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a novel inhibitor of human microsomal prostaglandin E
synthase-1, on prostanoid biosynthesis in human monocytes
in vitro**

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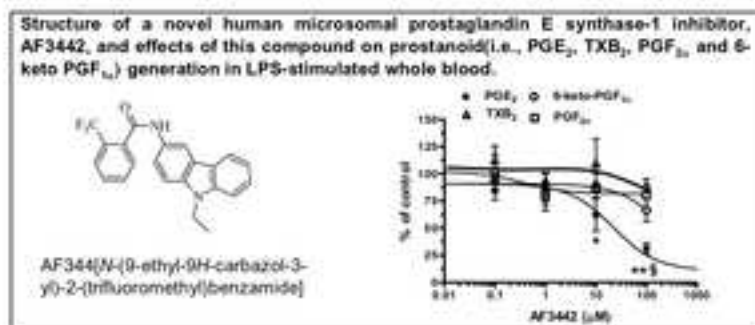
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5 **human monocytes in vitro**
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Abbreviations:

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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

1
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
5
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
13
14 and down-stream synthases in the presence of pharmacological inhibition by the novel mPGES-1
15
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
8
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
12
13 regulation of COX-2 and mPGES-1.

14
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
20
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
24
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
38
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].

42
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-9H-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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In the present study we aimed to characterize the contribution of mPGES-1 to PGH₂ metabolism in LPS-stimulated isolated human monocytes and whole blood by studying the synthesis of prostanoids and expression of COX-isozymes and down-stream synthases in the presence of pharmacological inhibition by AF3442. We choose to study the effect of the compound in human monocytes because they represent an important cell-target of the clinical efficacy of mPGES-1 inhibitors. Interestingly, we found that inhibition of PGE₂ generation at clinically relevant ranges was not associated with any significant change of other prostanoids in human monocytes in vitro. The functional relevance of this observation deserves to be investigated in vivo.

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 µg/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 10µM PGH₂ substrate.
9

10 AF3442 (0.01-10µM) 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 100µl 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 (0.1-100µM), 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 (300µM). 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 (1.5-2x10⁶ 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 (10µg/ml)
26 (derived from Escherichia Coli 026:B6) at 37°C for 24 h in the presence of DMSO vehicle or
27 increasing concentrations (0.01-100µM) of the mPGES-1 inhibitor, AF3442. This compound was
28 dissolved in DMSO and 2µl of vehicle or stock solution was added to 1ml of cell suspension. PGE₂,
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1 TXB₂ (the stable hydrolysis product of TXA₂), PGF_{2α} and 6-keto-PGF_{1α} (the hydrolysis product of
2 PGI₂) levels were measured in cell culture media by previously described and validated
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4 radioimmunoassay techniques [17, 19, 23], while COX-2, COX-1, mPGES-1, mPGES-2, cPGES,
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6 TXS and PGIS protein levels were evaluated in cell lysates by previously described Western blot
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8 techniques [17, 19].
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10 11 2.6. Isolation of human platelets 12 13

14 Platelet-rich plasma was prepared by centrifugation of citrated blood at 200g for 15 minutes and
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16 platelets were isolated from plasma as previously described [24, 25] and resuspended in RPMI-1640
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18 supplemented with 0.5% (v/v) FCS. Platelet suspensions (5x10⁸/ml; containing <1 leukocyte per 10
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20 000 platelets) were analyzed for the levels of COX-2, COX-1, mPGES-1, mPGES-2, cPGES and
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22 TXS in cell lysates by Western blot technique [17, 19].
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25 26 2.7. Effects of AF3442 on prostanoid generation in human whole blood 27 28

