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Cannabidiol inhibits cancer cell invasion via upregulation of tissue inhibitor of matrix metalloproteinases-1☆

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ABSTRACT

Although cannabinoids exhibit a broad variety of anticarcinogenic effects, their potential use in cancer therapy is limited by their psychoactive effects. Here we evaluated the impact of cannabidiol, a plant-derived non-psychoactive cannabinoid, on cancer cell invasion. Using Matrigel invasion assays we found a cannabidiol-driven impaired invasion of human cervical cancer (HeLa, C33A) and human lung cancer cells (A549) that was reversed by antagonists to both CB_1 and CB_2 receptors as well as to transient receptor potential vanilloid 1 (TRPV1). The decrease of invasion by cannabidiol appeared concomitantly with upregulation of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1). Knockdown of cannabidiol-induced TIMP-1 expression by siRNA led to a reversal of the cannabidiol-elicited decrease in tumor cell invasiveness, implying a causal link between the TIMP-1-upregulating and anti-invasive action of cannabidiol. P38 and p42/44 mitogen-activated protein kinases were identified as upstream targets conferring TIMP-1 induction and subsequent decreased invasiveness. Additionally, in vivo studies in thymic-aplastic nude mice revealed a significant inhibition of A549 lung metastasis in cannabidiol-treated animals as compared to vehicle-treated controls. Altogether, these findings provide a novel mechanism underlying the anti-invasive action of cannabidiol and imply its use as a therapeutic option for the treatment of highly invasive cancers.

1. Introduction

During the last decade several cell culture and animal studies have provided a great body of evidence for an antitumorigenic action of cannabinoids on the levels of cancer cell proliferation [1,2], apoptosis [3,4], metastasis [5], and angiogenesis [6–8]. Although cannabinoid-based drugs such as Δ^9-tetrahydrocannabinol (THC) are clinically used to palliate wasting, emesis and pain in cancer patients, a shortcoming for these and forthcoming indications clearly lies in the psychoactive adverse side effects of cannabinoids. For this reason, the interest in the non-psychoactive cannabinoid-mimetic compound cannabidiol has substantially increased in recent years. Meanwhile, a formulation including a 1:1 ratio of THC and cannabidiol has been approved for the pharmacotherapy of multiple sclerosis-related spasticity and pain in Canada [9].

The precise mechanism by which cannabidiol exerts its biological effects is still a matter of debate. Although cannabidiol displays a low affinity to CB_1 and CB_2 receptors [10], several effects of cannabidiol including modulation of cytokine release and macrophage chemotaxis [11], antiproliferative [12] as well as proapoptotic properties [13] have been shown to be mediated via CB_1 and/or CB_2 receptors. In other investigations, cannabidiol has even been reported to display antagonistic effects on CB_1 and CB_2 receptor [14], as well as synergistic effects on THC-mediated hypoactivity, hypothermia and impairment of spatial memory [15]. On the other hand increasing evidence suggests an indirect receptor activation by cannabidiol. In this context an inhibitory action on fatty acid amidohydrolase activity that confers release of anandamide and increased receptor-mediated signalling by this endocannabinoid has been demonstrated in diverse systems [16–18]. Additionally, the non-selective cation channel transient receptor potential 1 (TRPV1) was shown to be involved in

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Abbreviations: AM-251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; AM-630, (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl) (4-methoxyphenyl)methanone; cannabidiol, 2-((1S,6S)-3-methyl-6-(prop-1-en-2-yl) cyclohex-2-enyl)-5-pentylbenzene-1,3-diol; CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcriptase-polymerase chain reaction; siRNA, small-interfering RNA; THC, Δ^9-tetrahydrocannabinol; TIMP, tissue inhibitor of matrix metalloproteinase; TRPV1, transient receptor potential vanilloid 1; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.6-benzene disulfonate.

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cannabidiol-mediated antihyperalgesia [19] and inhibition of cancer cell proliferation [17]. Finally, diverse studies suggest the involvement of a hitherto undefined abnormal cannabidiol receptor in several actions of cannabidiol (for review see [20]).

In context with the antitumorigenic action of cannabinoids, modulation of cancer cell invasion has recently emerged as a topic of increasing interest [21–23]. Cancer cell invasion is a complex process of cell locomotion through degraded matrix components of microenvironmental spaces surrounding a tumor thereby contributing to tumor cell metastasis and angiogenesis. The level of tumor invasiveness and malignancy is mainly determined by a sensitive balance between collagen- and proteoglycan-degrading matrix metalloproteinases (MMPs) and their endogenous inhibitors, the tissue inhibitors of MMPs (TIMPs) (for review see Ref. [24]). Among the four distinct members of the TIMP family, elevated TIMP-1 was shown to mediate the anti-invasive effects of several anticarcinogenic drugs [23,25–29]. Furthermore, decreased TIMP-1 levels were demonstrated to correlate with high cancer invasiveness [30,31].

The present study focussed on a potential role of TIMP-1 upregulation in the anti-invasive action of cannabidiol and on a possible involvement of cannabinoid receptors and TRPV1 in this process. In view of recent studies demonstrating p38 and p42/44 mitogen-activated protein kinases (MAPKs) activation as intracellular signalling pathways leading to induction of TIMP-1 [32,33] as well as findings showing a cannabinoid receptor-dependent activation of MAPKs [3,34,35], our study also assessed a role of both MAPKs in cannabidiol-modulated invasion and TIMP-1 expression. Finally, the impact of cannabidiol on cellular invasion was confirmed by studying metastasis in vivo.

2. Materials and methods

2.1. Materials

(−)-Cannabidiol was purchased from Tocris (Bad Soden, Germany). AM-251, AM-630, capsazepine, PD98059 and SB203580 were bought from Alexis Deutschland GmbH (Grünberg, Germany). 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. DMSO, HEPES, NaCl, EDTA, Triton® X-100, and glycerol were bought from Applichem (Darmstadt, Germany). PMSF, leupeptin, aprotinin, picrinic acid, and formaldehyde were obtained from Sigma (Taufkirchen, Germany).

2.2. Cell culture

HeLa, C33A and A549 cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were grown in a humidified incubator at 37 °C and 5% CO_2. 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 cannabidiol) or 0.1% (v/v) dimethyl sulfoxide (DMSO) (for AM-251, AM-630, capsazepine, PD98059 and SB203580).

