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Effects of AF3442 [N-(9-ethyl-9H-carbazol-3-yl)-2-(trifluoromethyl)benzamide], a novel inhibitor of human microsomal prostaglandin E synthase-1, on prostanoid biosynthesis in human monocytes in vitro

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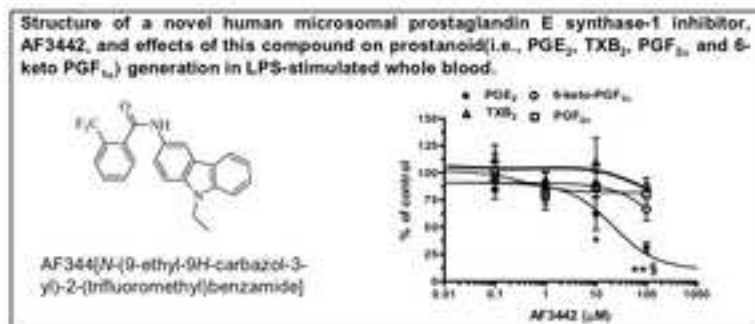
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5 **human monocytes in vitro**
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Abbreviations:

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2 mPGES-1, microsomal prostaglandin(PG)E synthase-1; LPS, *E. coli* lipopolysaccharide; TX,
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4 thromboxane; COX, cyclooxygenase; AF3442, [*N*-(9-ethyl-9*H*-carbazol-3-yl)-2-
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6 (trifluoromethyl)benzamide]; AA, arachidonic acid; PGI₂, prostacyclin; cPGES, cytosolic PGE
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8 synthase; tNSAIDs, traditional non-steroidal anti-inflammatory drugs; GI, gastrointestinal;
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10 MAPEG, membrane-associated proteins involved in eicosanoid and glutathione metabolism; TXS,
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12 TX synthase; PMSF, phenylmethylsulfonyl fluoride; FCS, Fetal Calf Serum; TBS, tris-buffered
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14 saline; SDS-PAGE, sodium dodecyl sulphate - polyacrylamide gel electrophoresis.
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Abstract

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2 Inhibitors of microsomal prostaglandin(PG)E synthase-1(mPGES-1) are being developed for the
3
4 relief of pain. Redirection of the PGH₂ substrate to other PG synthases, found both in vitro and in
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6 vivo, in mPGES-1 knockout mice, may influence their efficacy and safety. We characterized the
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8 contribution of mPGES-1 to PGH₂ metabolism in lipopolysaccharide(LPS)-stimulated isolated
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10 human monocytes and whole blood by studying the synthesis of prostanoids [PGE₂,
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12 thromboxane(TX)B₂, PGF_{2α} and 6-keto-PGF_{1α}] and expression of cyclooxygenase(COX)-isozymes
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14 and down-stream synthases in the presence of pharmacological inhibition by the novel mPGES-1
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16 inhibitor AF3442 [*N*-(9-ethyl-9*H*-carbazol-3-yl)-2-(trifluoromethyl)benzamide]. AF3442 caused a
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18 concentration-dependent inhibition of PGE₂ in human recombinant mPGES-1 with an IC₅₀ of
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20 0.06μM. In LPS-stimulated monocytes, AF3442 caused a concentration-dependent reduction of
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22 PGE₂ biosynthesis with an IC₅₀ of 0.41μM. At 1μM, AF3442 caused maximal selective inhibitory
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24 effect of PGE₂ biosynthesis by 61±3.3%(mean±SEM, P<0.01 versus DMSO vehicle) without
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26 significantly affecting other prostanoids (i.e., TXB₂, PGF_{2α} and 6-keto-PGF_{1α}). In LPS-stimulated
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28 whole blood, AF3442 inhibited in a concentration-dependent fashion inducible PGE₂ biosynthesis
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30 with an IC₅₀ of 29μM. A statistically significant inhibition of mPGES-1 activity was detected at 10
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32 and 100μM (38±14%, P<0.05, and 69±5%, P<0.01, respectively). Up to 100μM, the other
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34 prostanoids were not significantly affected. In conclusion, AF3442 is a selective mPGES-1 inhibitor
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36 which reduced monocyte PGE₂ generation also in the presence of plasma proteins. Pharmacological
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38 inhibition of mPGES-1 did not translate into redirection of PGH₂ metabolism towards other
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40 terminal PG synthases in monocytes. The functional relevance of this observation deserves to be
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42 investigated in vivo.
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57 **Keywords:** mPGE synthase-1, PGE₂, monocytes, inflammation, human whole blood
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1. Introduction

1 Prostanoids are biologically active derivatives of arachidonic acid(AA) released from membrane
2 phospholipids by phospholipases [1]. AA is transformed into prostaglandin(PG)H₂, through the
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7 activity of cyclooxygenase(COX) enzymes (i.e. COX-1 and COX-2), and subsequently PGH₂ is
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10 metabolized by terminal synthases to the biologically active prostanoids, i.e. prostacyclin(PGI₂),
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Several lines of evidence suggest that the mechanism of action of traditional non-steroidal anti-inflammatory drugs(tNSAIDs) and NSAIDs selective for COX-2 (named coxibs) for the treatment of pain and inflammatory joint disease, is mainly through the inhibition of COX-2-dependent PGE₂ [3]. Inhibition of constitutively expressed COX-1 in gastrointestinal(GI) tract and presumably in platelets by tNSAIDs seems to play a role in increased risk of upper GI bleeding/perforation [4]. In fact, coxibs which spare COX-1 are associated with lower GI toxicity [3]. Although the inhibition of COX-2-dependent PGE₂ generation by tNSAIDs and coxibs is effective in ameliorating symptoms of inflammation and pain, a small but consistent increased risk of myocardial infarction has been detected in NSAID users [5, 6, 7]. Such adverse reactions are probably linked to the inhibition of COX-2-derived PGI₂ in vascular endothelial cells in the absence of an almost complete and persistent inhibition of platelet COX-1-derived TXA₂ [5, 6, 7]. Thus, a great interest is in the development of new anti-inflammatory drugs with a safer profile. Inhibitors of mPGES-1 are being developed for relief of pain - similarly to NSAIDs - but with the potential advantage of not affecting or perhaps causing a beneficial impact on the cardiovascular system [8, 9]. Inhibitors of mPGES-1 might circumvent adverse GI side-effects of unselective COX inhibitors. However, in the stomachs of mice lacking mPGES-1 a shift from PGE₂ to all other prostanoids, i.e. TXA₂, PGD₂, PGF_{2α}, and PGI₂, was detected thus making extremely difficult to predict the clinical consequences of mPGES-

1 inhibition in the GI tract [10]. Further studies addressing this issue in appropriate animal models
2 are required.

3
4 mPGES-1 belongs to the MAPEG(membrane-associated proteins involved in eicosanoid and
5 glutathione metabolism) superfamily [11]. Similar to COX-2, mPGES-1 is highly up-regulated by
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7 proinflammatory stimuli and participates in elevated PGE₂ generation in inflammation [11]. It is
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9 functionally coupled with COX-2 [12], but differences in the promoter region between mPGES-1
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11 and COX-2 [13] suggest divergent transcriptional mechanisms responsible for the inducible
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13 regulation of COX-2 and mPGES-1.

