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**CYCLOOXYGENASE-2 AND TISSUE INHIBITOR OF MATRIX
METALLOPROTEINASES-1 CONFER THE ANTIMIGRATORY EFFECT OF
CANNABINOIDS ON HUMAN TRABECULAR MESHWORK CELLS[#]**

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Running title: Inhibition of trabecular meshwork cell migration by cannabinoids

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20 *Abbreviations:* AM-251, [N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-
21 1H-pyrazole-3-carboxamide]; AM-630, [(6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-
22 3-yl) (4-methoxyphenyl)methanone]; CB₁, cannabinoid receptor 1; CB₂, cannabinoid receptor
23 2; COX, cyclooxygenase; FCS, fetal calf serum; IOP, intraocular pressure; MA, R(+)-
24 methanandamide (R(+)-arachidonyl-1'-hydroxy-2'-propylamide); MMP, matrix
25 metalloproteinase; mPGES, microsomal prostaglandin E synthase; NSAID, non-steroidal
26 anti-inflammatory drug; NS-398, N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide;
27 PG, prostaglandin; POAG, primary open-angle glaucoma; RT-PCR, reverse transcriptase-
28 polymerase chain reaction; Δ^9 -THC, Δ^9 -tetrahydrocannabinol; TIMP, tissue inhibitor of matrix
29 metalloproteinase; WST-1, (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.6-
30 benzene disulfonate).
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ABSTRACT

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4 Cannabinoids have received considerable attention as potential antiglaucomatous drugs.
5
6 Recently, prostaglandins (PG) have been suggested to contribute to this effect. Within the
7
8 factors conferring the development of glaucoma, depletion of the aqueous humor outflow-
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10 regulating trabecular meshwork (TM) cells elicited by migration from the outflow system is
11
12 considered to play a pivotal role. This study therefore investigates the impact of two
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14 cannabinoids, Δ^9 -tetrahydrocannabinol and R(+)-methanandamide, on the migration of
15
16 human TM cells and the involvement of the PG-synthesizing enzyme cyclooxygenase-2
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18 (COX-2) and one of its potential downstream targets, the tissue inhibitor of matrix
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20 metalloproteinases-1 (TIMP-1), to this response. Using Boyden chamber assays
21
22 cannabinoids were shown to elicit an antimigratory effect that was reversed by antagonists
23
24 for CB₁ as well as CB₂ receptors and accompanied by upregulation of COX-2 and TIMP-1
25
26 expression and PGE₂ synthesis. Knockdown of cannabinoid-induced COX-2 or TIMP-1
27
28 expression by siRNA or inhibition of COX-2 activity by NS-398 led to a significant
29
30 suppression of this antimigratory action. Migration was also diminished by the major COX-2
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32 product PGE₂ and by recombinant TIMP-1. Experiments using selective E prostanoid (EP)
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34 receptor agonists and antagonists revealed that decreased migration by PGE₂, THC and MA
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36 was mediated via EP₂ and EP₄ receptors. Finally, the cannabinoid-mediated increases of
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38 TIMP-1 levels were abolished by NS-398 and PGE₂ was shown to elicit a concentration-
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40 dependent increase of TIMP-1. Collectively, this data demonstrate a COX-2-dependent
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42 upregulation of TIMP-1 conferring the antimigratory action of cannabinoids. A decreased
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44 migration reducing TM cell loss in glaucoma might be involved in the antiglaucomatous
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46 action of cannabinoids.
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1. INTRODUCTION

During past years cannabinoids have received considerable attention as potential antiglaucomatous drugs [1]. The intraocular pressure (IOP)-lowering action of Δ^9 -tetrahydrocannabinol (THC), the principal psychoactive component of marijuana, was first reported in 1971 [2]. In the following years, various human or animal studies have confirmed IOP decreases by marijuana, THC and classic cannabinoid derivatives [3–5]. Likewise, the endogenous cannabinoid, anandamide, and its stable analog, R(+)-methanandamide (MA), have been shown to cause IOP reduction [6,7]. Despite this, the molecular mechanism underlying the IOP-lowering and antiglaucomatous action of cannabinoids remains to be established. On the basis of inhibitor studies with non-steroidal antiinflammatory drugs (NSAIDs), the IOP-lowering effects of anandamide and THC [8,9], have been associated with the generation of prostaglandins (PG) which are known to facilitate the outflow of aqueous humor [10]. However, despite one study showing an upregulation of the PG-synthesizing enzyme cyclooxygenase-2 (COX-2) and a subsequent PG-dependent induction of matrix metalloproteinases (MMP) by cannabinoids in non-pigmented ciliary epithelial cells [11], no further mechanistic studies have been performed in this context so far.

Within the factors controlling IOP a pivotal role is ascribed to the trabecular meshwork (TM), a smooth muscle-like tissue with contractile properties in the anterior chamber angle of the eye that regulates the aqueous humor outflow [for review see 12]. Malfunctioning of TM may lead to elevation of intraocular pressure and development of glaucoma [13]. Moreover, depletion of TM cells is considered to play a pivotal role within the factors conferring the development of primary open-angle glaucoma (POAG). In fact, the age-related TM cell loss is even more pronounced in patients with primary open-angle glaucoma (POAG) than in age-matched normals [14–17]. In this context detachment from the trabeculae and migration from the outflow system stimulated by factors present in aqueous humor has been suggested as one mechanism conferring meshwork cell depletion in POAG [16–19].

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2 Recently, the TM has been implicated as a target of cannabinoid action. Accordingly, various
3 cannabinoids including anandamide [20], 2-arachidonylglycerol [21], noladin ether [22],
4 WIN55212-2 [23] and JWH015 [24] were shown to act on trabecular meshwork cells to
5 enhance aqueous humor outflow. In two further studies, cannabinoids have been
6 demonstrated to exhibit an inhibitory action on the migration of TM cells by virtue of CB₁
7 and/or CB₂ receptor activation [25,26].
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11 The present study investigates the mechanisms underlying the antimigratory action of
12 cannabinoids on human TM cells. Based on our previous investigations demonstrating
13 cannabinoids as potent inducers of COX-2 expression in diverse cell types [11, 27–31] and
14 on the above mentioned interference of NSAIDs with the IOP-lowering action of
15 cannabinoids, we were particularly interested in the role of COX-2 and one of its potential
16 downstream targets, the tissue inhibitor of matrix metalloproteinases-1 (TIMP-1), in this
17 process. Here we show that the antimigratory action of the phytocannabinoid THC and the
18 stable endocannabinoid analogue MA on human TM cells is conferred by a COX-2-
19 dependent upregulation of TIMP-1. Although more studies are needed, a decreased COX-2-
20 driven migration and consequent reduced TM cell loss could be one factor contributing to the
21 antiglaucomatous action of cannabinoids.
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2. MATERIALS AND METHODS

2.1. *Materials*

MA and recombinant human TIMP-1 were purchased from Calbiochem (Bad Soden, Germany). AM-251, AM-630, latanoprost acid, NS-398 and PGE₂ were bought from Alexis Deutschland GmbH (Grünberg, Germany). AH-6809, butaprost, 11-deoxy PGE₁, GW627368X, 17-phenyl- ω -trinor PGE₂, PGF_{2 α} , SC-19220, sulprostone and U-46619 were obtained from Cayman Chemical (Ann Arbor, MI, USA). Dulbecco's Modified Eagle's medium (DMEM) with 4 mM L-glutamine and 4.5 g/L glucose was from Cambrex Bio Science Verviers S.p.r.l. (Verviers, Belgium). Fetal calf serum (FCS) and penicillin-streptomycin were obtained from PAN Biotech (Aidenbach, Germany) and Invitrogen (Karlsruhe, Germany), respectively. Matrigel was obtained from BD Biosciences (Oxford, UK). Dimethyl sulfoxide (DMSO), EDTA, glycerol, HEPES, NaCl, Ponceau S, sodium dodecyl sulfate-polyacrylamide and Triton[®] X-100 were bought from Applichem (Darmstadt, Germany). Aprotinine, leupeptine, phenylmethylsulfonyl fluoride and THC were obtained from Sigma (Taufkirchen, Germany).

2.2. *Cell culture*

An immortalized simian virus (SV40) transformed human trabecular meshwork (HTM) cell line was kindly provided by Prof. Ernst Tamm, Regensburg, Germany [32]. Cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 μ g/ml streptomycin. The cells were grown in a humidified incubator at 37°C and 5% CO₂. All incubations were performed in serum-free medium. Phosphate-buffered saline was used as a vehicle for the tested substances with a final concentration of 0.1% (v/v) ethanol (for THC, MA, AH-6809, butaprost, 11-deoxy PGE₁, GW627368X, 17-phenyl- ω -trinor PGE₂, SC-19220 and sulprostone), 0.01% (v/v) DMSO (for AM-251, AM-630, NS-398) or 0.1% (v/v) DMSO (for PGF_{2 α} , latanoprost acid, U-46619).