29 Whole blood was drawn from 13 healthy volunteers (age range: 23-35 years). The study was
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31 approved by the local Ethics Committee and informed consent was obtained from each subject. The
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33 inhibitory effect towards constitutive PGE₂ generation was assessed by evaluating PGE₂ levels
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35 generated in whole blood allowed to clot for 60 minutes at 37°C. In clotting whole blood, PGE₂ is
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37 mainly produced by platelets in response to endogenously generated thrombin through the activity
38
39 of COX-1 [23-26] and cPGES and/or mPGES-2. In fact, mPGES-1 is not detectable in platelets
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41 (Figure 2). The parallel measurement of TXB₂ (the major product of endogenous AA metabolism in
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43 platelets), PGF_{2α} and 6-keto-PGF_{1α} was performed to verify a possible off-target effect of AF3442
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45 on COX-1. The effects of AF3442 on inducible PGE₂ generation were studied by assessing the
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47 levels of PGE₂ produced in heparinized human whole blood stimulated for 24 h with LPS
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49 (10μg/ml), a stimulus for the induction of both COX-2 and mPGES-1 in monocytes [17, 27]. The
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51 simultaneous measurements of TXB₂, PGF_{2α} and 6-keto-PGF_{1α} were performed to verify the impact
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53 of AF3442 on COX-2 and the possible redirection of the accumulated PGH₂ substrate towards other
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55 PG synthases. For the assessment of the effects on constitutive PGE₂ generation, peripheral venous
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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
5
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
12
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
27
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
31
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-
35
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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45 46 2.9. Statistical Analysis

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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
51
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
55
56 GraphPad InStat (version 3.00 for Windows). Values of P<0.05 were considered statistically
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significant. Concentration-response curves were fitted (using PRISM, GraphPad, version 5.00 for
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
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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
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8 been shown both in vivo and in vitro [8, 14]. This phenomenon might occur at different extent in
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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.
38

39 High levels of expression of TXS, together with efficiency of coupling between TXS and COX-2
40 [32, 33] might be the determinants which contribute to the dominant generation of inducible TXA₂
41 in isolated monocytes. In fact, it has been shown that TXS efficiently couples to COX-2 when small
42 amounts of AA are gradually supplied over a long period, such as in response to LPS [32, 33]. In
43 this scenario, mPGES-1 is not the dominant pathway of PGH₂ metabolism; thus its inhibition
44 translates into a limited accumulation of PGH₂ which explains our failure to detect redirection
45 towards TXB₂ and other prostanoids. This is different from the results obtained in thioglycollate-
46 elicited peritoneal macrophages stimulated in vitro with LPS [8, 14] showing that PGE₂ is the major
47 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

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14 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**
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31 **LPS-stimulated human monocytes.** Monocytes (1.5-2x10⁶ cell/ml) were incubated with LPS
32 (10 μ g/ml) at 37°C for 24 h in the presence of increasing concentrations (0.01-100 μ M) of the
33 mPGES-1 inhibitor, AF3442 or DMSO vehicle. COX-2, COX-1, mPGES-1, mPGES-2, cPGES,
34 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**
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68 **blood.** (A) Effects of AF3442 on prostanoid generation by whole blood COX-2-dependent pathway.
69 Increasing concentrations of AF3442 (0.01-100 μ M) or DMSO vehicle were incubated with 1ml of
70 heparinized human whole blood (drawn from healthy volunteers pre-treated with 300mg aspirin 48
71 h before sampling to reduce the contribution of platelet COX-1 to prostanoid generation) stimulated
72 with LPS (10 μ g/ml) for 24 h. Parallel assessment of PGE₂, TXB₂, PGF_{2 α} and 6-keto-PGF_{1 α} (by
73 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
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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.