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15 Deletion of mPGES-1 is associated with a profound inhibition of PGE₂ in vitro and in vivo and with
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17 a redirection of the PGH₂ substrate to other PG synthases, such as PGI₂ and PGD₂, which might
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19 participate to the safe cardiovascular profile [8, 9]. Discrepant results have been found on the
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21 possible shift of PGH₂ metabolism to TXA₂ [8, 14]. Whether redirection of PGH₂ metabolism
22
23 towards other PG synthases might be influenced by the extent of reduction of mPGES-1 and by
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25 cell-specific features is unknown.

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27 Development of biomarkers to predict drug responses is useful for a rational selection of doses for
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29 efficacy and toxicity. Endogenous mediators of inflammation might be used as biomarkers for
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31 therapeutic effects [15, 16]. Thus, the assessment of COX-2 inhibition by the evaluation of PGE₂
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33 levels in whole blood in response to *E. coli* lipopolysaccharide (LPS) (an index of inducible COX-2
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35 activity in monocytes) both in vitro and ex vivo (i.e. after dosing) [15, 17] is considered a promising
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37 biomarker of drug response (analgesia, anti-inflammatory effects) in humans [15, 16]. In fact, it has
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39 been shown that inhibition of LPS-induced PGE₂ by approximately 80% is sufficient to translate
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41 into clinical efficacy (analgesia) in humans [15].

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43 The design, synthesis and characterization of a series of carbazole benzamides led us to the
44
45 identification of several mPGES-1 inhibitors, among which AF3442[*N*-(9-ethyl-9*H*-carbazol-3-yl)-
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47 2-(trifluoromethyl)benzamide] was identified as a lead compound and selected for further
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49 characterization [18].
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2 In the present study we aimed to characterize the contribution of mPGES-1 to PGH₂ metabolism in
3 LPS-stimulated isolated human monocytes and whole blood by studying the synthesis of
4 prostanoids and expression of COX-isozymes and down-stream synthases in the presence of
5 pharmacological inhibition by AF3442. We choose to study the effect of the compound in human
6 monocytes because they represent an important cell-target of the clinical efficacy of mPGES-1
7 inhibitors. Interestingly, we found that inhibition of PGE₂ generation at clinically relevant ranges
8 was not associated with any significant change of other prostanoids in human monocytes in vitro.
9 The functional relevance of this observation deserves to be investigated in vivo.
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2. Materials and Methods

2.1. Materials

AF3442 (Figure 1) was synthesized and characterized in the Angelini Research Center [18]. MK-886 (3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2, 2-dimethylpropanoic acid) was purchased from Calbiochem (San Diego, CA, USA). RPMI-1640 and fetal calf serum (FCS) were purchased from GIBCO-Invitrogen (Carlsbad, CA, USA). Interleukin-1 β (IL-1 β), penicillin/streptomycin, L-glutamine, LPS (derived from Escherichia Coli 026:B6), anti-rabbit peroxidase-conjugated IgG were from Sigma-Aldrich (St. Louis, MO, USA). PGH₂ substrate, COX-1, mPGES-1, mPGES-2, cPGES, TXS, PGIS polyclonal antibodies were from Cayman Chemical (Ann Arbor, MI, USA); β -actin and anti-goat peroxidase-conjugated IgG were purchased from Santa Cruz (USA). COX-2 polyclonal antibody was kindly provided by Dr Stacia Kargman from Merk Frosst (Canada). Nucleofector Kit T was from Lonza (Cologne, AG, Germany). Ficoll-Paque and ECL plus were from GE Healthcare Life Sciences (Bucks, UK). Polyvinylidene fluoride (PVDF) membrane was purchased from Bio-Rad Laboratories (Hercules, CA).

2.2. Cloning and expression of human mPGES-1 in A549 cells

Cloning of human mPGES-1 was performed isolating mRNA from A549 (human lung adenocarcinoma) cells cultured in RPMI-1640 supplemented with 2% (v/v) FCS and stimulated with IL-1 β (10ng/ml) for 24 h. It was reverse transcribed into cDNA. Polymerase Chain Reaction amplification was then performed with specific primers [5'-CGGATCCGCCACCATGCCTGCCCACAGCCTG -3' (FW) and 5'-GCTCTAGAGTCACAGGTGGCGGGCCGC -3' (REV) containing BamHI and XbaI restriction sites respectively] and fragments of the expected 459 bp size were obtained, ligated in the bacterial expression vector pcDNA3.1 and transformed into JM109 competent cells. Plasmids were isolated and miniprep products from a few colonies were subjected to restriction analysis that confirmed the presence of the expected fragment pattern. Two clones were sequenced in comparison with a sequence from EMBL Databank (accession number AF027740) and a conservative mutation in

1 position 183 was found (a C replacing a T); however, the protein sequence alignment of the 2
2 clones with that of the reference mPGES-1 submitted in GenBank resulted in a 100% aminoacid
3 identity. A549 cell lines were transfected in order to obtain a stable cell line overexpressing
4 mPGES-1 enzyme. Transfection was performed in cuvettes by Nucleofector II (AMAXA)
5 instrument using Nucleofector Kit T according to the manufacturer's instructions. The recombinant
6 vector pcDNA3.1 (Neomycine resistant) ligated with human mPGES-1 was used for transfection.
7 Transfection efficiency was assessed transfecting cells with a GFP (green fluorescent protein)-
8 expressing vector and checking cell fluorescence by fluorescence microscopy after 4 and 24 h from
9 transfection. After 48 h the medium was replaced with medium containing 400µg/ml G418
10 (neomycin) as selecting agent. Cells were grown until neomycin selection in untransfected cells was
11 confirmed. Clones were amplified and subsequently tested for mPGES-1 expression by Western
12 blot [19] and for PGE₂ production by a validated radioimmunoassay [17]. Clone D8L was selected
13 for its mPGES-1 high expression level.
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31 *2.3. Preparation of human mPGES-1-containing microsomal fraction*

32 A549 and A549/human mPGES-1 (A549 clone D8L) cells maintained in culture were centrifuged,
33 washed once with PBS and suspended at 10×10^6 cells/ml in lysis buffer [TrisHCl 15mM; sucrose
34 0.25M; EDTA 0.1mM; phenylmethylsulfonyl fluoride (PMSF) 2mM; Aprotinin 10µg/ml;
35 Leupeptin 25µg/ml; glutathione 1mM]. The samples were kept on ice for 10 minutes. Then, the
36 cells were lysed by 4 x 30 seconds sonication pulses in a BANDELIN Soniplus sonifier at 70% of
37 maximum power, frequency 9. Cell debris was removed by centrifugation at 10 000g for 15 minutes
38 at 4°C. The supernatant was centrifuged at 250 000g for 1 h and the membrane pellets were finally
39 suspended in 0.1ml of PGEG plus buffer (i.e. potassium phosphate, 10mM, pH 7, glycerol, 20%,
40 EDTA, 0.1mM, glutathione, 1mM) containing PMSF 2mM, Aprotinin 10µg/ml, Leupeptin
41 25µg/ml, for each milliliter of lysis buffer. Total protein amount was determined by the Bradford
42 method (Bio-Rad Protein Assay) according to the manufacturer's instructions. Microsomal
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1 preparations were stored at -80°C until used. The presence of the mPGES-1 protein in the
2 microsomal preparation was confirmed by Western blot technique (Figure 3A).
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4 *2.4. Inhibition of recombinant human mPGES-1*