2.3. *Migration assay*

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3 The effect of test substances on the migration of TM cells was determined using Boyden
4 chambers according to the manufacturer's instructions (BD Biosciences, Oxford, UK). In this
5 assay, cellular motility is monitored by transmigration through transwell inserts (8 μ M pore
6 size) towards a chemoattractant. In brief, 1×10^5 cells in a volume of 500 μ l serum-free
7 DMEM were seeded into the upper sides of the transwell inserts and treated with test
8 substances or vehicles for the indicated times. DMEM containing 10% FCS was used as a
9 chemoattractant in the companion plate. Following incubation at 37°C and 5% CO₂
10 atmosphere for 24 h, the non-migrated cells on the upper surface of the inserts were
11 removed with a cotton swab. For calculation of migration, the viability of the migrated cells on
12 the lower side of uncoated chambers was determined by the colorimetric WST-1 test (Roche
13 Diagnostics, Mannheim, Germany). This cell viability test is based on the cleavage of the
14 tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.6-benzene
15 disulfonate) by mitochondrial succinate-tetrazolium-reductase in metabolically active cells.
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31 To exclude the possibility that the effect of cannabinoids on migration was an unspecific
32 cytotoxicity-related phenomenon, cell viability was analyzed after cannabinoid exposure in
33 quadruplicate. For this purpose, cells were seeded into 48-well plates at 1×10^5 cells per well
34 to match conditions of migration assays. Viability was measured subsequently using the
35 WST-1 test.
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2.4. *Wound-healing assay*

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46 To visualize migration human TM cells were seeded on 4-well chamber slides coated with
47 40.5 μ g Matrigel per well and were allowed to grow to complete confluence. A plastic pipette
48 tip was used for gently scratching the cell monolayer to create a cleared area. Subsequently,
49 cells were washed extensively with phosphate-buffered saline to remove cellular debris.
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51 Thereafter, cells were incubated with the indicated substances and the respective vehicles
52 daily, and wound closure was monitored for 72 h. Cell images of marked regions along the
53 wound area were obtained using an inverted microscope attached to a camera. To gain
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1 contrast cells were stained with Diff-Quick[®] according to the manufacturer's instructions
2 (Medion Diagnostics GmbH, Bidingen, CH). Wound-healing experiments were performed in
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4 duplicate.
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9 2.5. Quantitative RT-PCR analysis

10 Human TM cells were seeded into 24-well plates at a density of 1×10^5 cells per well and
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12 were grown to confluence. Following incubation of cells with the respective test compounds
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14 or its vehicles for the indicated times, cell culture media were removed and cells were lysed
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16 for subsequent RNA isolation. Total RNA was isolated using the RNeasy total RNA Kit
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18 (Qiagen, Hilden, Germany). β -Actin- (internal standard), COX-2 and TIMP-1 mRNA levels
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20 were determined by quantitative real-time RT-PCR using the TaqMan[®] RNA-to-C_T[™] Kit
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22 (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instruction.
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24 Primers and probe for human β -actin, COX-2, mPGES-1, mPGES-2 and TIMP-1 were Gene
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26 Expression Assay[™] products. (Applied Biosystems, Darmstadt, Germany).
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34 2.6. Western Blot Analysis

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36 For analysis of COX-2 and β -actin protein levels human TM cells were seeded into 6-well
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38 plates at a density of 2×10^5 cells per well and were grown to confluence. Following
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40 incubation with test substances or its vehicles for the indicated times, cell lysates were used
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42 for further analyses. Cells were lysed in solubilization buffer [50 mM HEPES pH 7.4, 150 mM
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44 NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl
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46 fluoride, 1 μ g/ml leupeptin, and 10 μ g/ml aprotinin], homogenized by sonication, and
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48 centrifuged at 10,000 g for 5 min. Cell culture media were used for Western blot analysis.
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51 Total protein in the cell culture media were measured using the bicinchoninic acid assay
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53 (Pierce, Rockford, IL, USA).
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57 For analysis of TIMP-1 protein levels human TM cells were seeded into 24-well plates at a
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59 density of 2×10^5 cells per well and were grown to confluence. Following the respective
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1 incubation time, cell culture media were collected and centrifuged at 500 x g. Cell culture
2 media were used for subsequent Western blot analysis of TIMP-1. Total protein was
3 determined using the bicinchoninic acid assay (Pierce, Rockford, IL, USA).
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7 Proteins were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel. Following
8 transfer to nitrocellulose and blocking of the membranes with 5% milk powder, blots were
9 probed with specific antibodies raised to COX-2 (BD Biosciences, Heidelberg, Germany), β -
10 actin (Calbiochem, Bad Soden, Germany) or TIMP-1 (New England BioLabs GmbH,
11 Frankfurt, Germany). Membranes were probed with horseradish peroxidase-conjugated Fab-
12 specific anti-mouse IgG (New England BioLabs GmbH, Frankfurt, Germany) for detection of
13 TIMP-1 and COX-2 protein expression. Antibody binding was visualized by enhanced
14 chemiluminescence Western blotting detection reagents (Amersham Biosciences, Freiburg,
15 Germany). Densitometric analysis of band intensities was achieved by optical scanning and
16 quantifying using the Quantity One 1-D Analysis Software (Biorad, Muenchen, Germany).
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18 Vehicle controls were defined as 100% for evaluation of changes in protein expression.
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32 In case of the COX-2 blots equal loading of lysates was ensured by hybridisation of
33 membranes with an antibody against β -actin. To ensure that equal amounts of protein in cell
34 culture media used for protein analysis of TIMP-1 had been transferred to the membrane,
35 proteins on Western blot membranes were stained with Ponceau S. To ascertain equal
36 protein loading in Western Blots of cell culture media obtained from upper Boyden chambers,
37 a band with a size of about 65 kDa that appeared unregulated is shown as a loading control
38 (LC) for protein analysis of cell culture media.
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48 For determination of cannabinoid receptors, membrane fractions of proteins were obtained
49 using the Proteo Extract™ Native Membrane Protein Extraction Kit according to the
50 manufacturer's instructions (Calbiochem, Bad Soden, Germany). Subsequently, membrane
51 fractions were concentrated using Microcon YM-10 centrifugal filter units (Millipore GmbH,
52 Schwalbach, Germany) with a 10-kDa cutoff with 12.000 x g for 45 minutes. The blots were
53 probed with antibodies raised to the CB₁- (Santa Cruz, Heidelberg, Germany) or the CB₂
54 receptor (Calbiochem, Bad Soden, Germany).
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2.7. *siRNA transfections*

Human TM cells were transfected with siRNA targeting CB₁, CB₂, COX-2, TIMP-1 or non-silencing siRNA (Eurogentec, Seraing, Belgium; Cat. No. OR-0030-neg) using RNAiFect[®] (Qiagen GmbH, Hilden, Germany). The target sequences of the siRNAs were as follows: 5'-gagcaatatgtcagtcactaa-3' (CB₁), 5'-ttccggaatcatctacaccta-3' (CB₂), 5'-aactgctcaacaccggaattt-3' (COX-2) and 5'-tcccatctttctccggacaa-3' (TIMP-1). The sequences of the siRNAs were controlled via BLAST search and did not show any homology to other known human genes. Transfections were performed according to the manufacturer's instructions. For migration assays cells grown to confluence were transfected with COX-2 siRNA (2.5 µg/ml), TIMP-1 siRNA (0.25 µg/ml) or non-silencing siRNA as respective negative control with an equal ratio (w/v) of RNA to transfection reagent for 24 h in DMEM supplemented with 10% FCS. Subsequently, cells were trypsinized, centrifuged, resuspended to a final density of 1 x 10⁵ cells per 500 µl in serum-free DMEM containing the same amounts of siRNA or non-silencing siRNA to provide constant transfection conditions. Afterward, cells were incubated for another 24-h period and migration was analyzed as described above. Transfection efficacy was monitored in parallel experiments by Western blot analysis as described above. For siRNA transfection targeting cannabinoid receptors, cells grown to confluence were transfected with CB₁, CB₂ or non-silencing siRNA (2.5 µg/ml) in serum-free DMEM for 72 h. Subsequently, membrane fractions were purified as described above.

2.8. *Determination of PGE₂*

Human TM cells were seeded into 24-well plates at a density of 2 x 10⁵ cells per well and were grown to confluence. Following incubation with test substances or its vehicles for the indicated times. PGE₂ concentrations in cell culture media were determined using a commercially available enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). PGE₂ amounts were normalized to protein levels and calculated as percent of vehicle control.

2.9. *Statistics*

Comparisons between groups were performed with Student's t test. All statistical analyses were undertaken using Prism 4.00 (GraphPad Software, San Diego, CA).

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3. RESULTS

3.1. *Antimigratory and COX-2-inducing action of THC in human TM cells*

The effect of THC on the migration of TM cells was determined using Boyden chambers. In this assay cellular motility is monitored by transmigration through transwell inserts towards FCS-containing medium being used as a chemoattractant. As shown in Fig. 1A, migration of human TM cells was diminished by a 24-h incubation with THC. Analysis of earlier incubation times (i.e., 4, 8, 12 h) revealed no antimigratory effect of THC (data not shown). Inhibition of migration by THC was shown to occur in a concentration-dependent manner and was even significant at concentrations as low as 0.01 μ M (Fig. 1A). To rule out the possibility that decreased migration by THC was an unspecific cytotoxicity-related phenomenon, cellular viability was measured. However, under experimental conditions very similar to those used for migration assays (1×10^5 cells per well, 24-h incubation) incubation with THC left viability of TM cells virtually unaltered (Fig. 1A). Moreover, the antimigratory action of THC was confirmed using the wound-healing assay. According to the phase-contrast images presented in Fig. 1B, THC concentration-dependently inhibited TM cell migration into the wounded area.