2.3. Matrigel invasion and migration assays

The effect of test substances on the invasiveness of cells was determined using a modified Boyden chamber technique with Matrigel-coated membranes according to the manufacturer's instructions (BD Biosciences, Oxford, UK). In this assay, tumor cells must overcome a reconstituted basement membrane by a sequential process of proteolytic degradation of the substrate and active migration. In brief, the upper sides of the transwell inserts (8 μm pore size) were coated with 28.4 μg Matrigel (BD Biosciences, Oxford, UK) per insert in 24-well plates. Trypsinized and pelleted cells were suspended to a final concentration of 5×10^5 cells in 500 μl serum-free DMEM in each insert and treated with a final concentration of 10 μM cannabidiol (except Fig. 1B and the data in Table 2) or ethanol vehicle for various incubation times. In Table 2 the half maximal effective concentration (EC_{50}) of cannabidiol in eliciting inhibition of Matrigel invasion was used for confirming key invasion experiments. To address the role of cannabinoid-activated receptors, specific antagonists to cannabinoid receptors (AM-251, AM-630) or TRPV1 (capsazepine) were used at a final concentration of 1 μM. In further experiments, inhibitors of p38 MAPK (SB203580) or p42/44 MAPK activation (PD98059) were used at a final concentration of 10 μM. Both receptor antagonists and inhibitors of MAPK pathways were tested vs. DMSO vehicles by adding to the cultures 1 h prior to starting the incubation with cannabidiol. Cannabidiol was added to the cultures without washing out the antagonists. DMEM containing 10% FCS was used as a chemoattractant in the companion plate. Following incubation in a humidified incubator at 37 °C and 5% CO_2 for the indicated times, the non-invading cells on the upper surface of the inserts were removed with a cotton swab, and the viability of the cells on the lower surface was measured by the colorimetric WST-1 test (Roche Diagnostics, Mannheim, Germany). This cell viability test is based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.6-benzene disulfonate) by mitochondrial succinate-tetrazolium-reductase in metabolically active cells.

For calculation of migration, the viability of cells on the lower side of uncoated invasion chambers was determined by the WST-1 test. Invasion was expressed as the invasion index, which is calculated as the absorbance at 490 nm of cells that invaded through Matrigel-coated Boyden chambers divided by absorbance of cells that migrated through uncoated control inserts with equal treatment ([invasion/migration] × 100%). For corroboration of the calculated invasion indices in one experiment (Fig. 3C), HeLa cells that had invaded through Matrigel-coated membranes were fixed and stained with Diff-Quick (Medion Diagnostics GmbH, Dügingen, Switzerland) and visualized using a microscope at 200× magnification.

To exclude the possibility that the effect of cannabidiol on invasion was an unspecific cytotoxicity-related phenomenon, cell viability was analyzed after cannabidiol exposure. For this purpose, cells were seeded into 48-well plates at 5×10^5 cells per well to match conditions of invasion assays or at 2.5×10^5, 1×10^5, 0.5×10^5, and 0.1×10^5 per well for testing lower cell densities in a volume of 500 μl DMEM per well and treated with 10 μM cannabidiol or ethanol vehicle for 72 h. Viability was measured subsequently using the WST-1 test.

The anti-invasive action of cannabidiol was confirmed in another experimental setup. To this end, HeLa cells were seeded at a final concentration of 2.5×10^6 cells in 10-cm dishes in DMEM containing 10% FCS. After 24 h cells were washed and treated with cannabidiol or vehicle in serum-free DMEM for an additional 48-h incubation period. Subsequently, trypsinized and pelleted cells were counted, suspended to a final concentration of 5×10^5 cells in 500 μl serum-free DMEM and subjected into each insert without further addition of test substances. Quantification of Matrigel invasion was performed after another 24-h incubation of HeLa cells in the Boyden chamber.

2.4. Quantitative RT-PCR analysis

HeLa cells were seeded into 48-well plates at a density of 5×10^5 cells per well. Following incubation of cells with cannabidiol or its vehicle for the indicated times, supernatants

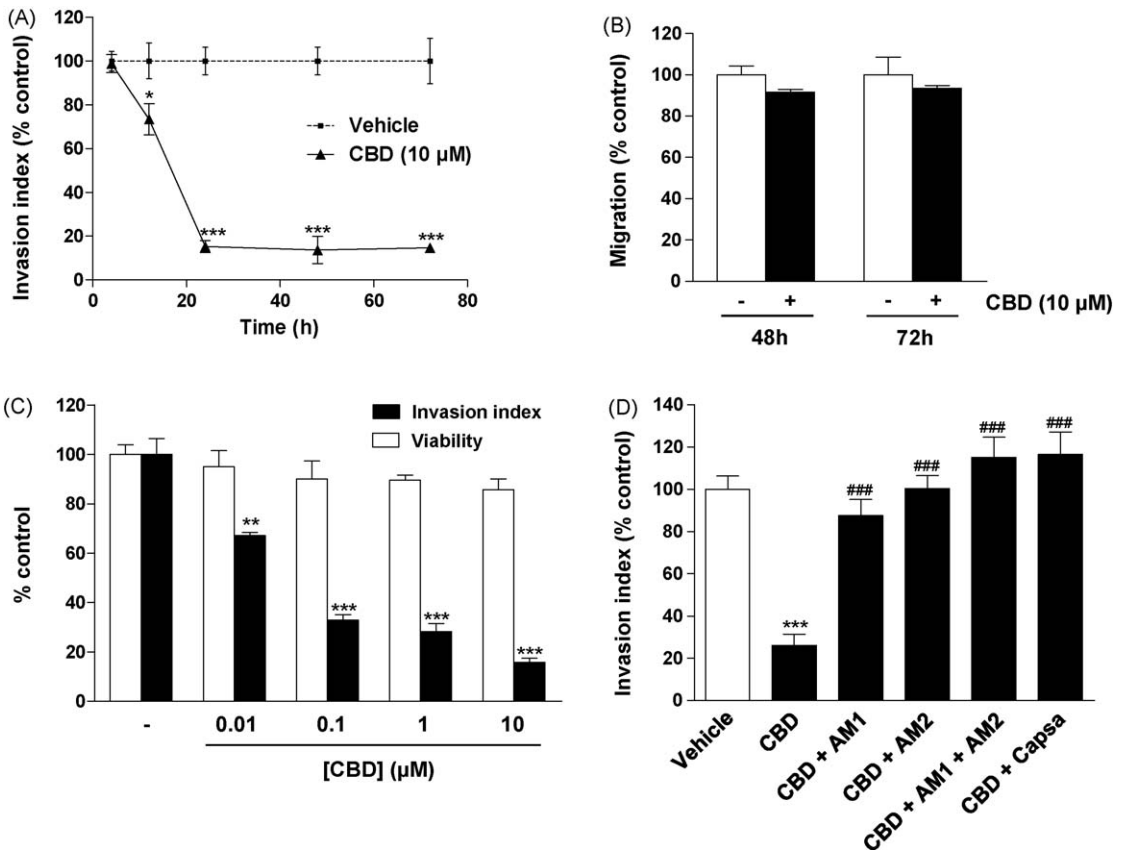

Fig. 1. Influence of cannabidiol (CBD) on HeLa cell invasion. (A) Time-dependency of Matrigel invasion of HeLa cells following stimulation of cells with 10 μM cannabidiol or vehicle over a 72-h incubation period. (B) Migration of HeLa cells through uncoated Boyden chambers after a 48- and 72-h stimulation with 10 μM cannabidiol or vehicle. (C) Concentration-dependency of cannabidiol's anti-invasive action (black bars) and its impact on cell viability (open bars) after a 72-h incubation period. (D) Effect of a 1-h pretreatment with AM-251 (AM1; CB_1 antagonist; 1 μM), AM-630 (AM2; CB_2 antagonist; 1 μM) and capsazepine (capsa; TRPV1 antagonist; 1 μM) on the anti-invasive action of cannabidiol (10 μM) after a 72-h incubation. Values are means ± SEM of $n = 4$ (A and B), $n = 3$–4 (C), and $n = 7$–8 (D) experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, vs. corresponding vehicle control; $^{\#\#\#}P < 0.001$ vs. cannabidiol-treated cells, Student's *t*-test.

were removed and cells were lysed for subsequent RNA isolation using the RNeasy total RNA Kit (Qiagen, Hilden, Germany). β-Actin- (internal standard) and TIMP-1 mRNA levels were determined by quantitative real-time RT-PCR as described [36]. Primers and probe for human TIMP-1 was an Assay-on-demand™-product (Applied Biosystems, Darmstadt, Germany).