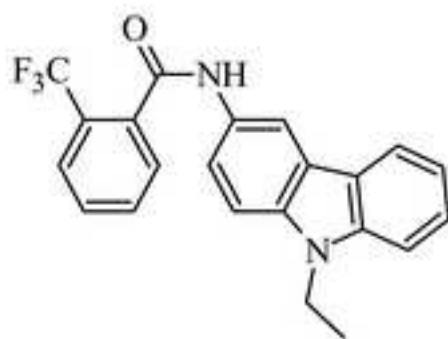


Figure 1

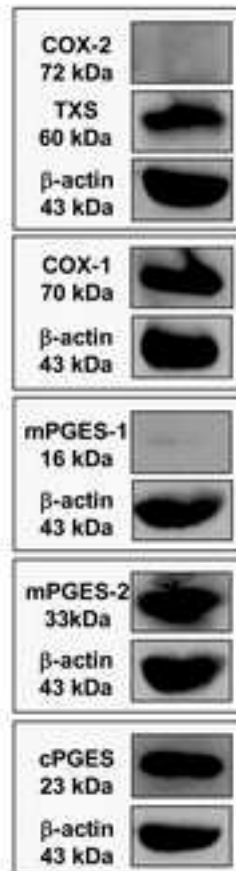


Figure 2