5 Aliquots of microsomal preparation from mPGES-1-overexpressing A549 clone D8L (30 $\mu\text{g}/\text{ml}$
6 protein concentration) were used for assessing the enzymatic activity using MultiPROBE II
7 Automated Liquid Handling System (Perkin Elmer, Waltham, MA, USA) in the presence of 2.5mM
8 reduced glutathione and 10 μM PGH₂ substrate.
9

10 AF3442 (0.01-10 μM) was pre-incubated with reaction mixture containing microsomal preparation
11 for 15 minutes at 4°C . Reaction was started by substrate addition (PGH₂) and stopped after 5
12 minutes at 4°C with 100 μl of stop solution (40mM ferric chloride and 0.4mM citric acid). Samples
13 were then subjected to PGE₂ quantification by previously described radioimmunoassay technique
14 [17]. MK-886 (0.1-100 μM), an inhibitor of MAPEG proteins, such as leukotriene C₄ synthase and
15 5-lipoxygenase activating protein (FLAP), affecting mPGES-1 activity at higher concentrations [20,
16 21] was assessed, as positive control.
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18 *2.5. Isolation of human monocytes and pharmacological treatment*

19 Human monocytes were separated from buffy coat by Ficoll-Paque, as previously described [17].
20 The contribution of COX-1 to prostanoid biosynthesis was minimized by pretreating the buffy coat
21 with aspirin (300 μM). Mononuclear cell layer (obtained by Ficoll-Hypaque density gradient
22 centrifugation) was treated with EDTA (2mM) for 15 minutes at 37°C to remove platelets
23 specifically adherent to the monocytes [22]. Cell suspensions routinely contained >90% monocytes
24 [17]. Monocytes (1.5-2 $\times 10^6$ cell/ml) cultured in RPMI-1640 supplemented with 0.5% (v/v) of FCS,
25 1% (v/v) penicillin/streptomycin and L-glutamine (2mM), were incubated with LPS (10 $\mu\text{g}/\text{ml}$)
26 (derived from Escherichia Coli 026:B6) at 37°C for 24 h in the presence of DMSO vehicle or
27 increasing concentrations (0.01-100 μM) of the mPGES-1 inhibitor, AF3442. This compound was
28 dissolved in DMSO and 2 μl of vehicle or stock solution was added to 1ml of cell suspension. PGE₂,
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TXB₂ (the stable hydrolysis product of TXA₂), PGF_{2α} and 6-keto-PGF_{1α} (the hydrolysis product of PGI₂) levels were measured in cell culture media by previously described and validated radioimmunoassay techniques [17, 19, 23], while COX-2, COX-1, mPGES-1, mPGES-2, cPGES, TXS and PGIS protein levels were evaluated in cell lysates by previously described Western blot techniques [17, 19].

2.6. Isolation of human platelets

Platelet-rich plasma was prepared by centrifugation of citrated blood at 200g for 15 minutes and platelets were isolated from plasma as previously described [24, 25] and resuspended in RPMI-1640 supplemented with 0.5% (v/v) FCS. Platelet suspensions (5x10⁸/ml; containing <1 leukocyte per 10 000 platelets) were analyzed for the levels of COX-2, COX-1, mPGES-1, mPGES-2, cPGES and TXS in cell lysates by Western blot technique [17, 19].

2.7. Effects of AF3442 on prostanoid generation in human whole blood

Whole blood was drawn from 13 healthy volunteers (age range: 23-35 years). The study was approved by the local Ethics Committee and informed consent was obtained from each subject. The inhibitory effect towards constitutive PGE₂ generation was assessed by evaluating PGE₂ levels generated in whole blood allowed to clot for 60 minutes at 37°C. In clotting whole blood, PGE₂ is mainly produced by platelets in response to endogenously generated thrombin through the activity of COX-1 [23-26] and cPGES and/or mPGES-2. In fact, mPGES-1 is not detectable in platelets (Figure 2). The parallel measurement of TXB₂ (the major product of endogenous AA metabolism in platelets), PGF_{2α} and 6-keto-PGF_{1α} was performed to verify a possible off-target effect of AF3442 on COX-1. The effects of AF3442 on inducible PGE₂ generation were studied by assessing the levels of PGE₂ produced in heparinized human whole blood stimulated for 24 h with LPS (10μg/ml), a stimulus for the induction of both COX-2 and mPGES-1 in monocytes [17, 27]. The simultaneous measurements of TXB₂, PGF_{2α} and 6-keto-PGF_{1α} were performed to verify the impact of AF3442 on COX-2 and the possible redirection of the accumulated PGH₂ substrate towards other PG synthases. For the assessment of the effects on constitutive PGE₂ generation, peripheral venous

1 blood samples were drawn from healthy volunteers when they had not taken any NSAID during the
2 2 weeks preceding the study. For the assessment of the effects on LPS-induced PGE₂ generation,
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4 heparinized peripheral venous blood samples were drawn from the same subjects pretreated with
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6 300mg aspirin 48 h before sampling to suppress the contribution of platelet COX-1 activity.
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9 Different concentrations (0.001-100μM) of AF3442 or DMSO vehicle (2μl) were incubated with
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11 whole blood samples and then allowed to clot at 37°C for 1 h or with heparinized whole blood
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13 samples in the presence of LPS (10μg/ml) at 37°C for 24 h. Prostanoids were measured in serum or
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15 plasma by previously described and validated radioimmunoassays [17, 19, 23]. In these assays the
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17 least detectable concentration was 1-2 pg/ml for all prostanoids.
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20 21 22 2.8. Western blot analysis

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24 Microsomal fractions or cell lysates samples were loaded (30 or 25-50 μg/lane, respectively) onto
25
26 4-15% SDS- PAGE and transferred to PVDF membrane. Membranes were saturated with a solution
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28 of 5% non-fat milk in tris-buffered saline-0.1% Tween-20 (TBS-Tween-20), and then incubated
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30 with anti-COX-2, anti-COX-1, anti-mPGES-1, anti-mPGES-2, anti-cPGES, anti-TXS, anti-PGIS, or
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32 anti-β-actin polyclonal antibodies for 1 h at room temperature [19]. Then, the membranes were
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34 washed in TBS-Tween-20 and incubated with the secondary antibodies: anti-goat peroxidase-
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36 conjugated IgG for β-actin or anti-rabbit peroxidase-conjugated IgG for the other proteins. Finally,
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38 the membranes were washed in TBS-Tween-20 and then all blots were developed by using ECL
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40 plus detection according to the manufacturer's instructions.
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48 Results are expressed as mean±SEM. For the experiments of LPS-stimulated monocytes and whole
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50 blood, the production of PGE₂, TXB₂, PGF_{2α} and 6-keto-PGF_{1α} was subtracted from the levels of
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52 the prostanoids measured in the presence of vehicle (DMSO). Statistical analysis was performed
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54 with Student's t test or one-way ANOVA and Newman-Keuls multiple comparison test, using
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56 GraphPad Instat (version 3.00 for Windows). Values of P<0.05 were considered statistically
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1 significant. Concentration-response curves were fitted (using PRISM, GraphPad, version 5.00 for
2 Windows, San Diego, CA) and IC₅₀ values were reported.
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3. Results

3.1. Effect of AF3442 on human recombinant mPGES-1

As shown in Figure 3B, AF3442 caused a concentration-dependent inhibition of PGE₂ generation by human recombinant mPGES-1 with an IC₅₀ of 0.06 μM. In the same microsomal preparation, MK-886, used as a positive control, inhibited PGE₂ generation with an IC₅₀ value of 4.1 μM (not shown), a comparable value to that found by Mancini et al. [20] (IC₅₀: 3.2 μM).