2.5. Western blot analysis

For determination of TIMP-1, MMP-2 and MMP-9 protein levels in Fig. 2B, cells grown to confluence in 48-well-plates were incubated with test substances or vehicles. Afterward, cell culture supernatants were centrifuged at 500 × *g* and used for Western Blot analysis. For all other blots, TIMP-1 was determined in supernatants collected from the upper Boyden chambers at the end of the respective invasion experiment. Total protein in the supernatants was measured using the bicinchoninic acid assay (Pierce, Rockford, IL, USA). For Western blot analysis of p38, phospho-p38, p42/44, and phospho-p42/44, cells grown to confluence in 6-well-plates were incubated with cannabidiol or its vehicle for the indicated times. Afterward, cells were washed, harvested, lysed in solubilization buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton® X-100, 10% (v/v) glycerol, 1 mM PMSF, 1 μg/ml leupeptin and 10 μg/ml aprotinin), homogenized by sonication, and centrifuged at 10,000 × *g* for 5 min. Supernatants were used for Western blot analysis. All proteins were separated on a 12% sodium dodecyl sulfate-polyacrylamide (Applichem, Darmstadt, Germany) gel. Following transfer to nitrocellulose and blocking of the membranes with 5% milk powder, blots were probed with specific antibodies raised to TIMP-1, MMP-2 and MMP-9 (all antibodies from Oncogene Research Products, San Diego, CA) or p38, phospho-p38, p42/44 and phospho-p42/44 (New England BioLabs GmbH, Frankfurt, Germany). Membranes were probed with horseradish peroxidase-conjugated Fab-specific anti-mouse IgG for detection of TIMP-1 and MMPs (New England BioLabs GmbH, Frankfurt, Germany) or anti-rabbit IgG for analysis of MAPK protein expression (New England BioLabs GmbH, Frankfurt, Germany). Densitometric analysis of TIMP-1 and MMP band intensities was achieved by optical scanning and quantifying using the Quantity One 1-D Analysis Software (Biorad, Muenchen, Germany). Vehicle controls were defined as 100% for evaluation of changes in protein expression. To ensure that equal amounts of protein in cell culture supernatants used for protein analysis of TIMP-1 and MMPs had been transferred to the membrane, proteins on Western blot membranes were stained with the fluorescent dye Roti-Green (Carl Roth, Karlsruhe, Germany) except for Fig. 2B, were membranes were stained with Ponceau red. To ascertain equal protein loading in Western Blots of supernatants obtained from upper Boyden chambers, a band with a size of about 65 kDa that appeared unregulated is shown as a loading control (LC) for protein analysis of supernatants.

2.6. SiRNA transfections

HeLa, C33A, and A549 cells were transfected with siRNA targeting the indicated sequence of TIMP-1 using RNAiFect® as the transfection reagent (Qiagen GmbH, Hilden, Germany) or negative control RNA (Eurogentec, Seraing, Belgium; Cat. No. OR-0030-neg). The target sequence of the TIMP-1 siRNA (Qiagen GmbH, Hilden,