In experiments addressing the impact of THC on the expression of COX-2 at the time of its antimigratory effect, the phytocannabinoid exhibited a concentration-dependent induction of COX-2 at the protein level (Fig. 1C). In further analyses an increase of COX-2 mRNA and PGE₂ levels by THC was shown (Fig. 1D). Following a 24-h treatment of cells with THC (1 μ M), average PGE₂ concentrations measured in cell culture medium were 1.16 ± 0.25 nmol/L (mean + SEM of n = 4 experiments). To examine the source of PGE₂ release, cells were treated with THC in the presence of the selective COX-2 inhibitor NS-398. NS-398 almost completely abolished THC-induced PGE₂ release, confirming an involvement of the COX-2 isozyme in this response (Fig. 1D). As COX-2-dependent regulation of PGE₂ release has been shown to be associated with upregulation of microsomal prostaglandin E₂ synthase-1 (mPEGS-1) [33], we additionally analysed the regulation of mPGES-1 and mPGES-2 mRNA.

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Real-time RT-PCR analyses performed after a 12-h treatment of TM cells with 1 μ M THC revealed an induction of both mPGES-1 and mPGES-2: 142.1% \pm 8.1% vs. 100% \pm 10.0% (vehicle control) for mPGES-1 (means \pm SEM of n = 4 experiments, $P < 0.05$), and 161.7% \pm 12.9% vs. 100% \pm 5.8% (vehicle control) for mPGES-2 (means \pm SEM of n = 3–4 experiments, $P < 0.05$)

3.2. *Involvement of cannabinoid receptors in the antimigratory and COX-2-inductive action of THC*

To investigate whether cannabinoid receptors are involved in THC-mediated reduction of TM cell migration and the concomitant induction of COX-2 expression, the impact of antagonists of CB₁ receptor (AM-251) and CB₂ receptor (AM-630) on THC's action was tested. Inhibitors were used at a concentration of 1 μ M which has been reported to be within the range of concentrations inhibiting CB₁- and CB₂-dependent events [34,35]. As shown in Fig. 2A, THC-induced inhibition of TM cell migration was totally prevented by both antagonists. This effect appeared to be further substantiated by the combination of CB₁- and CB₂ antagonists (Fig. 1A). In addition, an interference of AM-251 and AM-630 with the antimigratory effect of THC was observed in the wound-healing assay (Fig. 2B). With respect to COX-2 induction by THC, both CB receptor antagonists were shown to elicit a pronounced inhibition of THC-elicited COX-2 expression as assessed by Western blotting experiments (Fig. 2C). Finally, the expression of CB₁ and CB₂ receptors in TM cells was proven in respective membrane fractions (Fig. 2D). To further substantiate the specificity of the bands shown in the Western blots, knockdown of either CB₁ and CB₂ receptors was performed by transfection of siRNA targeting cannabinoid receptors. As shown in Fig. 2D both CB₁- and CB₂ siRNA substantially reduced the expression of its particular target, whereas leaving the expression of the respective non-targeted CB receptor virtually unaltered.

3.3. *Role of COX-2 in the antimigratory effect of THC*

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3 In a first attempt to link THC-induced COX-2 expression to the observed decrease of
4 migration, TM cells were treated with THC in the presence of NS-398 at a concentration
5 proven to interfere with THC-induced PGE₂ synthesis (Fig. 1D). As shown in Fig. 1A and B,
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7 NS-398 suppressed the antimigratory action of THC in both the Boyden chamber as well as
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9 in the wound-healing assay. The involvement of COX-2 in the antimigratory effect of THC
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11 was confirmed by experiments showing that transfection of cells with COX-2 siRNA
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13 completely inhibited the diminished migration by THC (Fig. 3C). At the concentration tested
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15 COX-2 siRNA was demonstrated to significantly interfere with THC-induced COX-2 protein
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17 expression (Fig. 3C). Control experiments revealed no significant effect of non-silencing
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19 siRNA on both the antimigratory and COX-2-inducing action of THC (Fig. 3C).
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3.4. *Antimigratory effect of the major COX-2 product PGE₂ and role of diverse EP receptors in this action*

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32 To further confirm the pivotal role of COX-2 in the antimigratory action of THC, additional
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34 experiments were performed using the major COX-2 product PGE₂. According to Fig. 4A,
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36 PGE₂ decreased the migrative behaviour of TM cells in a concentration-dependent and
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38 significant manner. By contrast, agonists to the FP receptor (PGF_{2α}, latanoprost) and the TP
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40 receptor (U-46619; stable analog of prostaglandin H₂ that mimicks thromboxan A₂ activity
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42 [36]) did not confer inhibition of TM cell migration (Fig. 4B). Analysis of the time-course of the
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44 antimigratory effect of 10 μM PGE₂ revealed a much faster action as compared to THC. The
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46 respective percentual values relative to control (100%) were as follows: 86.4% ± 6.3% at 4 h;
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48 63.9% ± 4.9% at 8 h ($P < 0.01$); 47.2% ± 5.9% at 12 h ($P < 0.001$) and 38.4% ± 1.3% at 24 h
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50 ($P < 0.001$). On the basis of this data, the 24-h incubation protocol conferring the maximum
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52 inhibition by PGE₂ was used for further analyses.
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59 In a first approach to determine the EP receptor subtype involved in PGE₂-mediated
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61 decrease of TM cell migration, different EP receptor agonists were tested. According to Fig.
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4C, the antimigratory action of PGE₂ was mimicked by butaprost (EP₂ receptor agonist) and 11-deoxy PGE₁ (EP₂/EP₄ receptor agonist), but not by the EP₁/EP₃ agonists 17-phenyl- ω -trior PGE₂ (EP₁ > EP₃) and sulprostone (EP₃ > EP₁).

To confirm a possible involvement of EP₂ and EP₄ receptor signaling in PGE₂-mediated decrease of TM cell migration, additional experiments were performed using the EP₁/EP₂ receptor antagonist, AH-6809, and the selective EP₄ receptor antagonist, GW627368X. As shown in Fig. 4D and 4E, both compounds significantly suppressed the antimigratory action of PGE₂. In contrast, the EP₁ receptor antagonist SC19220 was inactive in this respect (Fig. 4E). To provide evidence for a PGE₂-dependent mechanism underlying the antimigratory action of THC, the same receptor antagonists were coincubated with THC to address this issue. According to Fig. 4F the antimigratory effect of THC was reversed by GW627368X and AH-6809 (Fig. 4F). Again, this effect was not observed in the presence of SC19220 (Fig. 4F).

3.5. *Role of TIMP-1 in the antimigratory effect of THC*

To provide evidence for a downstream target of COX-2 in conferring the antimigratory action of THC on human TM cells, the role of TIMP-1 in this process was focused on in further experiments. Western blot analysis of cell culture media revealed a profound upregulation of TIMP-1 in the presence of 1 μ M THC after a 24- h incubation period (Fig. 5A,C,D).

In a first attempt to link the observed THC-mediated antimigratory effect with a potential upregulation of TIMP-1, TM cells were transfected with TIMP-1 siRNA. Monitoring of TIMP-1 secretion into the cell culture media confirmed a profound inhibition of TIMP-1 expression in cells transfected with TIMP-1 siRNA as compared to control samples (Fig. 5A). As further shown in Fig. 5A, knock-down of TIMP-1 expression led to an almost complete abrogation of the THC-mediated decrease of migration. By contrast, cultures treated with a non-silencing sequence exhibited the same migration pattern as control cells treated with transfection agent only (Fig. 5A).

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2 The upregulation of TIMP-1 protein levels as shown in Fig. 5A, C, and D was reflected by a
3 concomitant upregulation of TIMP-1 mRNA expression ($161.8\% \pm 7.3\%$ [THC at $1 \mu\text{M}$] vs.
4 $100.0\% \pm 5.3\%$ [vehicle control]; means \pm SEM of $n = 10$ experiments, $P < 0.001$) after a 24-
5 h incubation period.
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9 The functional relevance of TIMP-1 upregulation was substantiated by demonstrating that
10 recombinant TIMP-1 significantly inhibited TM cell migration at concentrations not interfering
11 with the viability of TM cells (Fig. 5B).
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16 The proposed function of TIMP-1 as a downstream target of COX-2 was focused on in
17 experiments using NS-398. In fact, inhibition of COX-2 activity by this compound was
18 associated with a significant reduction of THC-induced TIMP-1 protein levels (Fig. 5C).
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20 Moreover, the COX-2 product PGE₂ was shown to elicit a concentration-dependent induction
21 of TIMP-1 levels in cell culture media of TM cells (Fig. 5D). Finally and in line with the
22 migration and COX-2 data provided earlier (Fig. 2A,B,C), inhibitor experiments revealed
23 TIMP-1 induction by THC as an effect reversible by both CB₁ and CB₂ receptor antagonists
24 (Fig. 5E).
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36 3.6. *Confirmation of the proposed antimigratory mechanism by use of another* 37 *cannabinoid* 38 39