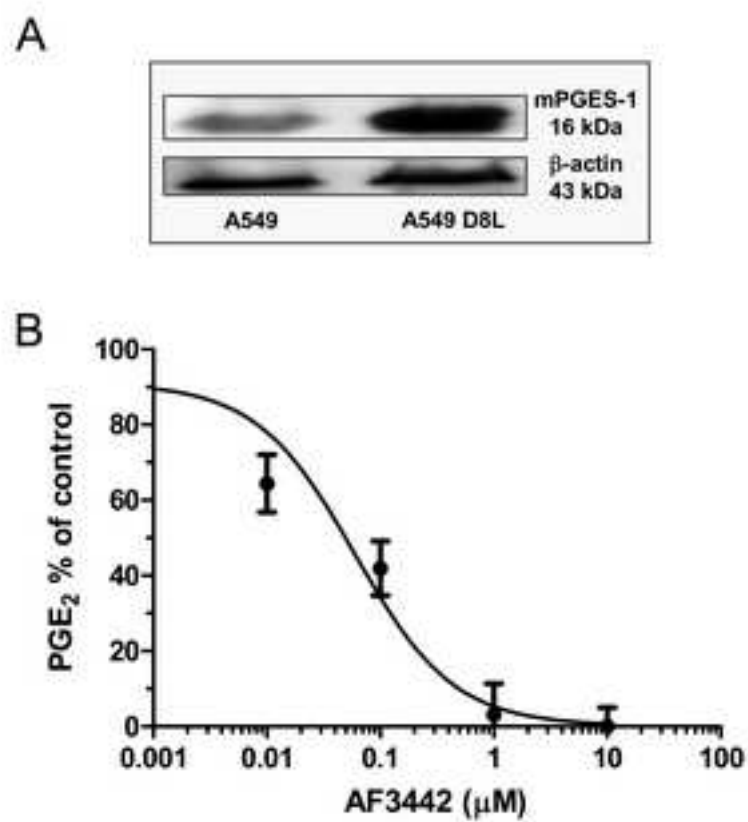


Figure 3

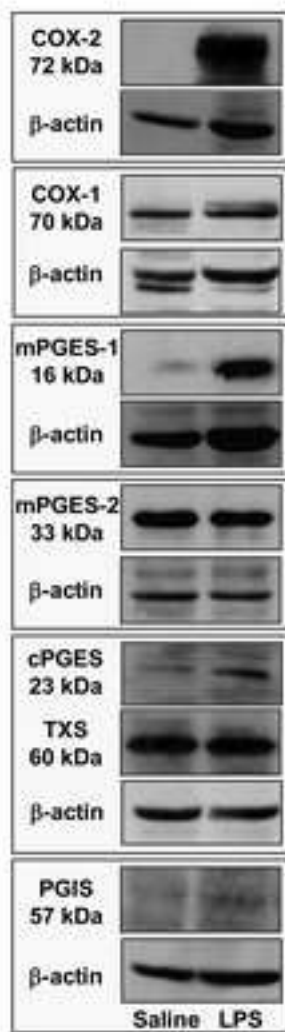


Figure 4

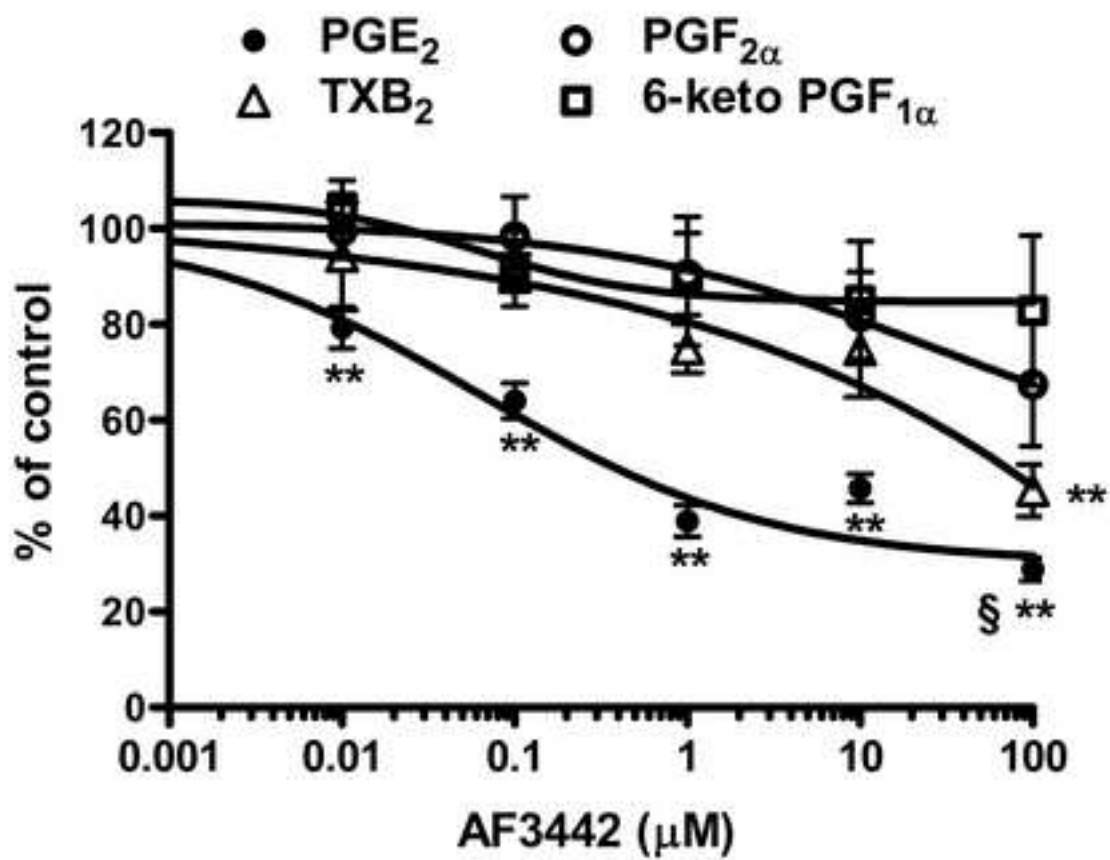


