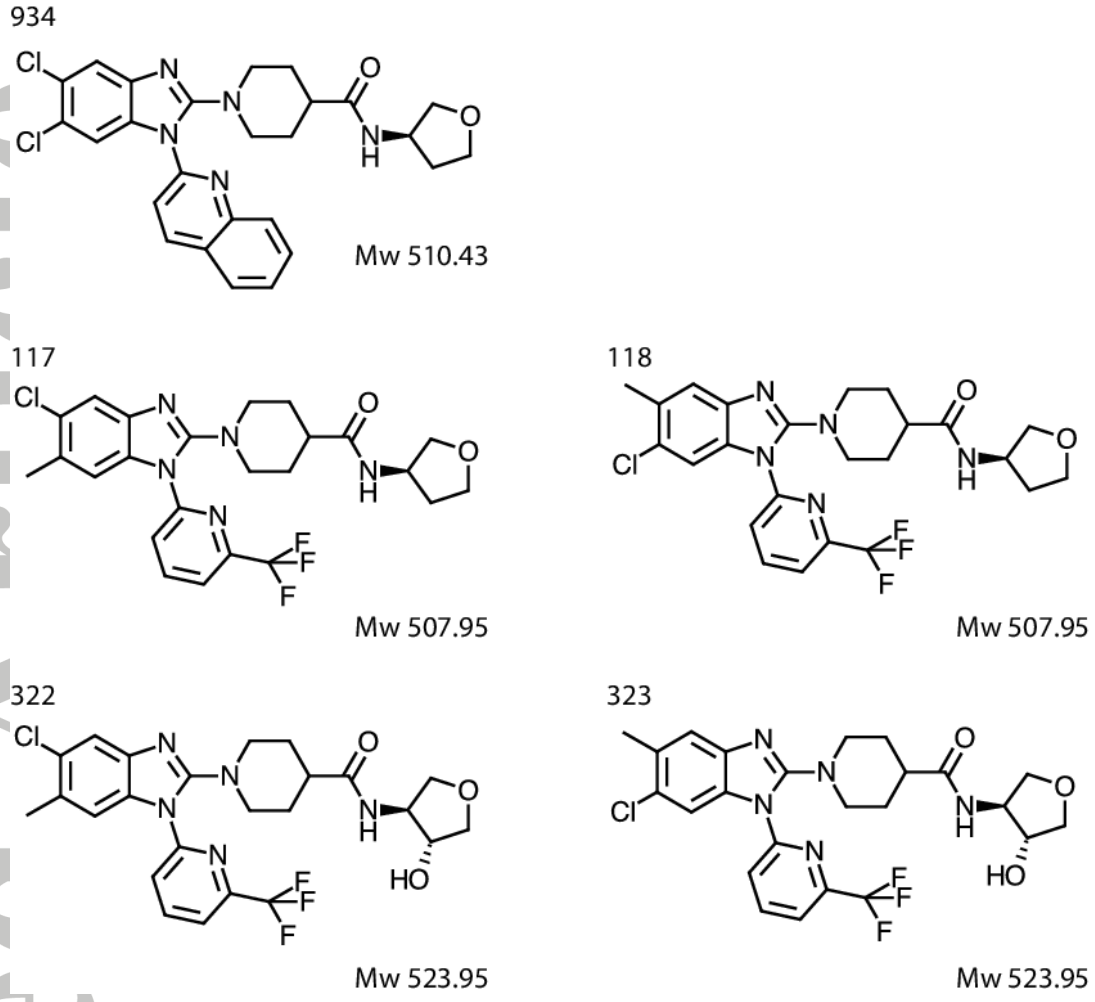

Figure 1. Chemical structures of small molecule mPGES-1 inhibitors.