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41 To determine whether the contribution of COX-2 and TIMP-1 to impaired migration of TM
42 cells was unique for THC or also shared by another cannabinoid, additional experiments
43 were performed with MA, a stable anandamide analogue. According to the data presented in
44 Fig. 6 and Table 1, MA elicited a concentration-dependent (Fig. 6A) and CB₁/CB₂ receptor-
45 dependent (Table 1) decrease of TM cell migration. As shown for THC, the antimigratory
46 effect of MA was confined to the investigated 24-h incubation time point and was not
47 observed earlier (i.e., at 4, 8, 12 h) (data not shown). This functional effect was accompanied
48 by a CB₁/CB₂ receptor-dependent upregulation of COX-2 expression and activity (Fig. 6B,C)
49 as well as TIMP-1 protein expression (Fig. 6F). As shown in Fig. 6C the MA-induced
50 increased release of PGE₂ into cell culture media was abolished in the presence of NS-398.
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1 After a 24-h treatment of cells with 10 μ M MA, average PGE₂ concentrations measured in
2 cell culture medium were 5.69 ± 0.32 nmol/L (mean \pm SEM of $n = 4$ experiments).
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4 Again, inhibition of MA-induced COX-2 activity by NS-398 (Table 1) or knockdown of TIMP-1
5 expression by TIMP-1 siRNA (Fig. 6D) led to a reversal of the cannabinoid-elicited decrease
6 in TM cell migration. As shown before for THC, the MA-mediated increase of TIMP-1 levels
7 in TM cell migration. As shown before for THC, the MA-mediated increase of TIMP-1 levels
8 was likewise abolished by NS-398 (Fig. 6E).
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10 The upregulation of TIMP-1 protein levels as shown in Fig. 6D, E, and F was reflected by a
11 concomitant upregulation of TIMP-1 mRNA expression ($204.1\% \pm 30.9\%$ [MA at 10 μ M] vs.
12 $100.0\% \pm 12.4\%$ [vehicle control]; means \pm SEM of $n = 4$ experiments, $P < 0.05$) after a 24-h
13 incubation period. A causal link between MA-induced PGE₂ release and inhibition of
14 migration via EP_{2/4} receptor activation is given by the reversal of the antimigratory effect of
15 MA by GW627368X and AH-6809 (Table 1). As shown before for PGE₂ and THC, SC19220,
16 on the other hand, was inactive in this respect (Table 1).
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4. DISCUSSION

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4 Recent investigations have provided evidence that cannabinoids mediate its IOP-lowering
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6 action, at least in part, through the release of endogenous PGs. However, the molecular
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8 mechanisms as well as the functional consequence of cannabinoids' PG-elevating action are
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10 poorly understood.

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13 The results from the present study show that newly expressed COX-2 confers the
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15 antimigratory effect of the phytocannabinoid THC and the stable anandamide analogue MA
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17 on human TM cells. There are several lines of evidence supporting this notion: First,
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19 significant elevations of COX-2 mRNA and protein as well as increased PGE₂ levels were
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21 observed at the time of the antimigratory effect. Second, inhibition of cannabinoid-induced
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23 PGE₂ formation with the selective COX-2 inhibitor NS-398 was associated with a reversal of
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25 the antimigratory action. Third, transfection of cells with COX-2 siRNA mimicked the
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27 inhibitory effect of NS-398 on THC-elicited decrease of migration. Fourth, an antimigratory
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29 effect was also elicited by PGE₂, the major prostanoid of the COX-2 pathway [37].
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34 In case of THC, the decrease of migration was even significant at concentrations as low as
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36 0.01 μ M (28% inhibition) and 0.1 μ M (37% inhibition). With reference to the fact that in
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38 humans average peak plasma concentrations of 0.046 μ M and 0.030 μ M can be obtained
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40 after oral doses of 15 and 20 mg THC [38], the effects of THC on cell migration were
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42 observed at therapeutically relevant concentrations.
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46 In the present study PGE₂ elicited inhibition of TM cell migration, whereas agonists to FP and
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48 TP receptors did not confer a comparable effect. PGE₂ is known to be a ligand of four PGE₂
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50 receptor subtypes, which mediate stimulation of phosphoinositol turnover with elevation in
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52 intracellular free calcium (EP₁- and some EP₃ receptors isoforms), activation of adenylyl
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54 cyclase activity resulting in elevation of intracellular cAMP (EP₂- and EP₄ receptors) or
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56 inhibition of adenylyl cyclase (EP₃ receptor) [39,40]. Using agonists and antagonists of the
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58 different EP receptor subtypes, evidence was provided suggesting that EP₂- and EP₄
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60 receptors, but not EP₁- and EP₃ receptors, are involved in the antimigratory action of PGE₂ on
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1 human TM cells. Therefore, a decreased migration was elicited by the EP₂ receptor agonist
2 butaprost and by the EP₂/EP₄ receptor agonist 11-deoxy PGE₁, whereas EP₁/EP₃ receptor
3 agonists (17-phenyl- ω -trior PGE₂, sulprostone) were virtually inactive in this respect. In
4 support of a causal link between EP₂/EP₄ receptor activation and diminished migration by
5 PGE₂, the EP₁/EP₂ receptor antagonist, AH-6809, and the selective EP₄ receptor antagonist,
6 GW627368X, were shown to significantly reduce the antimigratory effect of PGE₂. In
7 contrast, the selective EP₁ antagonist SC-19220 was devoid of a significant inhibitory effect
8 in this respect. Importantly, all three EP antagonists influenced the antimigratory action of
9 both tested cannabinoids in the same manner thereby confirming a causal link between
10 cannabinoid-induced PGE₂ release and inhibition of migration via EP_{2/4} receptor activation.
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13 In contrast to the delayed antimigratory effect of the tested cannabinoids which both require
14 synthesis of endogenous PGE₂, the antimigratory effect of exogenously added PGE₂
15 occurred much earlier suggesting an immediate action by direct EP receptor activation. In
16 our hands, relatively high concentrations of exogenous PGE₂ were necessary to confer
17 inhibition of migration. Given a significant antimigratory effect of PGE₂ at 0.1 μ M, the
18 endogenous levels of PGE₂ produced by THC- and MA-stimulated TM cells were 86- and 18-
19 fold lower. This apparent difference has been observed in several studies. Only recently,
20 Sirianni et al. [41] investigating the impact of COX-2 on aromatase activity presented data
21 suggesting that a high endogenous production of PGE₂ requires the addition of high
22 exogenous amounts of PGE₂ to observe a particular regulation.
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25 Additional experiments using selective antagonists of CB₁- (AM-251) or CB₂ receptors (AM-
26 630) revealed that both CB₁ and CB₂ confer cannabinoid-induced COX-2 expression and
27 subsequent decreased migration. With respect to the observed CB receptor dependency of
28 COX-2 expression by both cannabinoids, our data contradict previous observations from our
29 group showing a receptor-independent induction of COX-2 expression by both cannabinoids
30 in human non-pigmented ciliary epithelial [11] and neuroglioma cells [27–29,42] and for MA
31 in human cervical carcinoma cells [30]. Thus, dependent on the cell type, cannabinoids may
32 use either receptor-dependent or -independent pathways to confer increased synthesis of
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PGs which may on the one hand depend on extracellular activation of membrane-bound receptors and might otherwise rely on a lipid raft-dependent intracellular uptake [29].

Interestingly, besides its COX-2-inducing potency, THC also elicited a substantial increase of the mRNA expression of mPGES-1 and mPGES-2 mRNA both acting downstream to COX activity by producing PGE₂. Regarding mPGES-1 this finding was expected given that mPGES-1 upregulation is mostly coupled to COX-2 induction [33,43]. In case of mPGES-2 this enzyme is known to be constitutively expressed in a variety of cells and coupled to COX-1 and COX-2 with modest preference for COX-2 [43]. However, mPGES-2 has also been shown to be upregulated by diverse stimuli including angiotensin II and UVB light [44,45].

To provide evidence for a downstream target of COX-2/PGE₂ in conferring its antimigratory action on TM cells, the expression of TIMP-1 was focused on in further experiments. RT-PCR and Western blot analyses revealed an induction of TIMP-1 by cannabinoids in human TM cells. The proposed function of TIMP-1 as a downstream target of COX-2 was corroborated by several experimental outcomes. First, inhibition of COX-2 activity by NS-398 was associated with a significant reduction of cannabinoid-induced TIMP-1 levels. Second, a role for COX-2 as effector of TIMP-1 expression was supported by the observation that PGE₂ mimicked the stimulatory effect of cannabinoids on TIMP-1 expression. Third, the functional relevance of TIMP-1 upregulation by cannabinoids was substantiated by demonstrating a concentration-dependent and significant decrease of TM cell migration by recombinant TIMP-1.