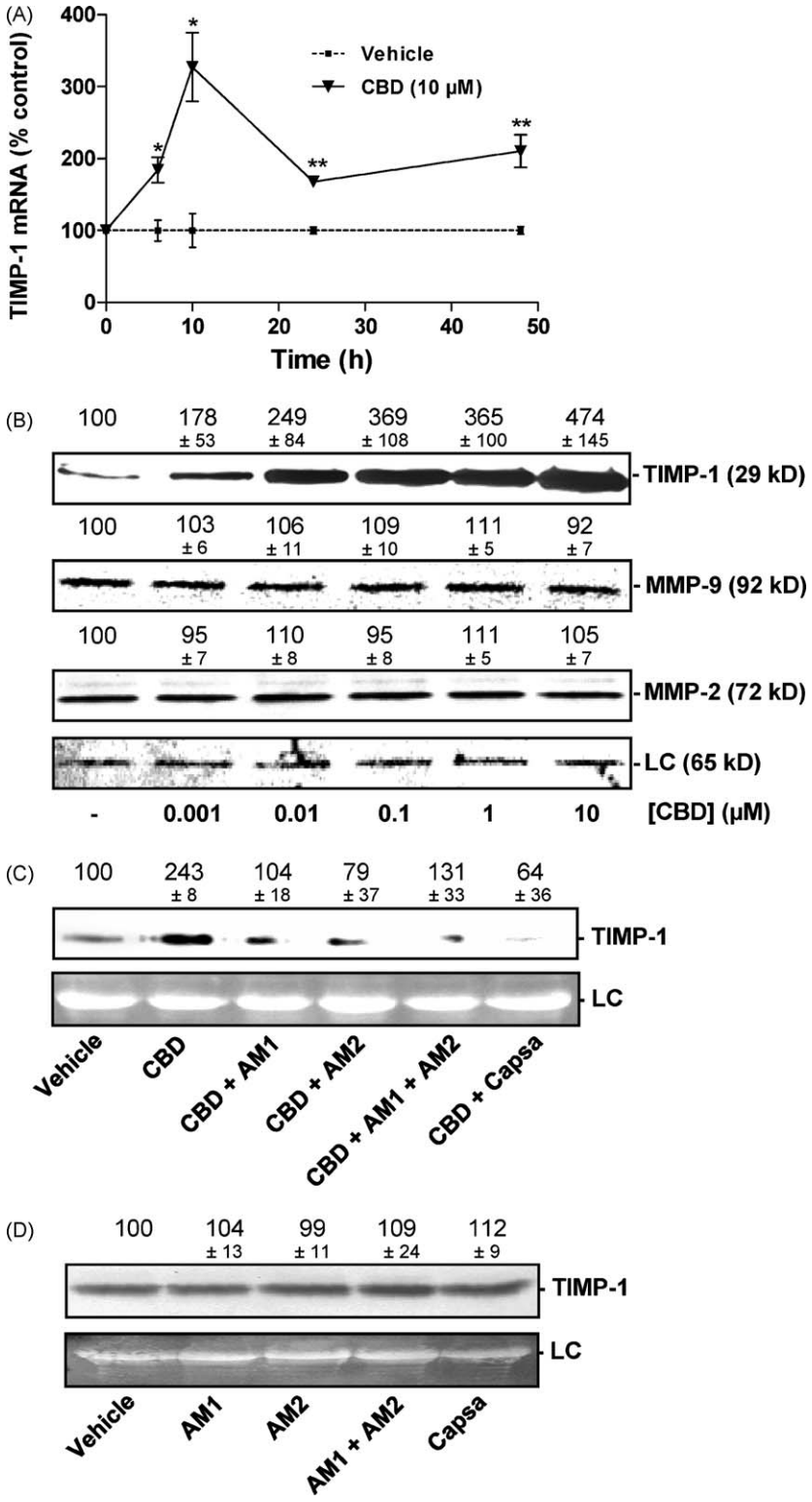

Fig. 2. Impact of cannabidiol (CBD) on TIMP-1 expression. (A) Time-course of TIMP-1 mRNA normalized to β-actin. Cells were incubated with 10 μM cannabidiol or its vehicle over a 48-h incubation period. (B) Concentration-dependent effect of cannabidiol (0.01–10 μM) or its vehicle on TIMP-1, MMP-2 and MMP-9 protein levels following a 72-h incubation of cells. (C) Influence of a 1-h preincubation with AM-251 (AM1; CB_1 antagonist; 1 μM), AM-630 (AM2; CB_2 antagonist; 1 μM) and capsazepine (capsa; TRPV1 antagonist; 1 μM) on cannabidiol-mediated TIMP-1 induction after a 72-h incubation period. (D) Influence of receptor antagonists on TIMP-1 expression without cannabidiol treatment after a 72-h incubation period. mRNA data (A) are means ± SEM of $n = 3$ experiments. $^{*}P < 0.05$; $^{**}P < 0.01$, vs. corresponding vehicle control (Student's t-test). Values above selected blots are means ± SEM obtained from densitometric analysis of $n = 4$ (B and C) or $n = 3$ (D) blots and represent percent control in comparison with vehicle-treated cells (100%) in the absence of test substance. Protein staining of supernatants is shown as loading control (LC).

Germany) was as follows: 5́-tcccatctttcttccggacaa-3́. A BLAST search revealed that the sequence selected did not show any homology to other known human genes. Transfections were performed according to the manufacturer's instructions. For invasion assays, cells grown to confluence were transfected with 0.25 μg/ml siRNA or non-silencing siRNA as 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 treated with trypsin for 3 min at 37 °C in a humidified incubator, centrifuged at 200 × *g*, resuspended to a final density of 5×10^5 cells in 500 μl of serum-free DMEM containing the same amounts of siRNA or non-silencing siRNA to provide constant transfection conditions, and seeded for invasion analysis as described above.

2.7. Mouse model of tumor metastasis

Athymic nude mice (NMRI-nu/nu) were purchased from Charles River GmbH (Sulzfeld, Germany). Mice were given injections of A549 cells (1×10^6 per 100 μl in PBS) through the lateral tail vein (day 1) and, after 24 h (day 2), were treated with cannabidiol (5 mg/kg body weight) or with vehicle intraperitoneally. Treatment protocols for the evaluation of the antitumorigenic action of cannabidiol in vivo developed by other groups revealed 5 mg cannabidiol per kg as an appropriate dose for this purpose [17]. In our experiments cannabidiol or vehicle were administered every 72 h. Mice were sacrificed on day 28 and one lung per animal was evaluated for experimental metastases. To contrast lung nodules, lungs were fixed in Bouin's fluid (saturated picrinic acid 15 ml, formadehyde 5 ml, glacial acetic acid 1 ml), and metastatic nodes were scored under a stereoscopic microscope in an investigator-blinded fashion. For histopathological examination lung samples were fixed in 4% formaline. Paraffin sections were stained with hematoxylin and eosin.

2.8. Statistics

All statistical analyses were undertaken using GraphPad Prism 3.00 (GraphPad Software, San Diego, CA). Results were considered to be statistically significant at $P < 0.05$.

To assess the half maximal effective concentration (EC_{50}) of cannabidiol in eliciting an anti-invasive response, concentration response curves were fitted by a log(agonist) vs. response regression with variable slope.

3. Results

3.1. Time-course and concentration-dependency of cannabidiol's inhibitory effect on HeLa cell invasion

HeLa cells showed a diminished invasion within the first 24 h of incubation with 10 μM cannabidiol that continued to increase over the investigated 72-h incubation period (Fig. 1A). To exclude an effect based on cell motility, transmigration through uncoated Boyden chambers was evaluated after 48 and 72 h. As shown in Fig. 1B, cannabidiol did not show a significant inhibitory effect on this parameter.

Moreover, cannabidiol caused a concentration-dependent decrease of invasion that was even significant at concentrations as low as 0.01 μM (Fig. 1C). Cellular viability measured under comparable experimental conditions (i.e., 48-well plates with 5×10^5 cells per well) was virtually unaltered in the presence of either cannabidiol concentration tested (Fig. 1C). As recently shown, the phytocannabinoid THC and the hydrolysis-stable endocannabinoid analog R(+)-methanandamide revealed a toxic influence on HeLa cells that was dependent on cellular density [23]. A similar pattern was observed for cannabidiol in HeLa cells. As compared with vehicle-treated cells, cell viability was significantly ($P < 0.001$, Student's *t*-test) lowered by a 72-h treatment with 10 μM cannabidiol at cellular densities lower than of 2.5×10^5 cells (Table 1).

To confirm the anti-invasive action of cannabidiol in another experimental setup, additional experiments were performed with HeLa cells that were first treated with cannabidiol or vehicle for 48 h and trypsinized, counted and adjusted to a final cellular density of 0.5×10^5 cells per insert thereafter. Quantification of invasiveness was performed after a further incubation of cells in Boyden chambers for another 24 h. As compared to vehicle [100 ± 17.2%], the invasion index became significantly decreased after pretreatment with cannabidiol at final concentrations of 0.01 μM [42.4% ± 1.5%; $P < 0.05$], 0.1 μM [32.1% ± 10.8%; $P < 0.05$], 1 μM [27.8% ± 2%; $P < 0.01$], and 10 μM [16.1% ± 4.2%; $P < 0.01$].

3.2. Involvement of cannabinoid receptors and TRPV1 in cannabidiol's anti-invasive action

As previously demonstrated, HeLa, A549 and C33A cells express CB_1 and CB_2 receptors as well as TRPV1 [23]. To investigate the role of cannabinoid receptors and TRPV1 in cannabidiol-mediated reduction of HeLa cell invasiveness, the impact of antagonists of CB_1 receptor (AM-251), CB_2 receptor (AM-630) and TRPV1 (capsazepine) on the cannabidiol-reduced invasion was tested. Inhibitors were used at a concentration of 1 μM which has been reported to be within the range of concentrations inhibiting CB_1-, CB_2- and TRPV1-dependent events [1,37]. As shown in Fig. 1D as well as in Table 2, cannabidiol-induced inhibition of HeLa cell invasion was prevented in the presence of AM-251, AM-630 or the combination of both antagonists that were added to the cultures 1 h prior to cannabinoid stimulation. Likewise, the TRPV1 antagonist capsazepine fully abolished the anti-invasive action of cannabidiol (Fig. 1D, Table 2). Cannabinoid receptor and TRPV1 antagonists tested without cannabidiol treatment left basal invasiveness virtually unaltered (data not shown).