3.2. Expression of COX-isozymes and down-stream synthases and prostanoid generation in LPS-stimulated human monocytes

As shown in Figure 4, in isolated human monocytes stimulated with LPS, COX-2, mPGES-1 were profoundly induced. A slight, but reproducible, increase of cPGES by only 2-fold was detected. In contrast, we detected a constitutive expression of COX-1, TXS, mPGES-2 which was not affected by LPS. PGIS was barely detectable (Figure 4). The spectrum of prostanoid generation (TXB₂, PGE₂, PGF_{2α} and 6-keto-PGF_{1α}) in monocytes both unstimulated and LPS-stimulated is reported in the Table. LPS caused a significant increase of their generation. TXB₂ was the major product of AA (increased by 370-folds) while 6-keto-PGF_{1α} was the minor one (increased by 10-folds). PGE₂ and PGF_{2α} were increased at a similar extent (120-folds) (Table).

3.3. Effects of AF3442 on prostanoid generation in LPS-stimulated human monocytes

Incubation of LPS-stimulated monocytes for 24 h with AF3442 caused a concentration-dependent reduction of PGE₂ biosynthesis with an IC₅₀ value of 0.41 μM (Figure 5). At 1 μM, AF3442 caused an inhibitory effect of 61±3.3% (P<0.01 versus DMSO vehicle) without significantly affecting the other prostanoids (TXB₂, PGF_{2α} and 6-keto-PGF_{1α}: 25.1±5.1%, 21.1±4.1%, 14.8±4.8% of inhibition, respectively) (Figure 5). Similar effects were found at 10 μM. In contrast, at 100 μM, we detected a higher reduction in the biosynthesis of PGE₂ (71.2±2.4%, P<0.05 versus 1 and 10 μM) (Figure 5). This additional inhibitory effect of PGE₂ was associated with a significant reduction of TXB₂ generation of 54.7±5.5% (P<0.01 versus DMSO vehicle). A lower reduction, but not

1 significant, was detected for $\text{PGF}_{2\alpha}$ and 6-keto- $\text{PGF}_{1\alpha}$ ($38.0\pm 8.9\%$ and $29.0\pm 8.8\%$, respectively)
2 (Figure 5).
3

4 These data suggest a selective, but incomplete, inhibitory effect of AF3442 on mPGES-1 activity up
5 to $10\mu\text{M}$. At $100\mu\text{M}$, PGE_2 reduction was associated with an inhibitory effect on TXB_2
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7 suggesting a possible off-target effect on COX-2 activity and/or expression.
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10 3.4. Effects of AF3442 on the expression of COX-isozymes and down-stream synthases in LPS- 11 stimulated human monocytes 12 13 14 15

16 As shown in Figure 6, AF3442 up to $10\mu\text{M}$ did not significantly affect the levels of COX-2, COX-
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18 1, mPGES-1, mPGES-2, cPGES and TXS. In contrast, at $100\mu\text{M}$, the compound caused a
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20 significant ($P<0.01$ versus control) reduction only of COX-2 levels by $31.4\pm 2.6\%$ (assessed as ratio
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22 of their optical density normalized to the optical density of β -actin).
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26 3.5. Effects of AF3442 on prostanoid generation in human whole blood assays 27 28

29 To study whether the compound affected mPGES-1 activity even in the presence of plasma
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31 proteins, we assessed the effect of AF3442 on prostanoid generation in LPS-stimulated whole
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33 blood. Incubation of heparinized whole blood with LPS ($10\mu\text{g/ml}$) resulted in a significant ($P<0.01$)
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35 enhancement of PGE_2 , TXB_2 , $\text{PGF}_{2\alpha}$ and 6-keto- $\text{PGF}_{1\alpha}$ production (Table). In this experimental
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37 model, PGE_2 and TXB_2 were the major prostanoid generated with PGE_2 that was higher than
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39 TXB_2 ($P<0.05$) (Table). As reported in Figure 7A, AF3442 inhibited in a concentration-dependent
40
41 fashion LPS-induced PGE_2 biosynthesis with an IC_{50} of $29\mu\text{M}$. This value was 70-fold higher than
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43 that found in isolated human monocytes (in the presence of low plasma protein level: 0.5% FCS)
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45 suggesting an important binding of the compound to plasma proteins. A significant and selective
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47 inhibition of mPGES-1 activity was detected at 10 and $100\mu\text{M}$ ($38\pm 14\%$, $P<0.05$ and $69\pm 5\%$,
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49 $P<0.01$ versus vehicle, respectively). Up to $100\mu\text{M}$, the other prostanoids were not significantly
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51 affected (Figure 7A).
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1 Then, we assessed the impact of AF3442 on thrombin-stimulated prostanoids which are generated
2 mainly from platelet COX-1 and constitutive down-stream synthases. In fact, in platelets inducible
3 COX-2 and mPGES-1 were not detectable (Figure 2). As shown in Figure 7B, the compound did
4 not affect thrombin-stimulated PGE₂ and also other prostanoids.
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9 Altogether, these results showed that the compound up to 100µM - causing a clinically relevant
10 degree of inhibition of PGE₂ by approximately 70% - is a selective inhibitor of mPGES-1 in human
11 whole blood.
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4. Discussion

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2 Inhibitors of mPGES-1 are potential, novel anti-inflammatory drugs with the possible advantage of
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4 not affecting or perhaps causing a beneficial impact on cardiovascular homeostasis [9]. However,
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6 redirection of the PGH₂ substrate to other PG synthases in the presence of mPGES-1 deletion has
7
8 been shown both in vivo and in vitro [8, 14]. This phenomenon might occur at different extent in
9
10 different cell types, thus, translating into the generation of prostanoids with opposite effects for the
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12 cardiovascular system [9]. It is unknown whether this effect occurs also in the presence of an
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14 incomplete inhibition of mPGES-1, as it would take place after drug treatment. Thus, we studied the
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16 contribution of mPGES-1 to PGH₂ metabolism in LPS-stimulated isolated human monocytes and
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18 whole blood in the presence of pharmacological inhibition by the novel mPGES-1 inhibitor,
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20 AF3442 [18]. We aimed to explore this effect in human monocytes because: i) they are the target
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22 for clinical efficacy; ii) they play a key role in atherogenesis through the generation of PGE₂, a
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24 mediator of inflammation, and TXA₂, a potent proaggregatory and vasoconstrictor agent [28].
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26 AF3442 affected LPS-induced mPGES-1 activity of human monocytes even in the presence of
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28 plasma proteins, i.e. in whole blood (Figure 7A). In whole blood at 100µM, it caused a clinically
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30 relevant degree of inhibition of PGE₂ by approximately 70% which was not associated with any
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32 significant change of the biosynthesis of other prostanoids.
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36 AF3442 is a molecule belonging to the carbazole class of compounds [18]. It was very potent
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38 towards recombinant human mPGES-1 (IC₅₀: 0.06 µM) and at 1µM, it completely suppressed the
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40 enzyme activity. In LPS-stimulated monocytes (in the presence of low concentrations of proteins,
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42 i.e. 0.5% FCS), AF3442 1µM caused a maximal, selective inhibition of PGE₂ of 61%. An almost
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44 comparable selective degree of inhibition of PGE₂ generation (by 69%) was obtained at 100µM of
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46 the compound in LPS-stimulated whole blood which suggests an extensive plasma protein binding
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48 by the compound. An important issue is whether this extent of inhibition of LPS-stimulated whole
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50 blood PGE₂ by AF3442 might be clinically relevant. We have previously looked at the degree of
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52 inhibition of LPS-stimulated whole blood PGE₂ by circulating peak concentrations of therapeutic
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1 doses of several NSAIDs [6]. Our results show that low-dose ibuprofen (1200 mg/day) caused a
2 maximal inhibition of 75% [6]. Moreover, it has been shown that therapeutic plasma concentrations
3 of analgesic doses of NSAIDs correlated with the IC₈₀ (drug concentration which inhibits enzyme
4 activity by 80%) for inhibition of LPS-stimulated whole blood PGE₂ production in vitro [15].
5
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7 Altogether these findings suggest the possible clinical efficacy of AF3442 which should be
8 confirmed by in vivo studies.
9