Figure 5

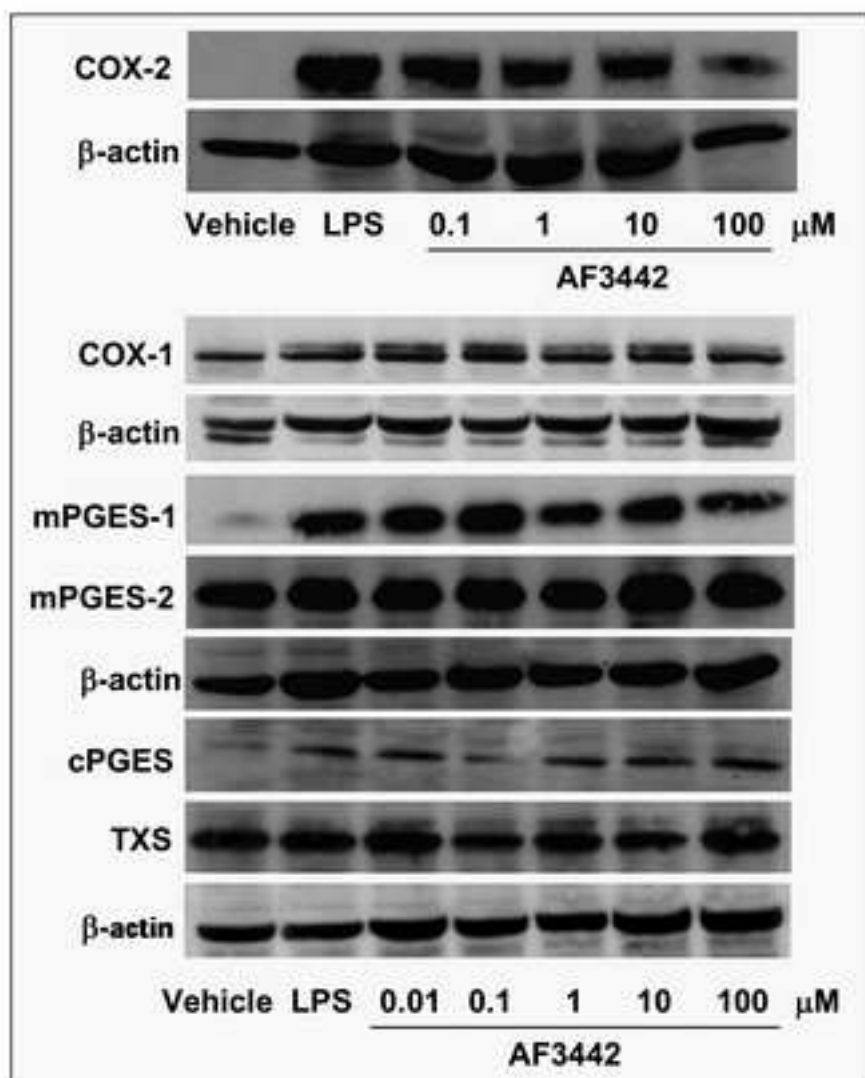


Figure 6

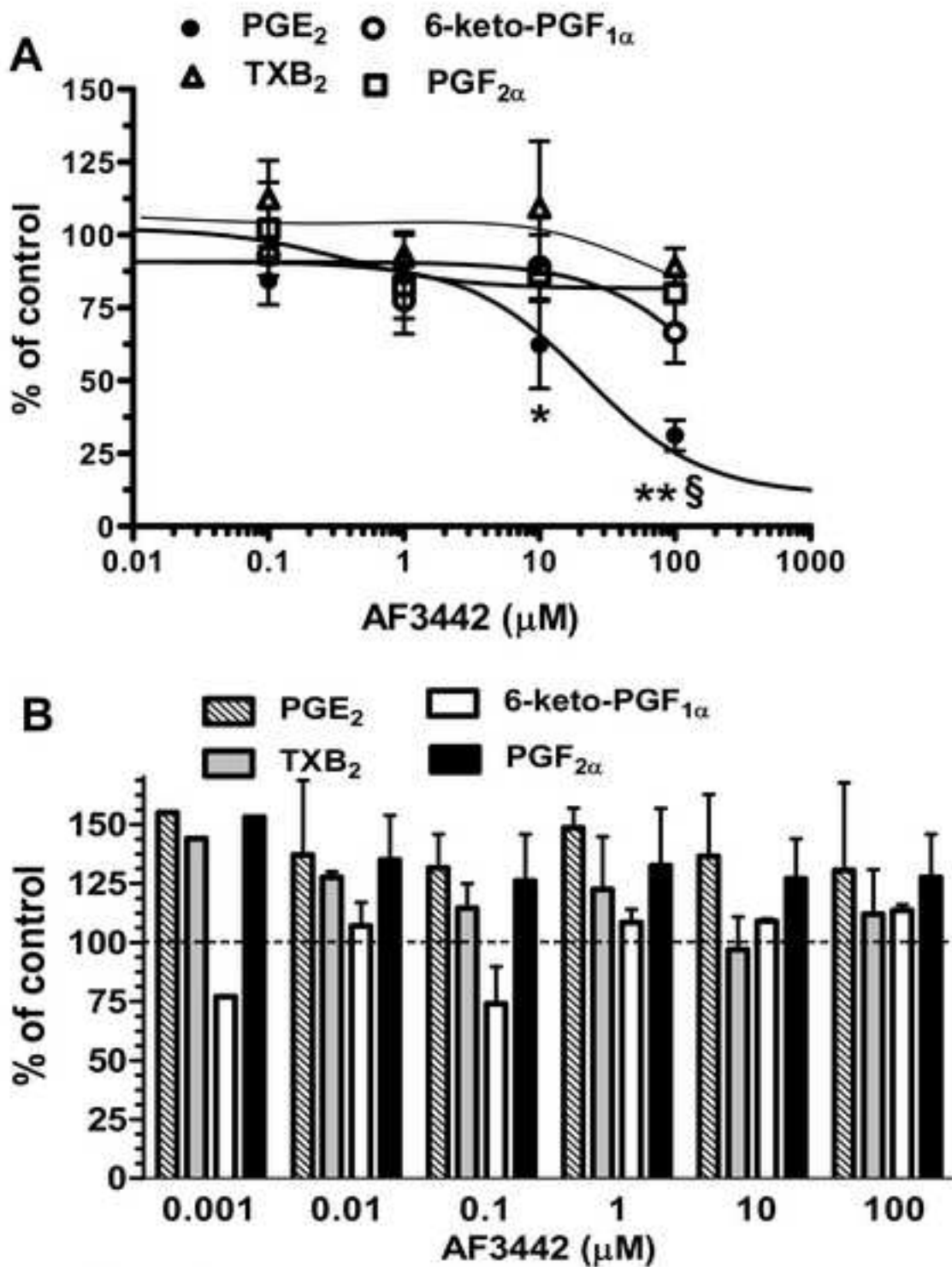


Figure 7