Table 1
Influence of cellular density on the viability of cannabidiol (CBD)-treated HeLa, A549 and C33A cells. Cells were seeded with the indicated number of cells per well of a 48-well plate in serum-free DMEM and treated with 10 μM cannabidiol or vehicle, respectively. After a 72-h incubation time, cellular viability was measured by WST-1. Values are means ± SEM of $n = 4$ experiments vs. corresponding vehicle control.

Seeded cell number	Cellular viability (%)					
	HeLa		A549		C33A	
	Vehicle	CBD	Vehicle	CBD	Vehicle	CBD
5.0×10^5	100 ± 8.9	98.3 ± 7.6	100 ± 8.4	90.7 ± 6.3	100 ± 5.2	82.8 ± 4.1*
2.5×10^5	100 ± 2.3	62.5 ± 2.6***	100 ± 4.1	54.8 ± 1.1***	100 ± 6.0	56.2 ± 3.6***
1.0×10^5	100 ± 1.1	2.0 ± 0.2***	100 ± 3.3	1.5 + 0.6***	100 ± 8.4	12.1 ± 2.6***
0.5×10^5	100 ± 6.9	1.8 ± 0.1***	100 ± 1.4	1.5 + 0.7***	100 ± 6.2	11.4 + 0.8***
0.1×10^5	100 ± 4.2	3.3 ± 0.2***	100 ± 17.5	1.0 ± 0.8***	100 ± 1.3	10.6 ± 4.4***

* $P < 0.05$, vs. corresponding vehicle control (Student's *t*-test).
*** $P < 0.001$, vs. corresponding vehicle control (Student's *t*-test).

Table 2
Involvement of cannabinoid receptors, TRPV1, p38 and p42/44 MAPKs and TIMP-1 in the anti-invasive action of 0.025 μM cannabidiol (CBD) on HeLa cells. Effect of a 1-h pretreatment of cells with AM-251 (CB_1 antagonist; 1 μM), AM-630 (CB_2 antagonist; 1 μM) and capsazepine (TRPV1 antagonist; 1 μM) on the anti-invasive action of 0.025 μM cannabidiol (upper set). Effect of a 1-h pretreatment with 10 μM SB203580 (inhibitor of p38 MAPK activity) and PD98059 (inhibitor of p42/44 MAPK activation) on the anti-invasive action of 0.025 μM cannabidiol (middle set), and effect of TIMP-1 siRNA (0.25 μg/ml) or non-silencing siRNA (0.25 μg/ml) on the anti-invasive action of 0.025 μM cannabidiol (lower set). Cells were incubated with cannabidiol for 72 h. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance of $n = 4$ experiments.

	Invasion Index (%)
Vehicle	100 ± 5.8
CBD	43.7 ± 2.1***
CBD + AM-251 (1 μM)	87.8 ± 0.9###
CBD + AM-630 (1 μM)	86.7 ± 5.3###
CBD + AM-251 (1 μM) + AM-630 (1 μM)	99.3 ± 6.6###
CBD + capsazepine (1 μM)	92.3 ± 4.9###
Vehicle	100 ± 7.4
CBD	42.8 ± 1.3***
CBD + SB203580 (10 μM)	77.9 ± 1.3###
CBD + PD98059 (10 μM)	115.4 ± 3.5###
Vehicle	100 ± 12.6
CBD	45.2 ± 1.4***
CBD + TIMP-1 siRNA	90.6 ± 3.6###
TIMP-1 siRNA	93.2 ± 6.2
CBD + non-silencing siRNA	45.2 ± 2.5
Non-silencing siRNA	103.5 ± 2.3

*** $P < 0.001$, vs. corresponding vehicle control.
$P < 0.001$, vs. cannabidiol (Student's t-test).

3.3. Effect of cannabidiol on the expression of TIMP-1, MMP-2 and MMP-9

In experiments addressing the impact of cannabidiol on the expression of TIMP-1, cannabidiol at a concentration of 10 μM exhibited a time-dependent induction of TIMP-1 mRNA that became first evident after a 6-h incubation period (Fig. 2A). In line with the invasion data, cannabidiol increased TIMP-1 protein levels in a concentration-dependent manner (Fig. 2B). Analysis of MMP-2 and MMP-9 revealed no alteration by either concentration of cannabidiol (Fig. 2B).

3.4. Impact of cannabinoid receptor- and TRPV1 antagonists on cannabidiol-elicited TIMP-1 induction

Experiments using cannabinoid receptor- and TRPV1 antagonists were performed to determine the receptor targets of cannabidiol involved in the induction of TIMP-1 expression. According to Western blot analyses from cell culture supernatants obtained from the upper Boyden chambers of the respective invasion experiments, cannabidiol-induced TIMP-1 expression was substantially reduced by antagonists to both CB_1 and CB_2 receptors (Fig. 2C). Furthermore, cannabidiol-induced TIMP-1 expression was abrogated by the TRPV1 antagonist capsazepine (Fig. 2C).

3.5. Effect of TIMP-1 knockdown on the anti-invasive action of cannabidiol

To further confirm a causal link between cannabidiol-mediated TIMP-1 induction and the accompanied decreased invasion, the expression of TIMP-1 was prevented by transfecting cells with TIMP-1 siRNA. As recently shown by our group, RNA interference with 0.25 μg/ml TIMP-1 siRNA elicits a reduction of cannabinoid-stimulated TIMP-1 expression without altering the basal level of HeLa cell invasiveness [23]. To study the functional impact of cannabidiol-induced TIMP-1 expression on the observed decrease of invasiveness, TIMP-1 knockdown experiments were therefore performed with a final concentration of 0.25 μg/ml TIMP-1 siRNA.

Referring to Fig. 3A and Table 2, knockdown of TIMP-1 expression led to a significant abrogation of cannabidiol-mediated decrease of invasion, whereas cultures treated with a non-silencing sequence exhibited the same invasion pattern as controls treated with transfection agent only. Monitoring of TIMP-1 secretion into the culture medium of the upper Boyden chamber confirmed a substantial inhibition of cannabidiol-induced TIMP-1 expression in cannabidiol-treated cells additionally transfected with TIMP-1 siRNA (Fig. 3A).

The impact of TIMP-1 siRNA transfection on tumor cell invasion was also confirmed by staining cells invaded through Matrigel-coated membranes after removal of non-invaded cells from the upper surface of the transwell membrane. The pictures in Fig. 3B demonstrate a significantly lower number of cells invaded in the presence of cannabidiol and a reversal of this effect when cells were concomitantly treated with TIMP-1 siRNA. Again, non-interfering sequences elicited an invasive behavior comparable to controls lacking siRNA.

3.6. Effect of blockade of MAPK pathways on cannabidiol's anti-invasive and TIMP-1-inducing action

In further experiments addressing the role of p38 and p42/44 MAPKs in the anti-invasive and TIMP-1-inducing action of cannabidiol, inhibitors of p38 MAPK activity (SB203580, 10 μM) and of p42/44 MAPK activation (PD98059, 10 μM) substantially inhibited both cannabidiol effects (Fig. 4A and B, Table 2). As previously described, SB203580 and PD98059 alone did not modulate baseline TIMP-1 expression and Matrigel invasion of HeLa cells [29].