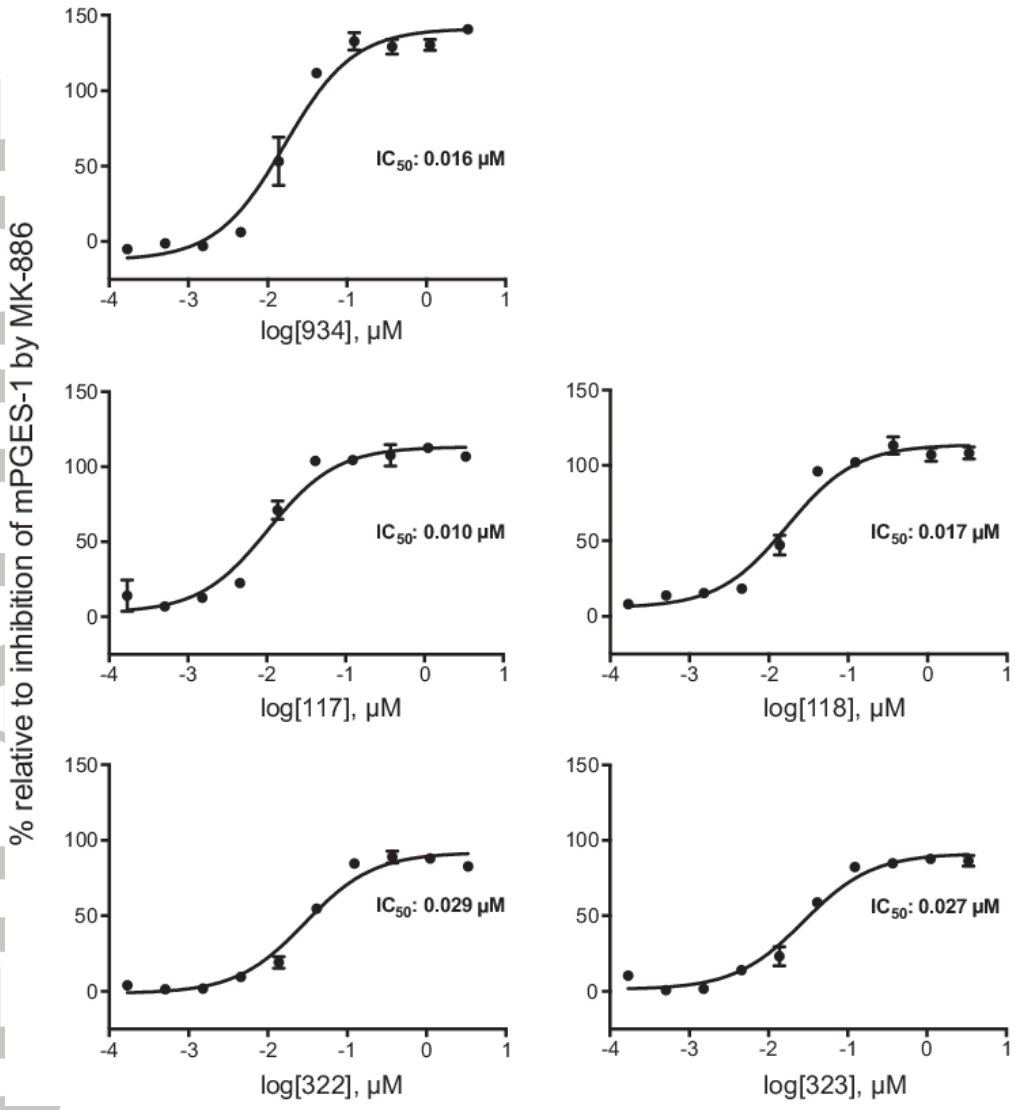

Figure 2. Inhibition of recombinant human mPGES-1 by 934, 117, 118, 322, and 323. Potency was determined by MDA-TBA assay. Data are presented as mean ± SD of technical duplicates from one representative experiment. The experiment was performed three times for 118 and four times for 934, 117, 322, and 323.