A relationship between COX-2 and TIMP-1 has also been established in other cell types. In support of the data presented here, an upregulation of TIMP-1 expression by COX-2-dependent PGs has been observed in human monocytes [46], human dendritic cells [47] and human non-pigmented ciliary epithelial cells [11]. In the latter cell type, cannabinoids elicited increased expression of TIMP-1 along with that of MMP-9 via a mechanism requiring prior induction of COX-2 [11]. Upregulation of TIMP-1 expression by cannabinoids has also been demonstrated in cervical carcinoma and lung cancer cells [48], albeit a possible COX-2-dependent pathway was not focussed on in these cells. Moreover, the antimigratory function

1 of TIMP-1 shown here is in line with other studies demonstrating a correlation between
2 increased TIMP-1 levels and impaired cellular migration [47,49–53].
3

4 Remarkably, the TIMP-1-dependent antimigratory action described in this study was
5 observed using uncoated Boyden chambers as experimental setup. This fact needs further
6 discussion given that TIMP-1 is traditionally regarded to impact the turnover of extracellular
7 matrix by inhibiting the activity of matrix-degrading MMPs thereby suppressing vascular
8 tumor growth, angiogenesis, migration and invasion [for review see 54]. A possible
9 explanation for this apparent discrepancy may be provided by the fact that, in addition to their
10 enzymatic activities, the TIMP-1-sensitive MMP-2 and MMP-9 can also influence cellular
11 migration by influencing cytoskeletal organization through an association with adhesion
12 receptors [55]. In line with this notion, several other groups have also found alterations in
13 migration elicited by a modulation of the MMP/TIMP system even when uncoated chambers
14 were used [50,53,55,56]. A second explanation may lie in the outcome of recent
15 observations showing that TIMP-1 and other members of the TIMP family are multifunctional
16 and can act either directly through cell surface receptors or indirectly through modulation of
17 protease activity [57].
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36 Collectively, our data indicate a novel mechanism by which cannabinoids could prevent
37 POAG-associated TM cell migration and subsequent cell loss. As in vitro analysis of TM cell
38 migration has the severe limitation of being remote from the physiological and pathologic
39 events in vivo, further in vivo analyses are necessary to confirm our observations. Apart from
40 the clarification of the signaling events elicited by cannabinoids in human TM cells and a
41 novel pathway possibly contributing to the antiglaucomatous action of cannabinoids, our
42 study extends previous investigations on the role and function of intraocular COX-2 in
43 glaucoma. According to a post-mortem analysis of human eyes, the expression of the PG-
44 synthesizing COX-2 is lost in the non-pigmented ciliary epithelium of patients with end-stage
45 POAG [58]. In line with this notion, the amounts of outflow-facilitating PGE₂ are significantly
46 lower in aqueous humor of patients with POAG or steroid-induced glaucoma as compared to
47 cataract patients [58]. On the basis of these results, we recently proposed an induction of
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2 intraocular COX-2 by certain established (latanoprost) and potential (cannabinoids)
3 antiglaucomatous drugs [11,59] as a mechanisms contributing to its IOP-lowering action. The
4 data presented here confirm this assumption.
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7 In summary, we have demonstrated for the first time that cannabinoids are inductors of a
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9 COX-2/TIMP-1 pathway in human TM cells that confers an antimigratory action. Although
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11 more studies are needed, activation of COX-2 expression and subsequent formation of
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13 TIMP-1-inducing PGs could be an important mechanism by which cannabinoids exert its
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15 antiglaucomatous action.
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6. FIGURE LEGENDS

Figure 1

Impact of THC on migration, viability and COX-2 expression of TM cells. *A*, Concentration-dependent effect of THC on migration and viability of TM cells following incubation with THC or vehicle for 24 h. *B*, Phase contrast images of migration of cells into wounded areas. Following a 72-h incubation with THC or vehicle migrated cells were stained with Diff-Quick® (Medion Diagnostics GmbH, Bidingen, CH) and documented under a 200 x magnification. *C*, Concentration-dependent effect of THC on COX-2 protein expression following incubation with THC or vehicle for 24 h. *D*, Effect of a 24-h incubation with THC (1 μ M) on COX-2 mRNA expression and PGE₂ synthesis by HTM cells and impact of NS-398 (1 μ M, 30 min pretreatment) on PGE₂ induction by THC. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means \pm SEM of n = 4–8 (*A*), and n = 4 (PGE₂) or n = 7–8 (mRNA) (*D*) experiments. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, vs. corresponding vehicle control; # *P* < 0.05 vs. THC-treated cells, Student's t-test. Values above the blot (*C*) are means \pm SEM obtained from densitometric analysis of n = 3 blots and represent percent control in comparison with vehicle-treated cells (100%) in the absence of test substance. Equal loading of lysates is indicated by hybridisation of membranes with an antibody against β -actin.

Figure 2

Involvement of cannabinoid receptors in the antimigratory and COX-2-inductive action of THC. *A,B,C*, Effect of AM-251 (CB₁ antagonist; 1 μ M) and AM-630 (CB₂ antagonist; 1 μ M) on the antimigratory (*A,B*) and COX-2-inducing (*C*) effect of THC. Cells were pretreated with the respective receptor antagonist (all tested at a final concentration of 1 μ M) for 30 min and incubated with THC (1 μ M) for another 24 h (*A, C*). Phase contrast images of migration of cells into wounded areas are shown following a 72-h incubation with daily treatment of cells with vehicle, THC or THC in combination with cannabinoid receptor antagonists as indicated.

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Migrated cells were stained with Diff-Quick[®] (Medion Diagnostics GmbH, Bidingen, CH) and documented under a 200 x magnification (B). D, Expression of CB₁ and CB₂ receptors in human TM cells. Membrane fractions obtained from cells treated with vehicle, CB₁-, CB₂- or nonsilencing siRNA at a final concentration of 2.5 µg/ml were used for monitoring cannabinoid receptors (D). Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means ± SEM of n = 8 (A) experiments. ****P* < 0.001, vs. corresponding vehicle control; #### *P* < 0.001 vs. THC-treated cells, Student's t-test. Values above the blot (C) are means ± SEM obtained from densitometric analysis of n = 4 blots and represent percent control in comparison with vehicle-treated cells (100%) in the absence of test substance. Equal loading of lysates is indicated by hybridisation of membranes with an antibody against β-actin.

Figure 3

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Role of COX-2 in the antimigratory effect of THC. A,B Cells were pretreated with the selective COX- inhibitor NS-398 (1 µM) for 30 min and subsequently treated with THC (1 µM) for another 24 h (A). Phase contrast images of migration of cells into wounded areas are shown following a 72-h incubation with daily treatment of cells with vehicle, THC or THC in combination with NS-398 as indicated. Migrated cells were stained with Diff-Quick[®] (Medion Diagnostics GmbH, Bidingen, CH) and documented under a 200 x magnification (B).

C, left panel, Effect of COX-2 knockdown on the THC-modulated migration of HTM cells and concomitant changes in COX-2 protein expression. Cells were transfected with 2.5 µg/ml COX-2 siRNA or non-silencing siRNA (nonsi) for 24 h. Subsequently, 1 x 10⁵ cells per 500 µl serum-free DMEM were subjected into Boyden chambers containing the same amounts of siRNA or non-silencing siRNA for another 24 h in presence of THC or vehicle. C, right panel, Monitoring of COX-2 knockdown was performed in parallel experiments in 6-well plates under the same conditions. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means ± SEM of n = 7–8 (A), n = 4 (C, left panel) experiments. ****P* < 0.001, vs. corresponding vehicle control; ## *P* < 0.01, #### *P* <

0.001 vs. THC-treated cells, Student's t-test. Values above the blot (C) are means \pm SEM obtained from densitometric analysis of $n = 3$ blots and represent percent control in comparison with vehicle-treated cells (100%) in the absence of test substance. Equal loading of lysates is indicated by hybridisation of membranes with an antibody against β -actin.

Figure 4

Antimigratory effect of the major COX-2 product PGE₂ and role of diverse EP receptors in this action. *A*, Concentration-dependent effect of THC on migration and viability of TM cells following incubation with PGE₂ or vehicle over a 24-h incubation period. *B*, Effect of PGF_{2 α} , latanoprost and U-46619 (all at a final concentration of 10 μ M) on migration and viability of TM cells following a 24 h-incubation period. *C*, Effect of 17-phenyl- ω -trinor PGE₂ (EP₁/EP₃ receptor agonist, EP₁ > EP₃), butaprost (EP₂ receptor agonist), sulprostone (EP₁/EP₃ receptor agonist, EP₃ > EP₁) and 11-deoxy PGE₁ (EP₂/EP₄ receptor agonist) on migration and viability of TM cells following a 24 h-incubation period. All EP agonists were tested at a final concentration of 1 μ M. *D,E,F*, Effect of AH-6809 (EP₁/EP₂ receptor antagonist, 10 μ M), GW627368X (EP₄ receptor antagonist, 1 μ M) and SC19220 (EP₁ receptor antagonist, 1 μ M) on the antimigratory effect of PGE₂ (*D,E*) or THC (*F*). Cells were pretreated with the respective receptor antagonist for 1 h and incubated with PGE₂ (10 μ M, *D,E*) or THC (1 μ M, *F*) for another 24 h. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means \pm SEM of $n = 4-8$ (*A*), $n = 4$ (*B*), $n = 4$ (*C*, viability), $n = 12$ (*C*, migration), $n = 3-4$ (*D*) and $n = 4$ (*E,F*) experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, vs. corresponding vehicle control; # $P < 0.05$, ### $P < 0.001$ vs. PGE₂-treated cells, Student's t-test.