Additional inhibitor experiments were performed to confirm a causal link between receptor activation and MAPK phosphorylations. As shown in Fig. 4C, cannabidiol-induced activation of p38 and p42/44 MAPKs at two early time points following stimulation. According to Fig. 4D, both activations were substantially suppressed by CB_1- and CB_2 antagonists as well as by the TRPV1 antagonist.

3.7. Role of TIMP-1 in the anti-invasive action of cannabidiol in other tumor cell lines

To exclude that the demonstrated effects are restricted to HeLa cells, experiments were also performed in human lung carcinoma cells (A549, Fig. 5) and in another human cervical carcinoma cell line (C33A, Fig. 6).

Like in HeLa cells, addition of cannabidiol resulted in a profound suppression of invasion through Matrigel-coated transwell plates in A549 (Fig. 5) and C33A cells (Fig. 6). Decreased invasiveness was accompanied by increased TIMP-1 secretion with both events being significantly suppressed by a 1-h preincubation with antagonists of CB_1 (AM-251, 1 μM) and CB_2 receptors (AM-630, 1 μM) alone or in combination and by a TRPV1 antagonist (capsazepine, 1 μM) in A549 (Fig. 5A) and C333A cells (Fig. 6A).

Furthermore, in both cell lines the blockade of p38 or p42/44 pathways led to a significant reconstitution of the cannabidiol-mediated decrease of Matrigel invasion (Figs. 5B and 6B). Like in HeLa cells, TIMP-1 levels in cell culture supernatants of the upper transwell inserts revealed an inverse correlation between invasiveness and TIMP-1 levels (Figs. 5B and 6B).

To further confirm the causal link between cannabidiol-mediated decrease of invasiveness and elevated TIMP-1 expression, additional RNA interference experiments were performed by targeting TIMP-1 expression with 0.25 μg/ml siRNA and equal concentrations of non-silencing siRNA in both cell lines after a 72-h incubation with 10 μM cannabidiol. As expected, transfection of

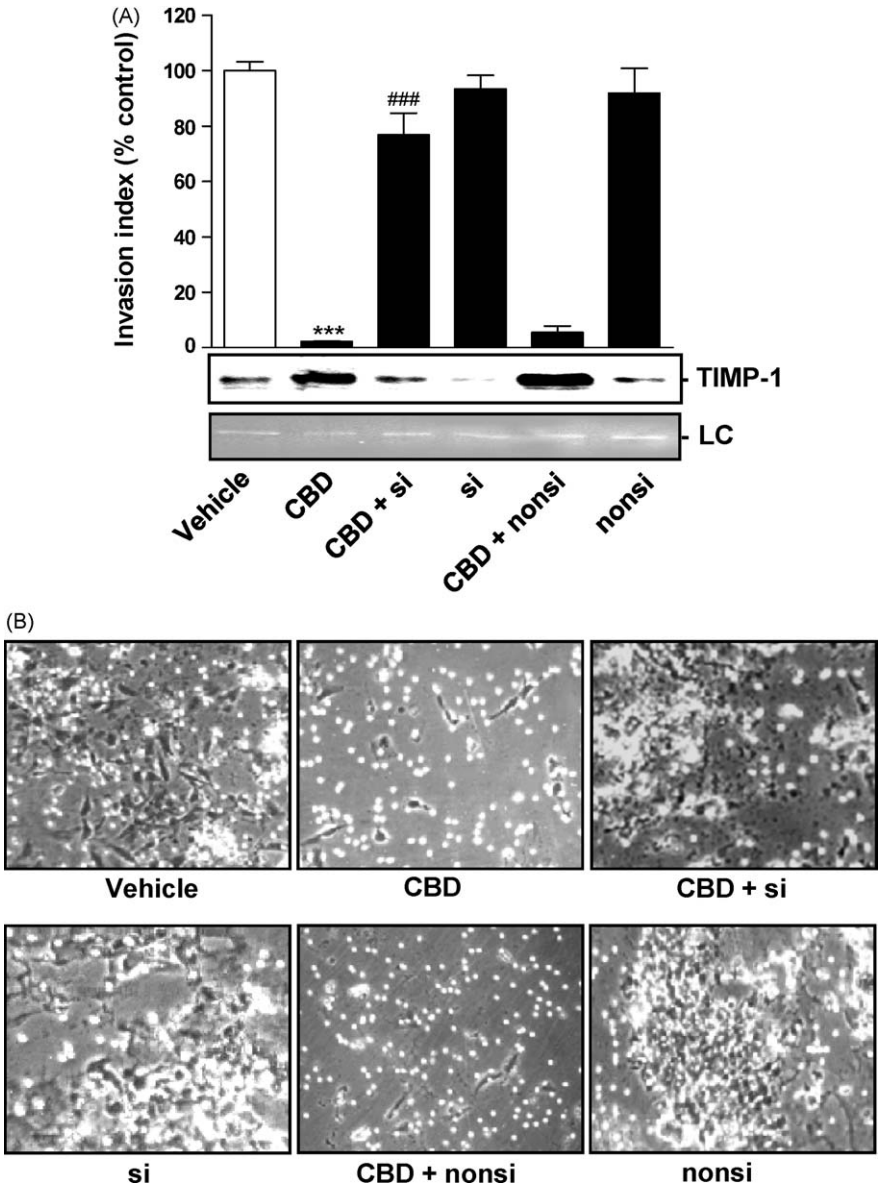

Fig. 3. Role of TIMP-1 in cannabidiol's (CBD) effect on HeLa cell invasion. (A) Effect of TIMP-1 siRNA (0.25 μg/ml) on cannabidiol's action on cell invasion and TIMP-1 protein expression. HeLa cells were transfected with TIMP-1 siRNA at a final concentration of 0.25 μg/ml siRNA (si) or with non-silencing siRNA (nonsi) for 24 h. Subsequently, cells were placed into invasion chambers, retransfected with the indicated type of siRNA or suspension buffer to provide constant knockdown conditions and incubated with 10 μM cannabidiol or vehicle for a further 72 h. Protein staining of supernatants is shown as loading control (LC). (B) Microscopy of HeLa cells from different treatment groups that invaded through Matrigel-coated membranes. Cells were stained with Diff-Quick® (Medion Diagnostics GmbH, Büdingen, CH) and documented under a 200× magnification. Percent control (A) represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means ± SEM of $n = 4$ experiments. $^{***}P < 0.001$, vs. corresponding vehicle control; $^{###}P < 0.001$, vs. cannabidiol (Student's *t*-test).

A549 and C33A cells with TIMP-1 siRNA led to inhibition of cannabidiol's effects on invasion and corresponding TIMP-1 expression (Figs. 5C and 6C).

Measurement of cellular viability with the WST-1 test revealed no significant toxicity by cannabidiol in A549 cells ($P = 0.411$) and only a weak toxic effect in C33A cells ($P = 0.039$). For viability analysis under conditions comparable to invasion assays, cells were treated with 10 μM cannabidiol for 72 h at a density of 5×10^5 cells per well in a 48-well plate. The toxic effect became highly significant ($P < 0.001$, Student's *t*-test) when cells of both lines were seeded at cell numbers of $\leq 2.5 \times 10^5$ per well (Table 1).