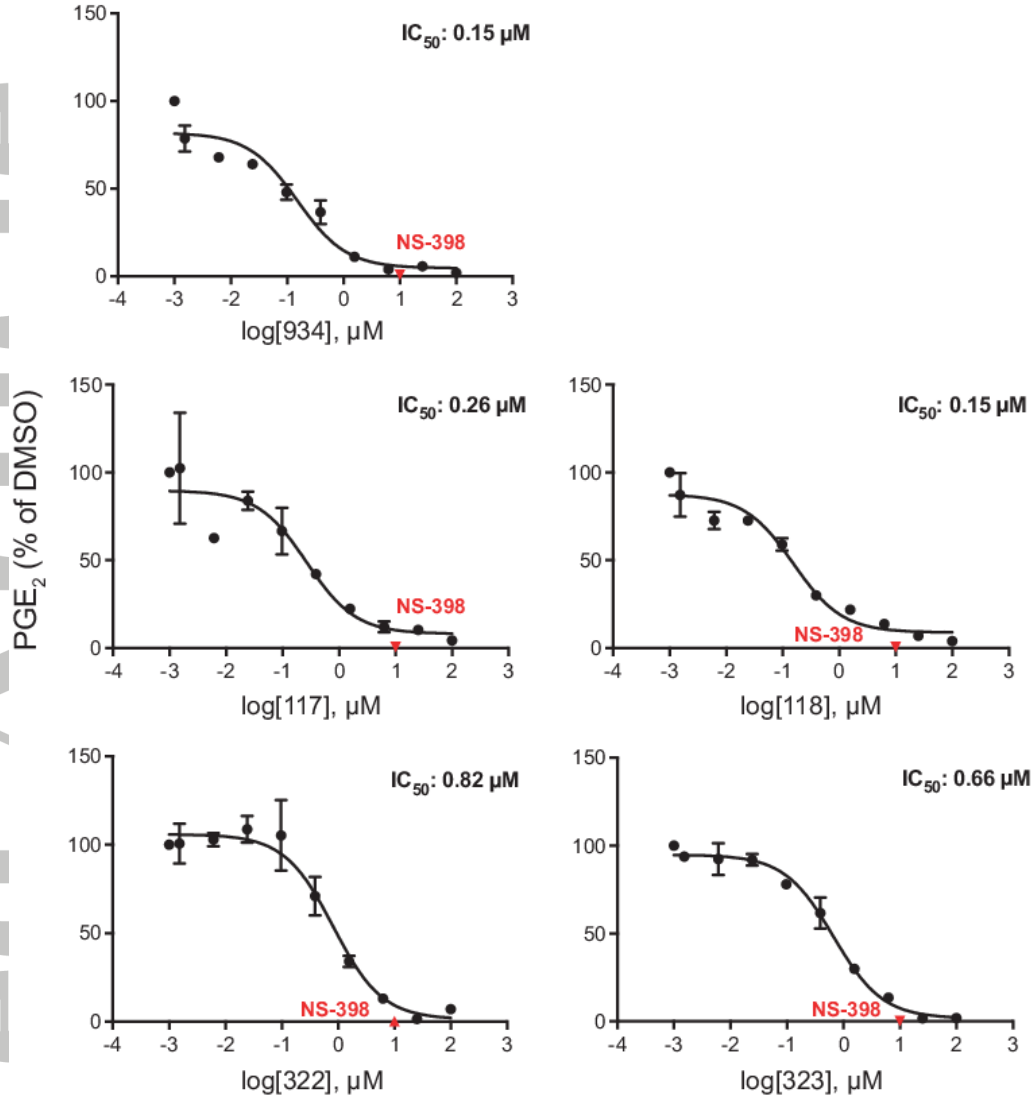

Figure 3. Inhibition of PGE_2 synthesis in intact A549 cells. A549 cells cultured in 2% FBS were treated with IL-1β (10 ng/ml) for 24 h in the presence of 934, 117, 118, 322, and 323 at indicated concentrations or vehicle control (1% DMSO). NS-398 at 10 µM was used as positive control. PGE_2 in supernatants was measured by EIA. Data are presented as mean ± SD of technical duplicates. The absolute PGE_2 concentration in the vehicle control was 10.8 ±0.4 ng/ml for 934, 5.0 ± 0.5 ng/ml for 117 and 118, and 19.7 ± 1.8 ng/ml for 322 and 323. The experiment was performed once.