Figure 5

Role of TIMP-1 in the antimigratory effect of THC. *A*, Effect of TIMP-1 knockdown on the THC-modulated migration of TM cells and concomitant changes in TIMP-1 protein levels in cell culture media. Cells were transfected with 0.25 μ g/ml TIMP-1 siRNA or non-silencing

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siRNA (nonsi) for 24 h. Subsequently, 1×10^5 cells per 500 μ l serum-free DMEM were subjected into Boyden chambers containing the same amounts of siRNA or non-silencing siRNA for another 24 h in the presence of THC or vehicle (upper panel). Monitoring of TIMP-1 knockdown was performed in parallel experiments in 24-well plates under comparable conditions. *B*, Concentration-dependent effect of recombinant TIMP-1 on migration and viability of TM cells following incubation with TIMP-1 or vehicle for 24 h. *C*, Effect of NS-398 (selective COX-2 inhibitor; 1 μ M) on the TIMP-1-inducing action of THC. *D*, Effect of AM-251 (CB₁ antagonist; 1 μ M) and AM-630 (CB₂ antagonist; 1 μ M) on the TIMP-1-inducing action of THC. In *C* and *D* cells were pretreated with inhibitor/antagonists for 30 min and incubated with THC (1 μ M) for another 24 h. *E*, Concentration-dependent effect of PGE₂ on TIMP-1 protein levels in cell culture media following incubation with PGE₂ or vehicle for 24 h. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance of $n = 4$ (*A*) and $n = 4$ (*B*) experiments. $**P < 0.01$; $***P < 0.001$, vs. corresponding vehicle control; $#### P < 0.001$ vs. THC-treated cells, Student's t-test. Values above the blots (*A*, lower panel, *C*, *D*, *E*) are means \pm SEM obtained from densitometric analysis of $n = 4$ (*A*), $n = 5$ (*C*), $n = 5$ (*D*) and $n = 4$ (*E*) blots and represent percent control in comparison with vehicle-treated cells (100%) in the absence of test substance. Protein staining of cell culture media is shown as loading control (LC).

Figure 6

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Role of COX-2 and TIMP-1 in the antimigrative action of MA. *A*, Concentration-dependent effect of MA on migration and viability of HTM cells following incubation with THC or vehicle for 24 h. *B*, Effect of AM-251 (CB₁ antagonist; 1 μ M) and AM-630 (CB₂ antagonist; 1 μ M) on the increased COX-2 protein expression by MA. Cells were pretreated with the respective receptor antagonist (all tested at a final concentration of 1 μ M) for 30 min and incubated with MA (10 μ M) for another 24 h. *C*, Effect of a 24-h incubation with MA (10 μ M) on COX-2 mRNA expression and PGE₂ synthesis by HTM cells and impact of NS-398 (1 μ M, 30 min pretreatment) on PGE₂ induction by MA. *D*, Effect of TIMP-1 knockdown on the MA-

1 modulated migration of HTM cells and concomitant changes in TIMP-1 protein levels in cell
2 culture media. Cells were transfected with 0.25 $\mu\text{g/ml}$ TIMP-1 siRNA or non-silencing siRNA
3 for 24 h. Subsequently, 1×10^5 cells per 500 μl serum-free DMEM were subjected into
4 Boyden chambers containing the same amounts of siRNA or non-silencing siRNA for another
5 24 h in presence of MA (10 μM) or vehicle (upper panel). Monitoring of TIMP-1 knockdown
6 was performed in parallel experiments in 24-well plates under comparable conditions. E,F,
7 Effect of NS-398 (selective COX-2 inhibitor; 1 μM), AM-251 (CB₁ antagonist; 1 μM) and AM-
8 630 (CB₂ antagonist; 1 μM) on the TIMP-1-inducing action of MA. Cells were pretreated with
9 inhibitor/antagonists for 30 min and incubated with MA (10 μM) for another 24 h. Percent
10 control represents comparison with vehicle-treated cells (100%) in the absence of test
11 substance of $n = 4$ (A,C,D) experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, vs.
12 corresponding vehicle control; ### $P < 0.001$ vs. MA-treated cells, Student's t-test. Values
13 above the blots (B,D,E,F) are means \pm SEM obtained from densitometric analysis of $n = 4$
14 (B), $n = 3$ (D), $n = 5$ (E) and $n = 3$ (F) blots and represent percent control in comparison with
15 vehicle-treated cells (100%) in the absence of test substance. In Panel B equal loading of
16 lysates is indicated by hybridisation of membranes with an antibody against β -actin. In
17 Panels D, E and F protein staining of cell culture media is shown as loading control (LC).
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Table 1

Effect of AM-251 (CB₁ antagonist; 1 μM), AM-630 (CB₂ antagonist; 1 μM), NS-398 (selective COX-2 inhibitor; 1 μM), GW627368X (EP₄ receptor antagonist, 1 μM), AH-6809 (EP₁/EP₂ receptor antagonist, 10 μM) and SC19220 (EP₁ receptor antagonist, 1 μM) on the antimigratory effect of MA. Cells were pretreated with antagonists/inhibitors for 30 min and incubated with MA (10 μM) for another 24 h. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance of n = 4 experiments. ***P* < 0.01; ****P* < 0.001, vs. corresponding vehicle control; ## *P* < 0.01; ### *P* < 0.001 vs. MA-treated cells, Student's t-test.

	Migration (%)
Vehicle	100.0 ± 12.8
MA	23.8 ± 14.4 **
MA + AM-251	98.5 ± 6.1 ##
MA + AM-630	96.6 ± 10.8 ##
MA + AM-251 + AM-630	104.6 ± 5.6 ##
Vehicle	100.0 ± 3.3
MA	38.0 ± 4.1 ***
MA + NS-398	95.3 ± 11.4 ##
NS-398	100.7 ± 1.8
Vehicle	100 ± 3.6
MA	36.4 ± 4.4 ***
MA + GW627368X	83.9 ± 8.6 ##
MA + AH6809	97.1 ± 6.0 ###
MA + SC19220	21.3 ± 4.8