3.8. Impact of cannabidiol on lung metastases

We further investigated the impact of cannabidiol on tumor cell metastasis in vivo in a mouse model. For this purpose, A549 cells were injected intravenously through the lateral tail vein of athymic nude mice. According to Fig. 7, the number of lung metastatic nodules was significantly lower in cannabidiol-treated animals as compared to vehicle-treated controls. The decrease of nodules was from an average of 6 nodules in vehicle-treated nude mice to an average of 1 nodule in cannabidiol-treated mice yielding an 84% inhibition of metastasis.

4. Discussion

An increasing number of data obtained both in vivo and in vitro implicates a possible role for cannabinoids as systemic cancer therapeutics. Although cannabinoids virtually lack side effects of contemporarily used anticancer drugs such as chemotherapeutics-induced emesis and collateral toxicity, cannabinoid's therapeutical use is limited by their psychotropic side effects. Therefore, recent

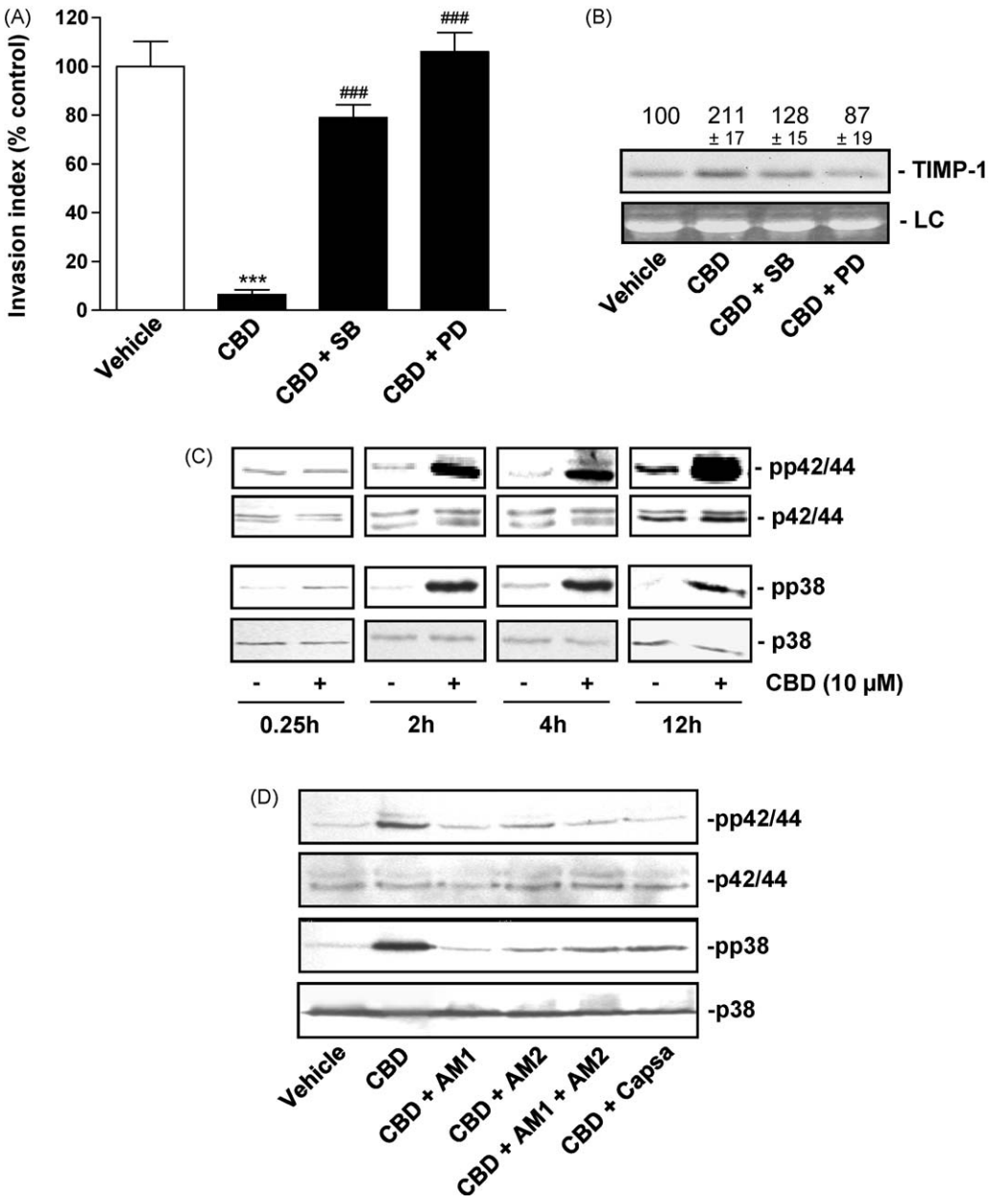

Fig. 4. Role of p38 and p42/44 mitogen-activated protein kinase (MAPK) pathways in the effects of cannabidiol (CBD) on HeLa cell invasion and TIMP-1 expression. (A) Effect of a 1-h pretreatment with 10 μM SB203580 (SB; inhibitor of p38 MAPK activity) and 10 μM PD98059 (PD; inhibitor of p42/44 MAPK activation) on the anti-invasive action of cannabidiol (10 μM) after a 72-h incubation period. (B) Effect of the indicated treatment on TIMP-1 protein levels after a 72-h incubation period. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means ± SEM of $n = 3$–4 experiments (A), $^{***}P < 0.001$, vs. corresponding vehicle control; $^{\#\#\#}P < 0.001$, vs. cannabidiol (Student's *t*-test). Values above the representative blot are means ± SEM obtained from densitometric analysis of $n = 7$ blots (B) and represent percent control in comparison with vehicle-treated cells (100%) in the absence of test substance. Protein staining of supernatants is shown as loading control (LC). (C) Effect of cannabidiol on activation of p38 and p42/44 MAPK after 0.25–12 h. (D) Effect of a 1-h pretreatment with AM-251 (AM1; 1 μM), AM-630 (AM2; 1 μM), and capsazepine (Capsa; 1 μM) on phosphorylation of p38 and p42/44 MAPKs after a 2-h incubation with 10 μM cannabidiol. To analyze MAPK phosphorylations, immunoblots were probed with antibodies directed against the phosphorylated form of p38 or p42/44. Equal loading of lysates was ensured by probing membranes with antibodies against the nonphosphorylated forms of p38 and p42/44 MAPKs.

research focussed on non-psychoactive cannabinoid-mimetic compounds such as cannabidiol.