10 Failure of AF3442 to completely suppress inducible PGE₂ generation could be explained by the
11 contribution of other PGES, such as mPGES-2 which may couple to COX-2, to the synthesis of this
12 prostanoid [29]. Alternatively, it is possible that some PGH₂ isomerized to PGE₂, non-
13 enzymatically [30, 31]. However, in murine macrophages, in presence of deletion of mPGES-1,
14 PGE₂ generation was reduced by more than 90% demonstrating that mPGES-1 activity is a key
15 factor in LPS-induced PGE₂ production, in this cell-type [8, 14].
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29 Unexpectedly, we showed that in the presence of an incomplete suppression of mPGES-1, shunting
30 of COX-2-derived PGH₂ to other prostanoids did not occur. This finding was showed both in whole
31 blood and in isolated monocytes.
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36 In human monocytes, LPS induced the generation of TXB₂, PGE₂, PGF_{2α} and extremely low levels
37 of PGI₂. TXB₂ was the dominant product of monocytes exceeding by 10-fold PGE₂.
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41 High levels of expression of TXS, together with efficiency of coupling between TXS and COX-2
42 [32, 33] might be the determinants which contribute to the dominant generation of inducible TXA₂
43 in isolated monocytes. In fact, it has been shown that TXS efficiently couples to COX-2 when small
44 amounts of AA are gradually supplied over a long period, such as in response to LPS [32, 33]. In
45 this scenario, mPGES-1 is not the dominant pathway of PGH₂ metabolism; thus its inhibition
46 translates into a limited accumulation of PGH₂ which explains our failure to detect redirection
47 towards TXB₂ and other prostanoids. This is different from the results obtained in thioglycollate-
48 elicited peritoneal macrophages stimulated in vitro with LPS [8, 14] showing that PGE₂ is the major
49 prostanoid and deletion of mPGES-1 caused redirection towards TXB₂ and PGI₂, but TXB₂
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1 predominated. However, Boulet et al. [10] studied the shunting of PGH₂ to alternative prostanoid
2 pathways in the presence of complete and incomplete loss of mPGES-1 gene and they did not detect
3 a significant redirection towards TXB₂ in peritoneal macrophages under similar experimental
4 conditions. The reason of these discrepant results is unclear. It has been suggested that the use of
5 mice with different genetic background might have played a role [14].

6
7 We propose that differential expression of influx and efflux transport proteins may influence PGH₂
8 redirection towards other PG synthases. Excess PGH₂ may be immediately released outside the cell
9 after intracellular biosynthesis - probably through the action of MRP4/ABCC4 (multidrug
10 resistance protein 4 adenosine triphosphate-binding cassette, subfamily C, member 4) [34] or simple
11 diffusion - and then it may be reuptaken, probably via the prostaglandin transporter [35, 36]. Thus,
12 shunting of PGH₂ to other prostanoids may be dependent on the expression of transport proteins in
13 different cell types. This hypothesis will be explored in a specific study.

14
15 In order to verify the occurrence of PGH₂ redirection in the presence of mPGES-1 inhibition in a
16 more physiologic system, we studied the effect of AF3442 in LPS-stimulated whole blood where
17 TXB₂, PGE₂, PGF_{2α} and 6-keto-PGF_{1α} are mainly from monocytes through the activity of induced
18 COX-2 [17]. In this system, PGE₂ is the major prostanoid generated which was only slightly more
19 abundant than TXB₂ (Table). The lower concentration of PGE₂ generated in isolated monocytes
20 versus whole blood may suggest the contribution of extramonocyte mPGES-1 to the generation of
21 PGE₂ in whole blood. In particular, neutrophil mPGES-1, which has been shown to be
22 constitutively expressed [37], might act a part. However, even in whole blood where PGE₂ was the
23 major prostanoid, we did not detect significant redirection of PGH₂ metabolism towards other
24 prostanoids. These results may suggest that accumulated PGH₂ is rapidly released and not reuptaken
25 by blood cells. Released PGH₂ may bind to plasma proteins with avidity thus preventing
26 transcellular metabolism of PGH₂ between blood cells and leading to its degradation.

27
28 Deletion of mPGES-1 retards atherogenesis and limits aortic aneurysm formation in
29 hyperlipidaemic mice [8, 9]. However, it does not predispose to thrombogenesis and has a limited

1 impact on blood pressure compared to inhibition of COX-2 [8, 9]. These favourable effects for the
2 cardiovascular system, in the presence of deletion of mPGES-1, may at least in part be due to
3
4 augmented excretion of major metabolites of PGD₂ (PGD-M) and PGI₂ (PGI-M) [8, 9, 38].
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7 Enhanced synthesis of PGI₂ in mPGES-1 deleted murine macrophages and vascular smooth muscle
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9 cells in vitro, was found by Wang et al. [8]. We did not confirm this phenomenon in human
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11 monocytes by the novel mPGES-1 inhibitor in vitro. However, it should be pointed out that in these
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13 cells PGI₂ was generated at very low-concentrations which is consistent with the very low levels of
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15 expression of PGIS detected in monocytes (Figure 4). The occurrence of increased biosynthesis of
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17 PGI₂ in vivo by pharmacological inhibition of mPGES-1 should be verified.
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21 In human monocytes, in the absence of plasma proteins, a concentration of AF3442 100µM, i.e.
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23 100-fold higher than that causing a maximal inhibition of mPGES-1, was associated with a small
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25 off-target effect on COX-2 expression. Whether down-regulation of COX-2 levels by 35% involved
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27 a transcriptional and/or a posttranscriptional mechanism remains to be investigated.
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31 In conclusion, we have demonstrated that a selective mPGES-1 inhibitor reduced monocyte PGE₂
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33 generation at clinically relevant ranges (i.e. approximately 70%) also in presence of plasma
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35 proteins. In human monocytes, mPGES-1-dependent PGH₂ metabolism represents a major
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37 metabolic route of PGE₂ generation, while other PGESs or non-enzymatic formation of PGE₂
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39 represent minor pathways of this prostanoid biosynthesis. Pharmacological inhibition of mPGES-1
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41 did not translate into redirection of PGH₂ metabolism towards other terminal PG synthases in
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43 monocytes. The functional consequences of the phenotype induced by pharmacological inhibition
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45 of mPGES-1 deserve to be investigated in vivo in suited experimental animals.
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Footnotes