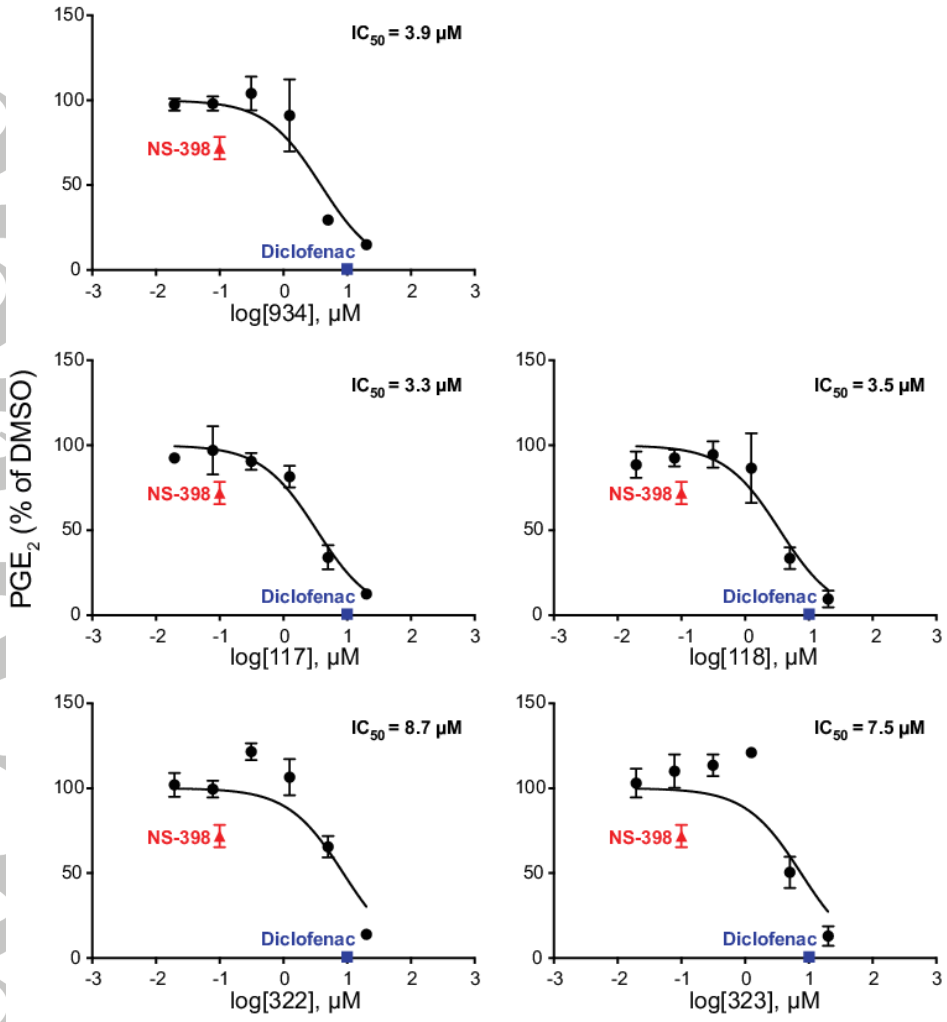

Figure 4. Inhibition of PGE_2 synthesis in human whole blood. Freshly drawn blood was incubated with compounds at various concentrations or vehicle control (DMSO) for 30 min and then treated with LPS (10 µg/ml) for 24 h, when plasma was recovered. Diclofenac at 10 µM and NS-398 at 0.1 µM were used as reference compounds. PGE_2 concentration was measured by LC-MS/MS. Data are presented as mean ± SD of technical duplicates from one representative experiment. The absolute PGE_2 production in the vehicle control was 45.7 ± 3.7 ng/ml. The compounds were tested in two experiments.