Figure

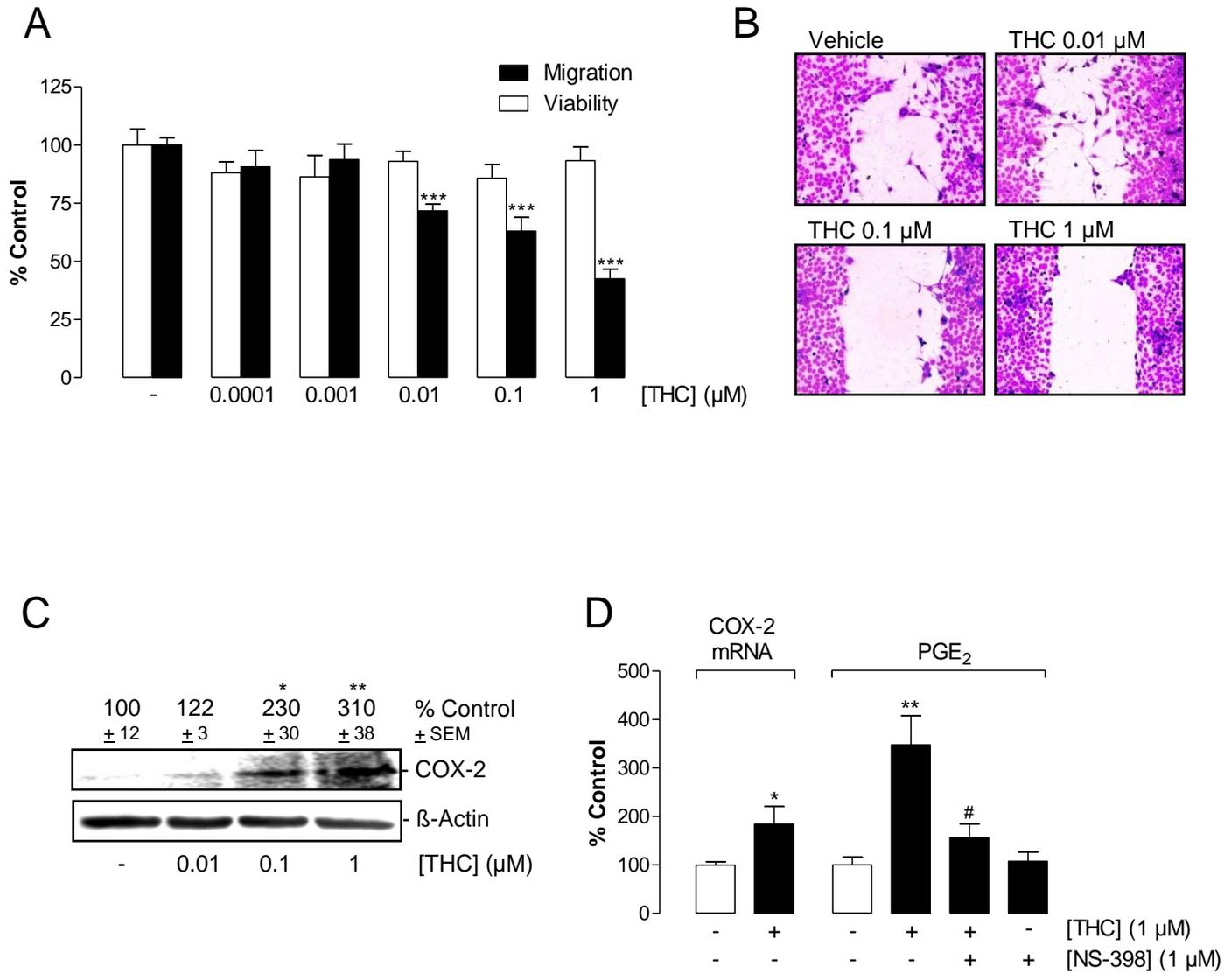
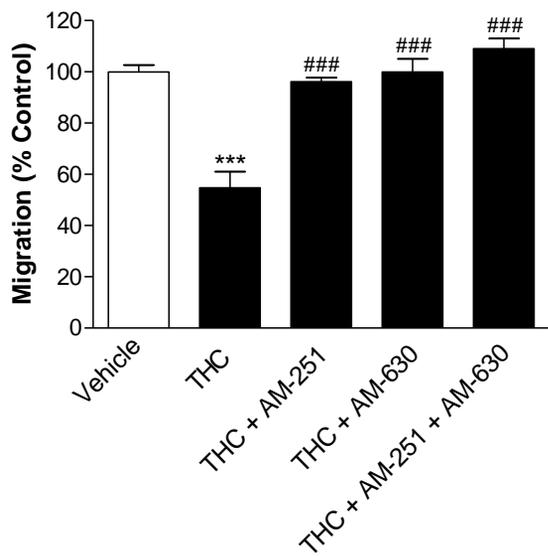
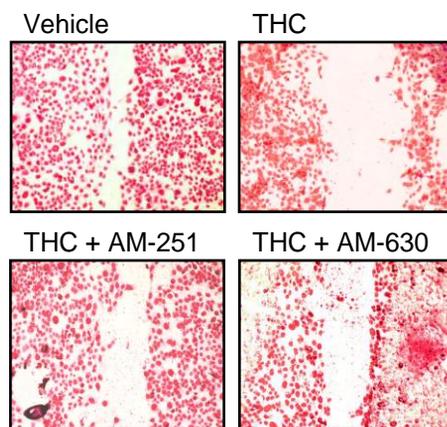
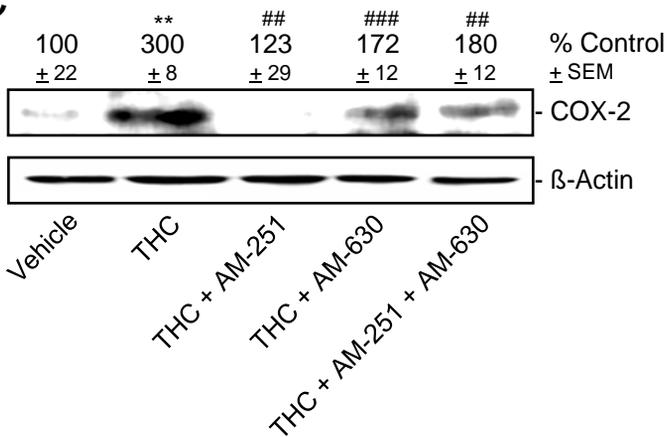
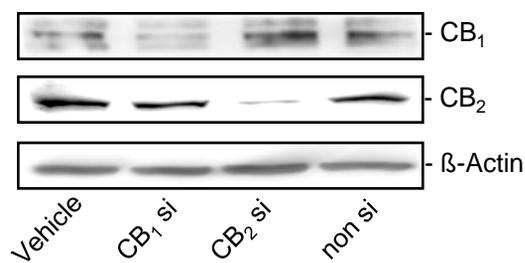
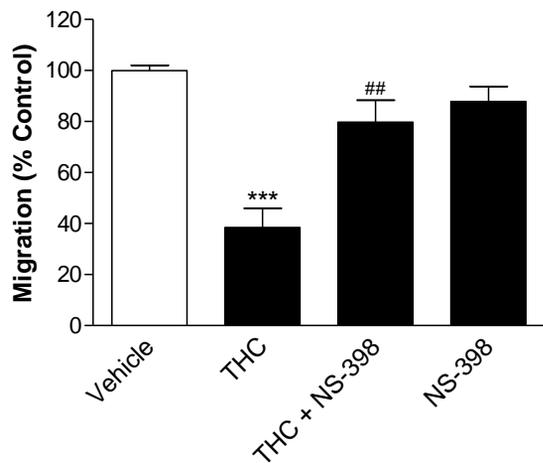
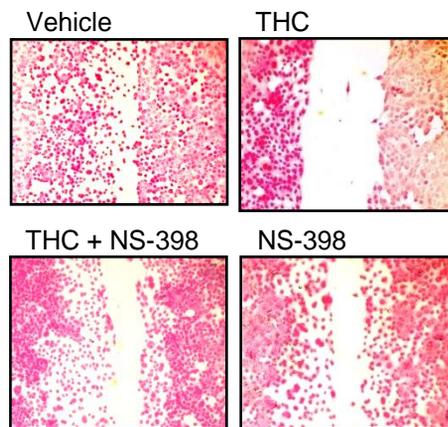
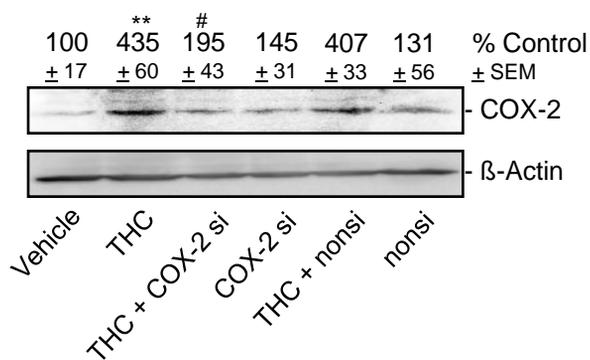
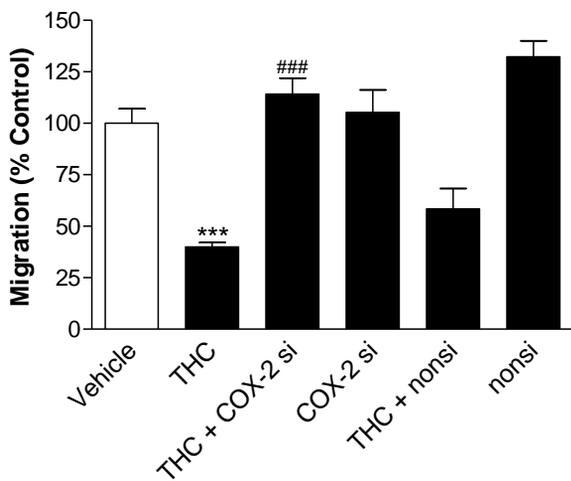