Conflict of interest

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Legends for figures

Figure 1. Structure of AF3442 [N-(9-ethyl-9H-carbazol-3-yl)-2-(trifluoromethyl)benzamide]

Figure 2. Expression of COX-isozymes and down-stream synthases in isolated human

platelets. Platelet-rich plasma was prepared by centrifugation of citrated blood at 200g for

15 minutes and platelets were isolated from plasma as previously described [24, 25] and

resuspended in RPMI-1640 supplemented with 0.5% (v/v) FCS (5×10^8 platelets per ml; containing

<1 leukocyte per 10 000 platelets). Platelets were lysed and 25 μ g of total proteins were analyzed for

COX-1, COX-2, TXS, cPGES, mPGES-2, mPGES-1 and β -actin levels by SDS-PAGE and

Western blot analysis [19]. The blot used for detection of COX-2 was stripped (stripping solution

was from Chemicon International, USA) and reprobred for TXS. β -actin was used as protein loading

control. The figure is representative of 3 different experiments.

Figure 3. Effect of AF3442 on microsomal preparations of A549 (human lung

adenocarcinoma) cell line overexpressing human mPGES-1. (A) Western blot analysis of

mPGES-1 in A549 (human lung adenocarcinoma) cell line and in those transfected with the

recombinant vector pcDNA3.1 (Neomicine resistant) ligated with human mPGES-1 (clone D8L);

(B) inhibitory effect of AF3442 on PGE₂ generation in microsomal preparations of A549 clone

D8L. AF3442 (0.01-10 μ M) was pre-incubated with reaction mixture containing microsomal

preparations (30 μ g/ml protein concentration) for 15 minutes at 4°C, then the reaction was started

by substrate addition (PGH₂) and stopped after 5 minutes at 4°C with 100 μ l of stop solution (40mM

ferric chloride and 0.4mM citric acid). Samples were then subjected to PGE₂ quantification by

radioimmunoassay. Results were reported as percentage of control (DMSO vehicle) (mean \pm SEM)

from 3-4 separate experiments.

Figure 4. Expression of COX-isozymes and down-stream synthases in LPS-stimulated human

monocytes. Human monocytes were separated from buffy coat and $1.5-2 \times 10^6$ cell/ml were cultured

in RPMI-1640 supplemented with 0.5% (v/v) FCS and incubated without (saline) or with LPS

(10 μ g/ml) at 37°C for 24 h. Then, monocytes were lysed and 50 μ g of total proteins were loaded

1 onto 4-15% SDS-PAGE and analyzed by Western blot technique for COX-2, COX-1, TXS, cPGES,
2 mPGES-1, mPGES-2, PGIS and β -actin expression. β -actin was used as protein loading control.
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4 The figure is representative of 3 different experiments.
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7 **Figure 5. Effects of AF3442 on prostanoid generation in LPS-stimulated human monocytes.**

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9 Monocytes were separated from buffy coat which was pretreated with aspirin 300 μ M for 5 minutes
10 to suppress the contribution of COX-1 to prostanoid generation. Increasing concentrations of
11 AF3442 (0.01-100 μ M) or DMSO vehicle were incubated with human monocytes (1.5-2x10⁶
12 cell/ml) in the presence of LPS (10 μ g/ml) for 24 h at 37°C. PGE₂, TXB₂, PGF_{2 α} and 6-keto-PGF_{1 α}
13 levels were assayed in culture media by specific radioimmunoassays [17, 19, 23]. Results were
14 reported as percentage of control (DMSO vehicle) (mean \pm SEM) from 3 separate experiments.
15 §P<0.05, PGE₂ 1 μ M and 10 μ M versus 100 μ M; **P<0.01, PGE₂ 0.01-100 μ M and TXB₂ 100 μ M
16 versus control.
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29 **Figure 6. Effects of AF3442 on the levels of COX-isozymes and down-stream synthases in**

30 **LPS-stimulated human monocytes.** Monocytes (1.5-2x10⁶ cell/ml) were incubated with LPS
31 (10 μ g/ml) at 37°C for 24 h in the presence of increasing concentrations (0.01-100 μ M) of the
32 mPGES-1 inhibitor, AF3442 or DMSO vehicle. COX-2, COX-1, mPGES-1, mPGES-2, cPGES,
33 TXS and β -actin expression levels were evaluated in monocyte lysates by Western blot techniques.
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66 **Figure 7. Effects of AF3442 on LPS- and thrombin-induced prostanoids in human whole**

67 **blood.** (A) Effects of AF3442 on prostanoid generation by whole blood COX-2-dependent pathway.
68 Increasing concentrations of AF3442 (0.01-100 μ M) or DMSO vehicle were incubated with 1ml of
69 heparinized human whole blood (drawn from healthy volunteers pre-treated with 300mg aspirin 48
70 h before sampling to reduce the contribution of platelet COX-1 to prostanoid generation) stimulated
71 with LPS (10 μ g/ml) for 24 h. Parallel assessment of PGE₂, TXB₂, PGF_{2 α} and 6-keto-PGF_{1 α} (by
72 radioimmunoassays) was performed to verify the selective inhibitory effect of the compound
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2 towards mPGES-1 and the possible redirection of PGH₂ metabolism to other PG synthases. Results
3 were reported as % of control (DMSO vehicle) (mean±SEM) from 3 separate experiments.

4 *P<0.05, PGE₂ (% of control) at AF3442 10µM versus vehicle (DMSO); **P<0.01, PGE₂ (% of
5 control) at AF3442 100µM versus vehicle (DMSO); §P<0.01, PGE₂ versus PGF_{2α}, TXB₂ and 6-
6 keto-PGF_{1α} (% of control), at AF3442 100µM. (B) Effects of AF3442 on prostanoid generation by
7 whole blood COX-1-dependent pathway. AF3442 (0.001-100µM) or DMSO vehicle were
8 incubated with 1ml of whole blood samples (drawn from healthy volunteers who did not take any
9 NSAIDs for 2 weeks before blood collection) that were allowed to clot for 60 minutes to assess in
10 vitro selective inhibiting effect of AF3442 towards COX-1-dependent prostanoids mainly of platelet
11 origin [23, 26]. Since platelets do not express mPGES-1, PGE₂ generation may occur by the activity
12 of cPGES and/or mPGES-2. Thus, this assay allowed to verify the possible effects of the compound
13 towards other PGE synthases. PGE₂, TXB₂, PGF_{2α} and 6-keto-PGF_{1α} levels were measured by
14 specific radioimmunoassays. Results were reported as % of control (DMSO vehicle) (mean±SEM)
15 from 3 separate experiments for AF3442 0.01-100µM and from 2 experiments for AF3442
16 0.001µM.
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Table. Prostanoid levels in LPS-stimulated human monocytes and whole blood.