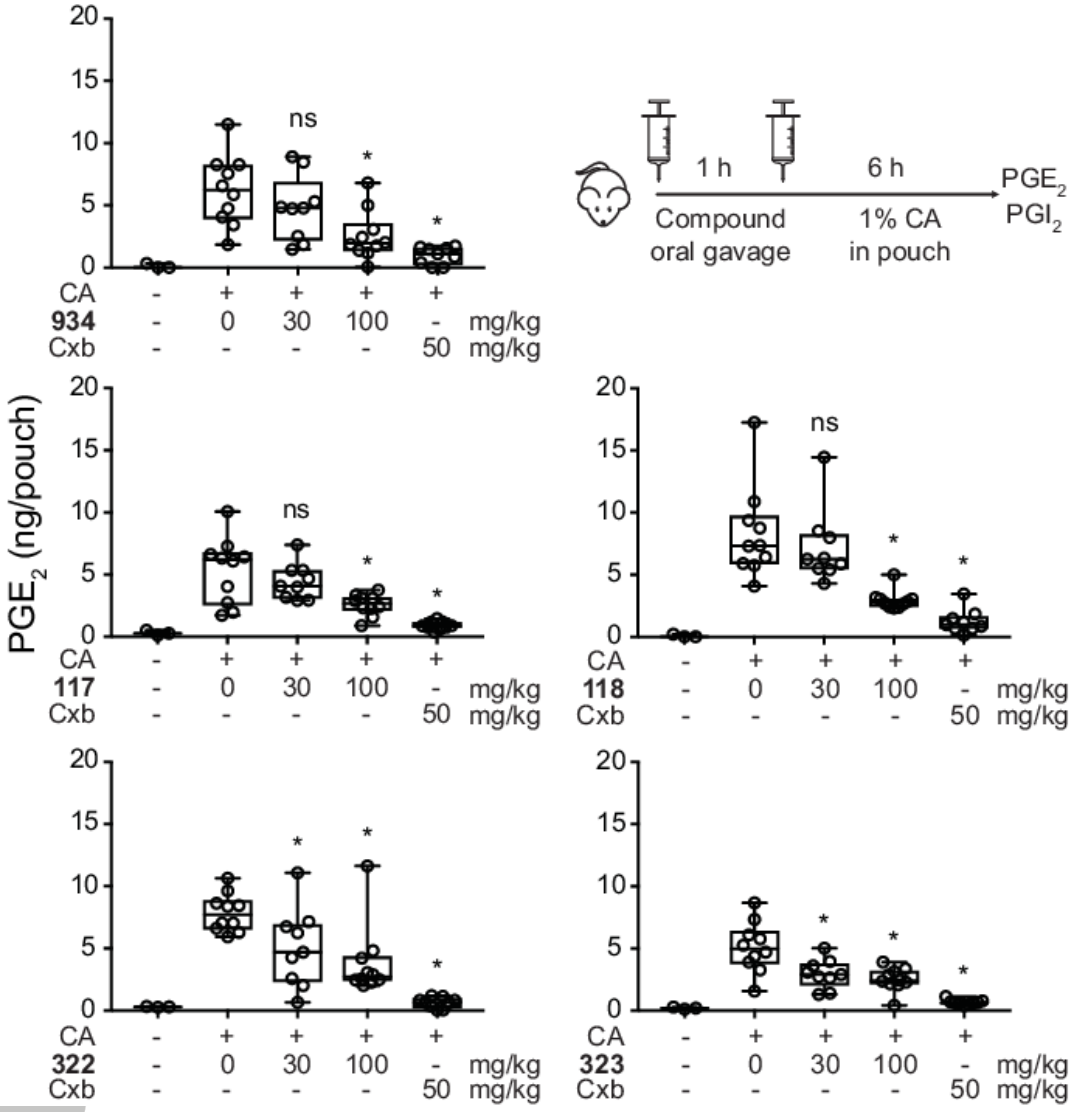

Figure 5. *In vivo* inhibition of mPGES-1. PGE_2 concentrations were measured in air pouch exudates without induction (n=3), with 1% CA induction (n=10), with 1% CA induction and mPGES-1 inhibitor (934, 117, 118, 322, and 323) at two doses (30 mg/kg, n=9 and 100 mg/kg, n=10), or COX-2 inhibitor celecoxib (Cxb, 50 mg/kg, n=9). Inhibitors were administered *p.o.* 1 h before induction. The effect of the inhibitors on PGE_2 concentrations were compared to 1% CA-induced air pouch 6 h post induction (*P<0.05).