Fig. 1

A**B****C****D****Fig. 2**

A**B****C****Fig. 3**

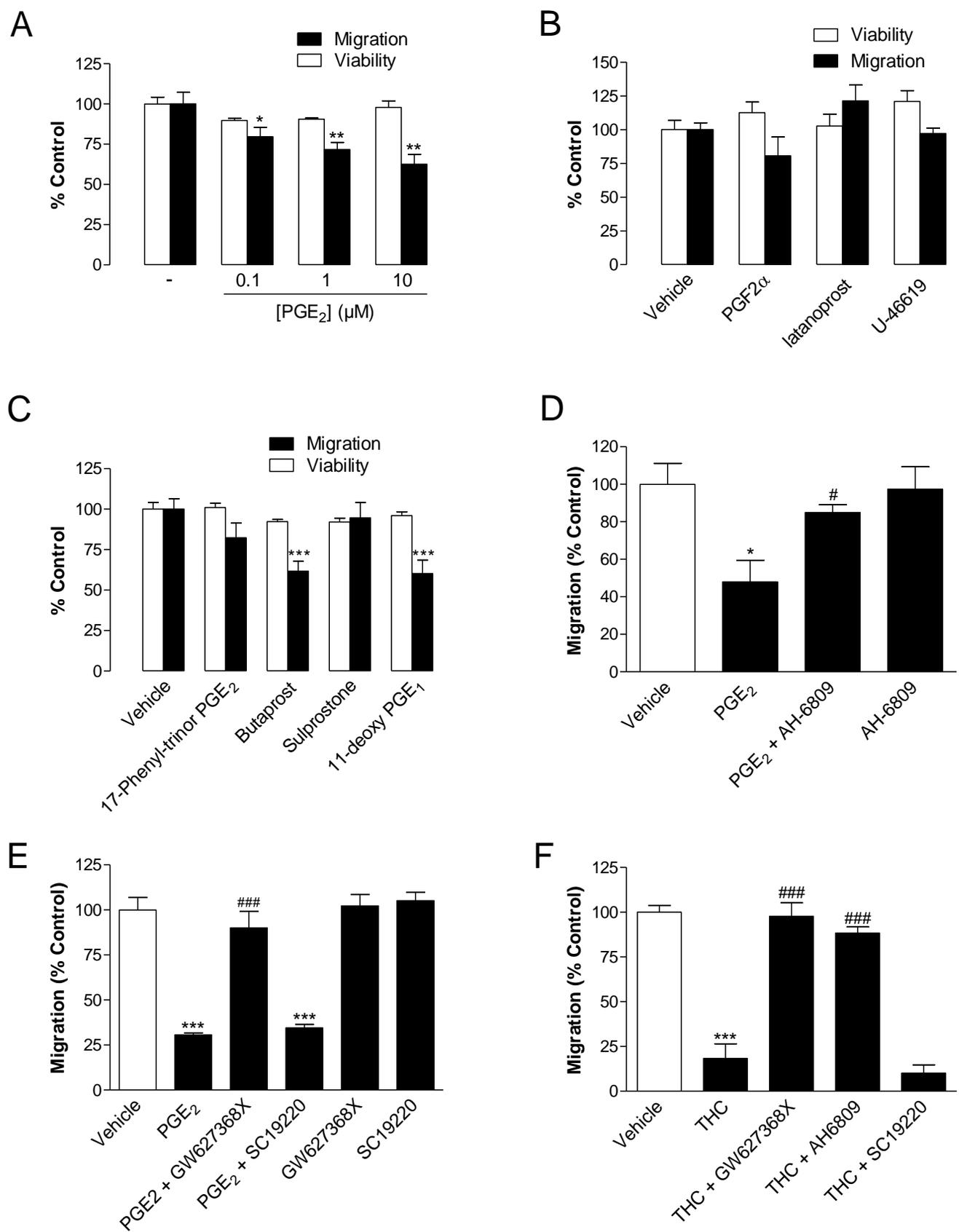
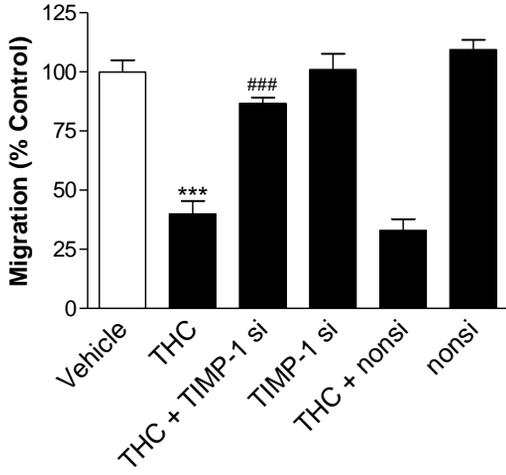
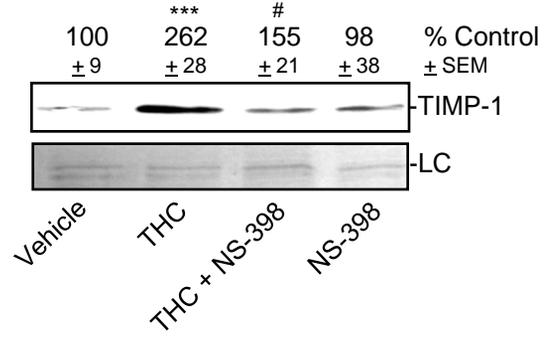


Fig. 4

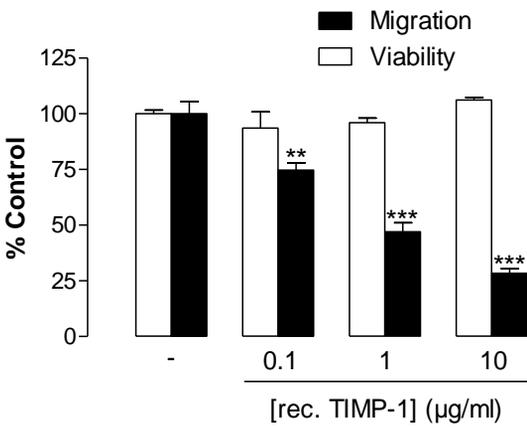
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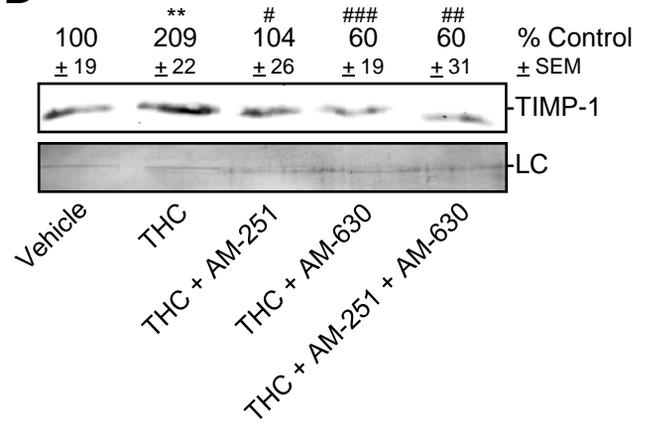
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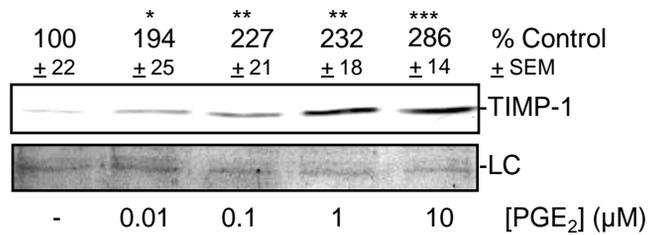


Fig. 5

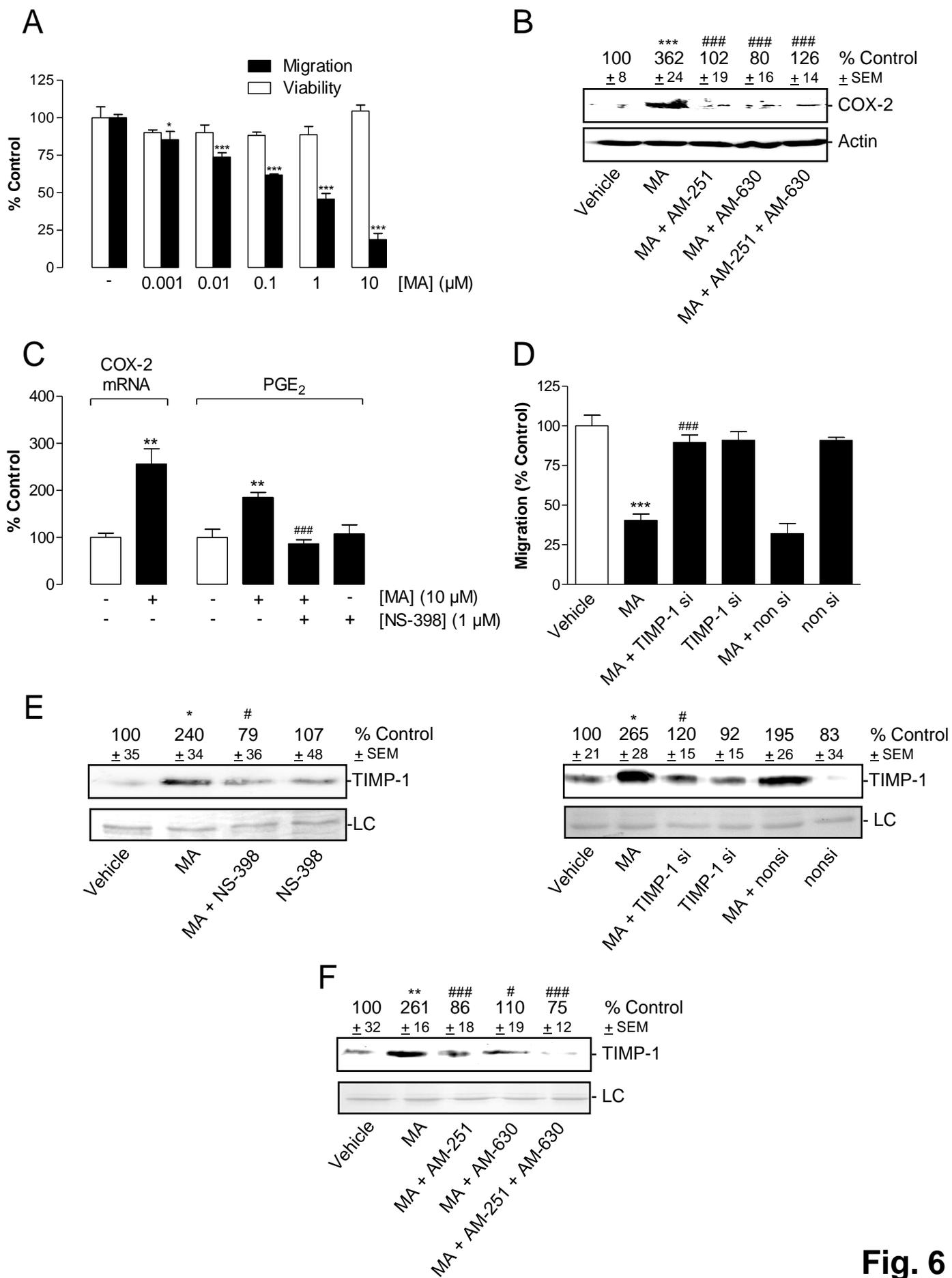


Fig. 6