Prostanoid	Monocytes (ng/10 ⁶ cells)		Whole blood (ng/ml)	
	Saline	LPS	Saline	LPS
TXB ₂	0.080±0.011	29.7±4.90*	0.890±0.240	27.08±3.66**
PGE ₂	0.030±0.005	3.70±0.59*	0.840±0.190	38.37±8.03**§
PGF _{2α}	0.022±0.004	2.69±0.61*	0.500±0.050	5.83±0.64**
6-keto-PGF _{1α}	0.025±0.010	0.30±0.03**	0.005±0.003	0.39±0.09**

Values are reported as mean±SEM (n=3 for monocytes and n=9-13 for whole blood). *P<0.05 and

**P<0.01 versus saline; §P<0.05 versus TXB₂.

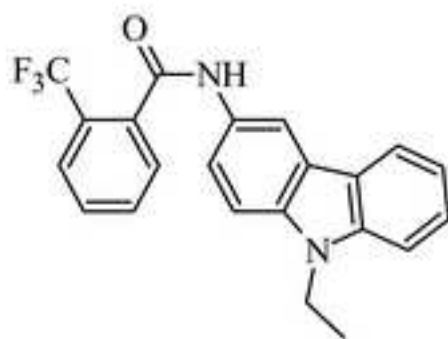


Figure 1

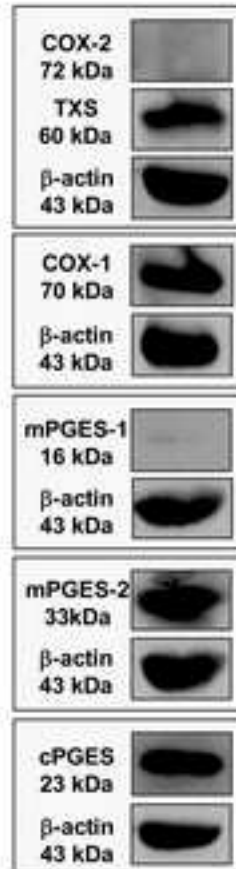


Figure 2

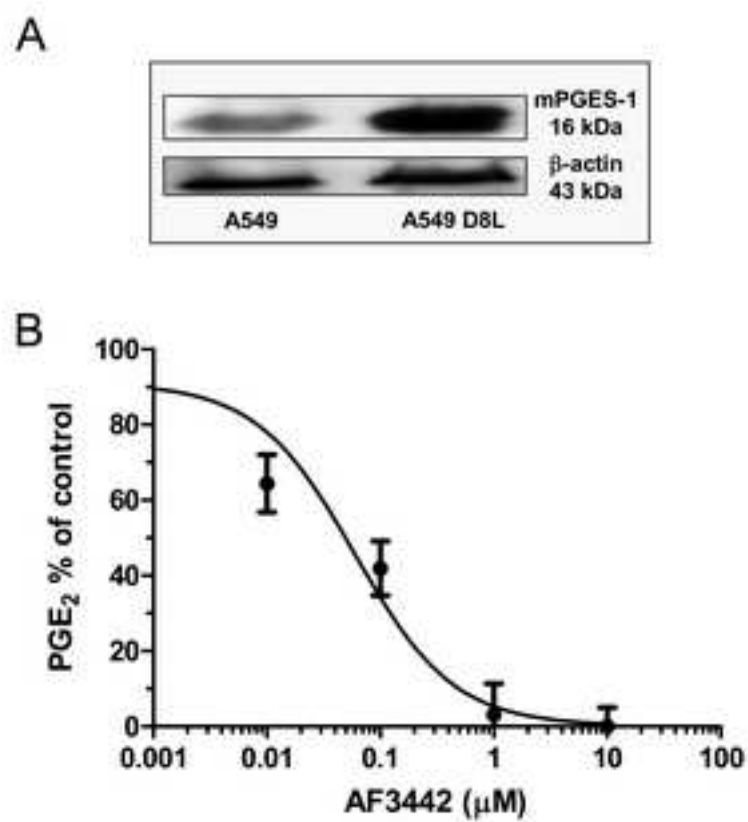


Figure 3

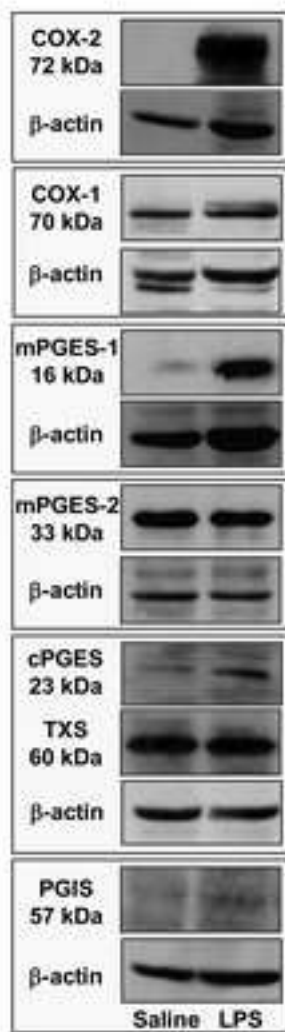


Figure 4

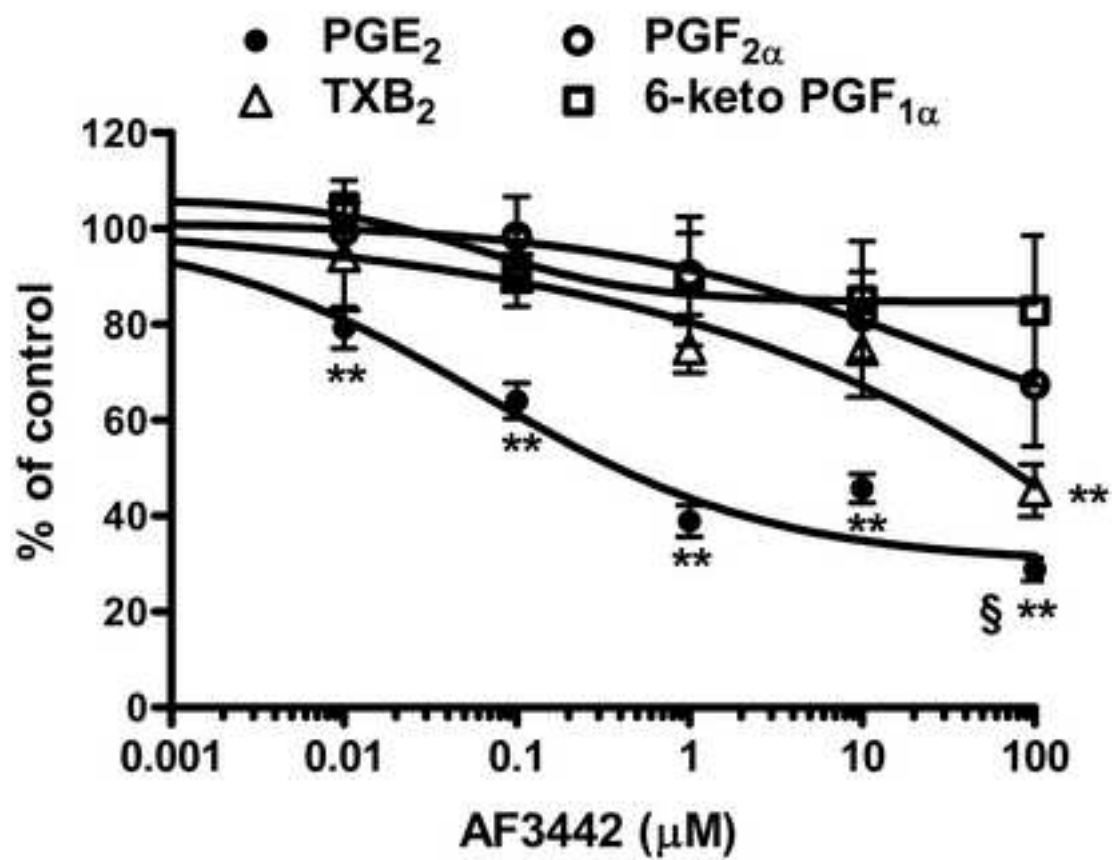


Figure 5

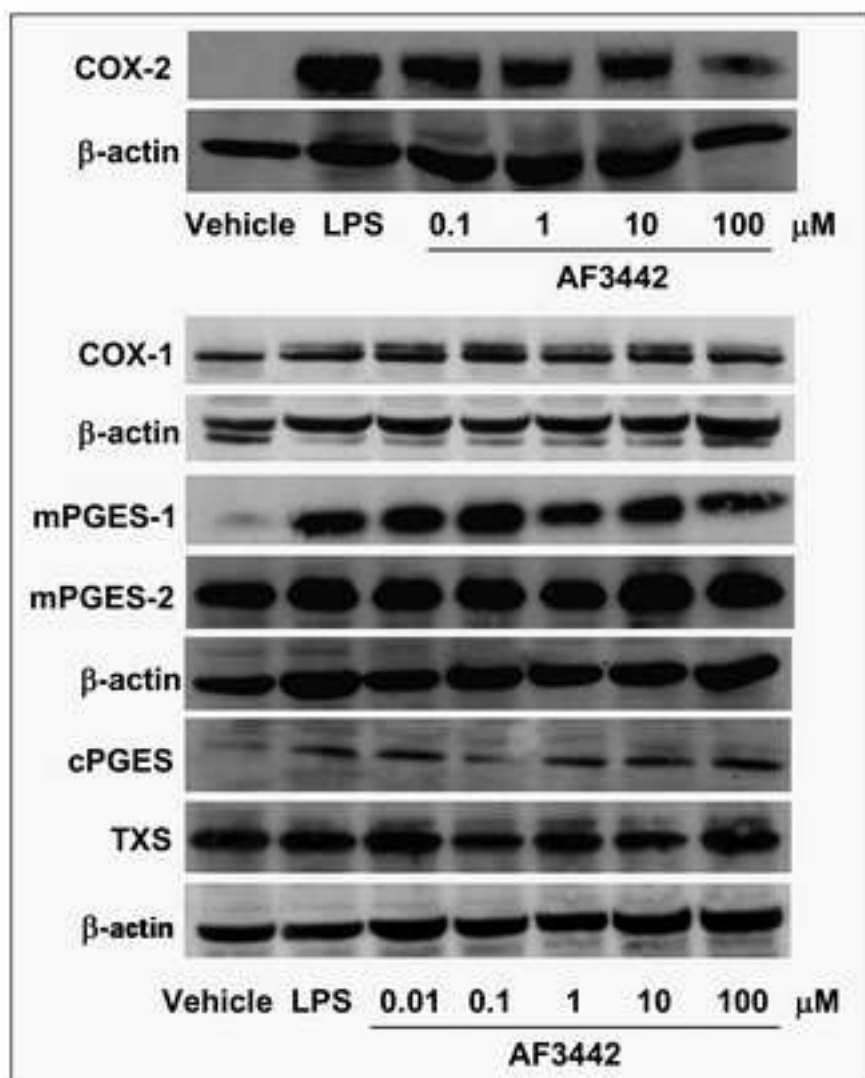


Figure 6

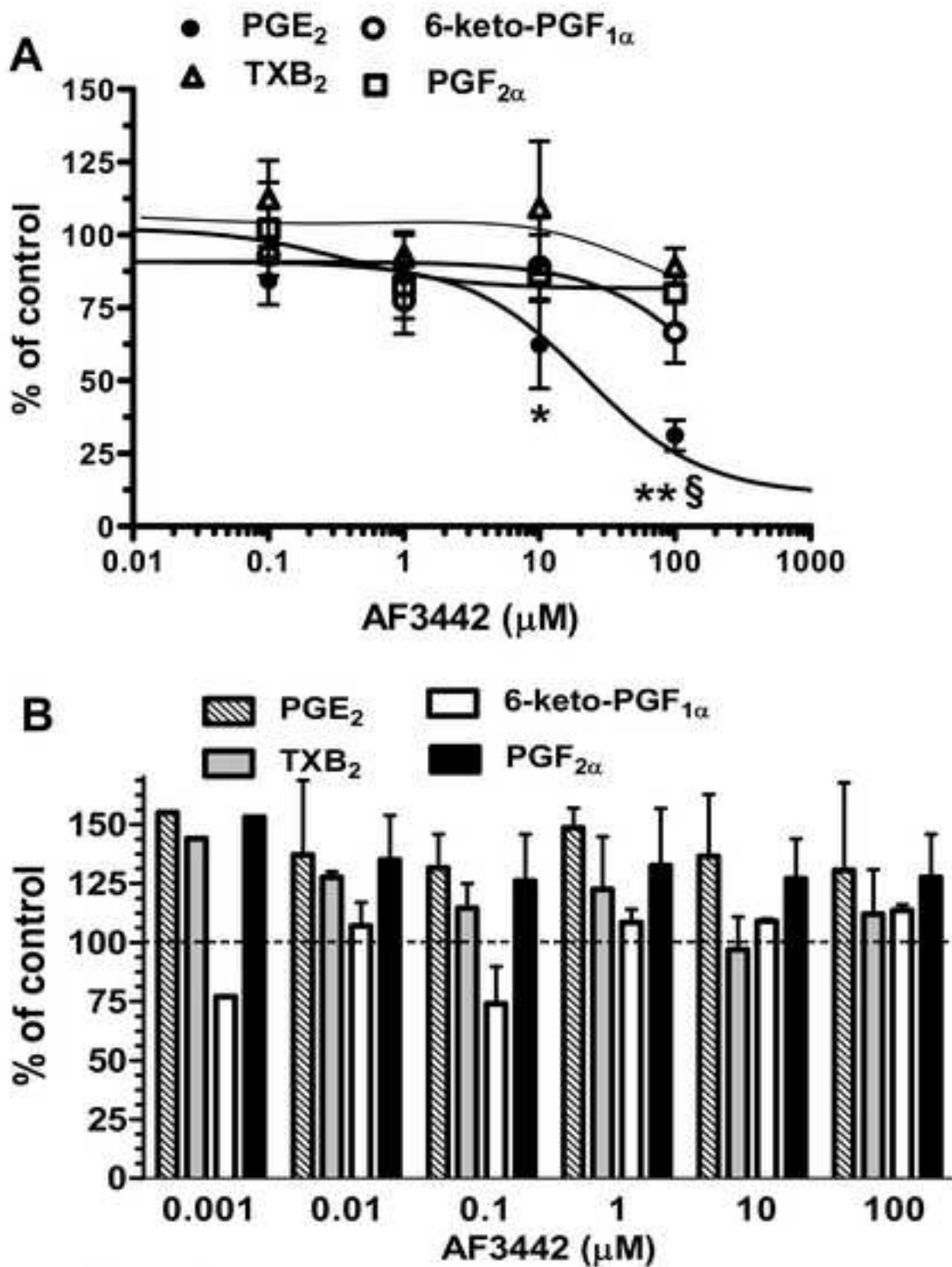


Figure 7