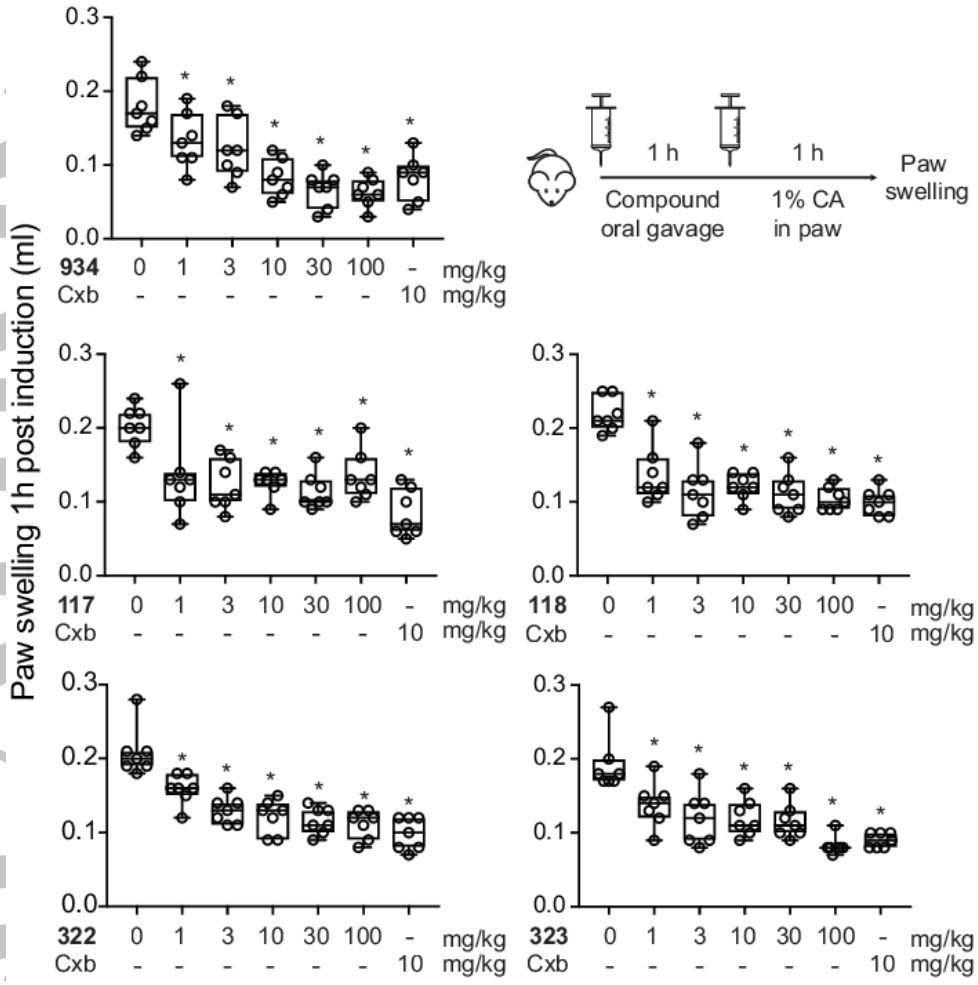

Figure 6. *In vivo* effect of mPGES-1 inhibition on inflammation. Paw swelling was recorded in rats treated with CA (n=7) and mPGES-1 inhibitors 934, 117, 118, 322, and 323 at different doses (*p.o.*, 1 h before CA induction; 1, 3, 10, 30, and 100 mg/kg, n=7). Celecoxib was used as reference compound (10 mg/kg, n=7). The effect of the inhibitors 1 h post induction on paw swelling was compared to vehicle treated controls (*P<0.05).

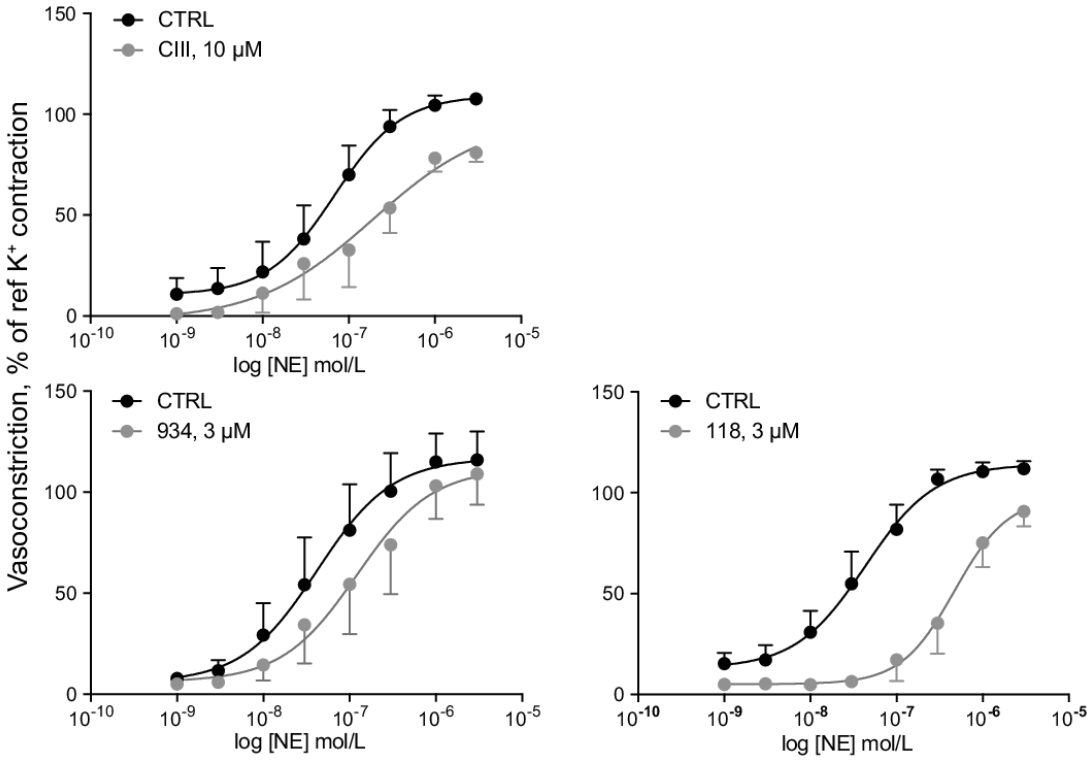

Figure 7. The effect of mPGES-1 inhibition on norepinephrine (NE)-induced vasoconstriction in resistance size arteries. Concentration-response curves are expressed as % of potassium contraction before (CTRL, black) and after incubation for 30 min with mPGES-1 inhibitors CIII, 118, or 934 (grey). Data is presented as mean ± SD with n=5. Significant reduction in vasoconstriction was seen with 118 ($P<0.05$) comparing individual EC_{50} values (concentration of NE where 50% constriction is obtained) and performing paired t-test.