In the present study we identified a cannabinoid receptor- and TRPV1-triggered expression of TIMP-1 as an important mediator of the anti-invasive action of cannabidiol. Cannabidiol was shown to decrease HeLa cell invasion in a time- and concentration-dependent manner. Following a 72-h incubation period, the decreased invasiveness was significant at a cannabidiol concentration as low as 0.01 μM which elicited a 33% inhibition when compared to vehicle control. Although this effect was less pronounced as compared to an equimolar concentration of THC which yielded a 68% inhibition of invasion in the same experimental setup [23], the anti-invasive effects of cannabidiol described here occurred in a range of therapeutically relevant concentrations. Accordingly, referring to clinical data on healthy volunteers obtained from studies with Sativex™ (1:1 ratio of THC and cannabidiol) applied at a 10-mg buccal dose or at self-titrated doses during chronic therapy, cannabidiol peak plasma concentrations of 0.01 μM or up to 0.05 μM can be achieved in vivo [38]. Moreover, mean cannabidiol plasma levels of 0.036 μM were analyzed following a 6-week oral treatment with cannabidiol at doses of 10 mg/kg/day [39].

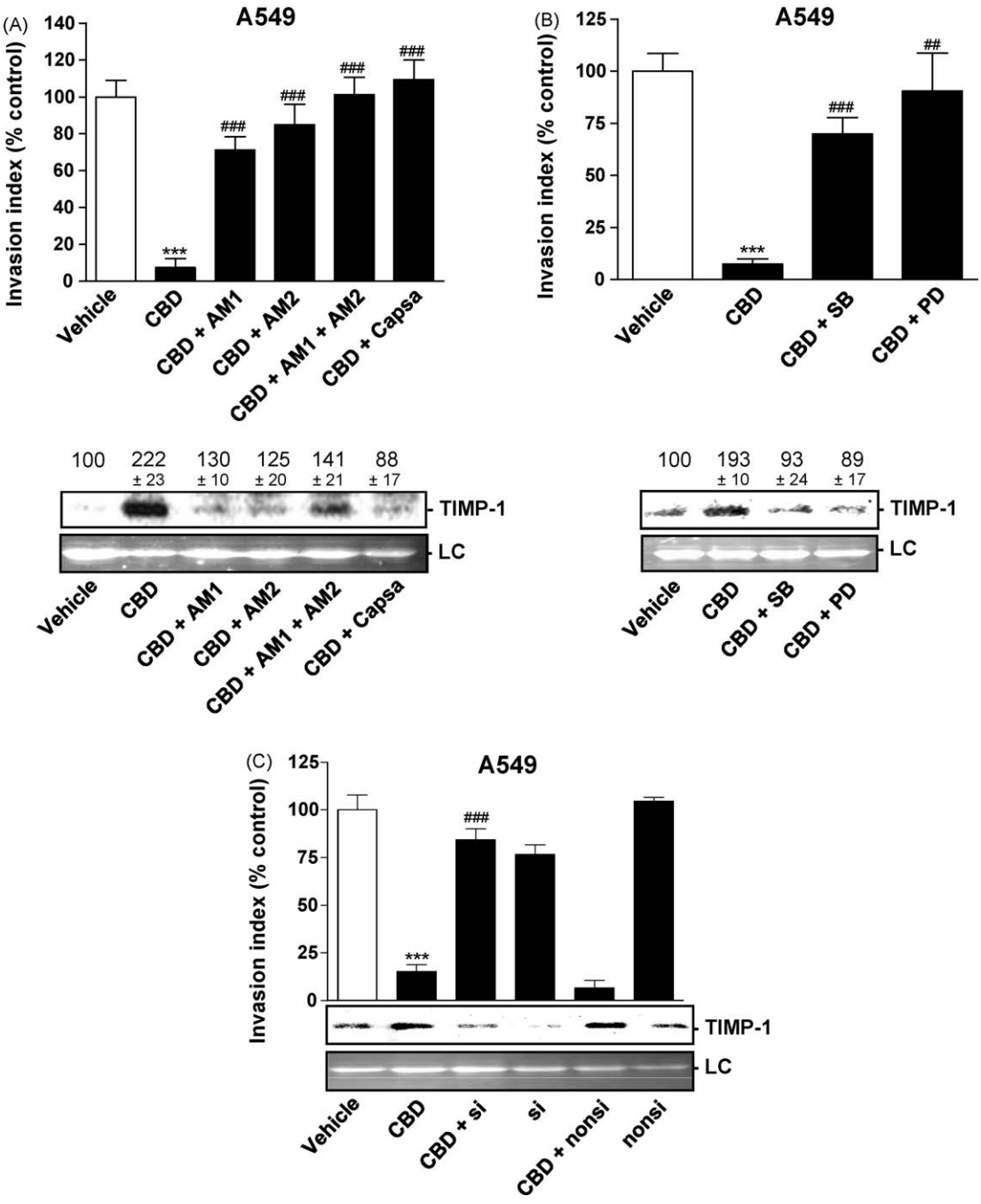

Fig. 5. Involvement of cannabinoid receptors, TRPV1, p38 and p42/44 MAPKs in the TIMP-1-dependent anti-invasive action of cannabidiol (CBD) on A549 cells. (A) Effect of a 1-h pretreatment of cells with AM-251 (AM1; CB_1 antagonist; 1 μM), AM-630 (AM2; CB_2 antagonist; 1 μM) and capsazepine (capsa; TRPV1 antagonist; 1 μM) on the anti-invasive (A, upper panel) and TIMP-1-inducing action of 10 μM cannabidiol (A, lower panel) after a 72-h incubation period. (B) Effect of a 1-h pretreatment with 10 μM SB203580 (SB; inhibitor of p38 MAPK activity) and 10 μM PD98059 (PD; inhibitor of p42/44 MAPK activation) on the anti-invasive action of 10 μM cannabidiol (B, upper panel) and on TIMP-1 protein levels (B, lower panel) after a 72-h incubation period. (C) Effect of TIMP-1 siRNA (0.25 μg/ml) on cannabidiol's action on A549 cell invasion (C, upper panel) and TIMP-1 expression (C, lower panel) after a 72-h incubation period. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance of $n = 3$–4 (A), and $n = 4$ (B and C) experiments. $^{***}P < 0.001$, vs. corresponding vehicle control; $^{###}P < 0.001$; $^{##}P < 0.01$, vs. cannabidiol (Student's *t*-test). Values above the representative TIMP-1 blots are means ± SEM obtained from densitometric analysis of $n = 4$ (A) or $n = 5$ (B) blots and represent percent control in comparison with vehicle-treated cells (100%) in the absence of test substance. Protein staining of supernatants is shown as loading control (LC).

With reference to a number of data reporting an anti-invasive effect of TIMP-1 on cancer cell invasion [23,25–31], the present work focussed on the causal link of cannabinoid receptor- and TRPV1-mediated activation of MAPK pathways and subsequent TIMP-1 induction as upstream effectors of cannabidiol-mediated suppression of cancer cell invasion. Several lines of evidence presented in this study suggest TIMP-1 induction as a major event underlying cannabidiol's anti-invasive action. First, antagonists of CB_1 and CB_2 alone or in combination as well as an antagonist of TRPV1 caused a significant suppression of both cannabidiol-induced TIMP-1 expression and activation of the MAPKs p38 and p42/44, and a significant reconstitution of the subsequent suppression of cancer cell invasion. Second, inhibitors of p38 and p42/44 MAPKs pathway that have previously been demonstrated as upstream regulators of TIMP-1 [32,33] elicited a comparable reconstitution of cancer cell invasiveness and inhibition of TIMP-1 induction by cannabidiol. Third, and

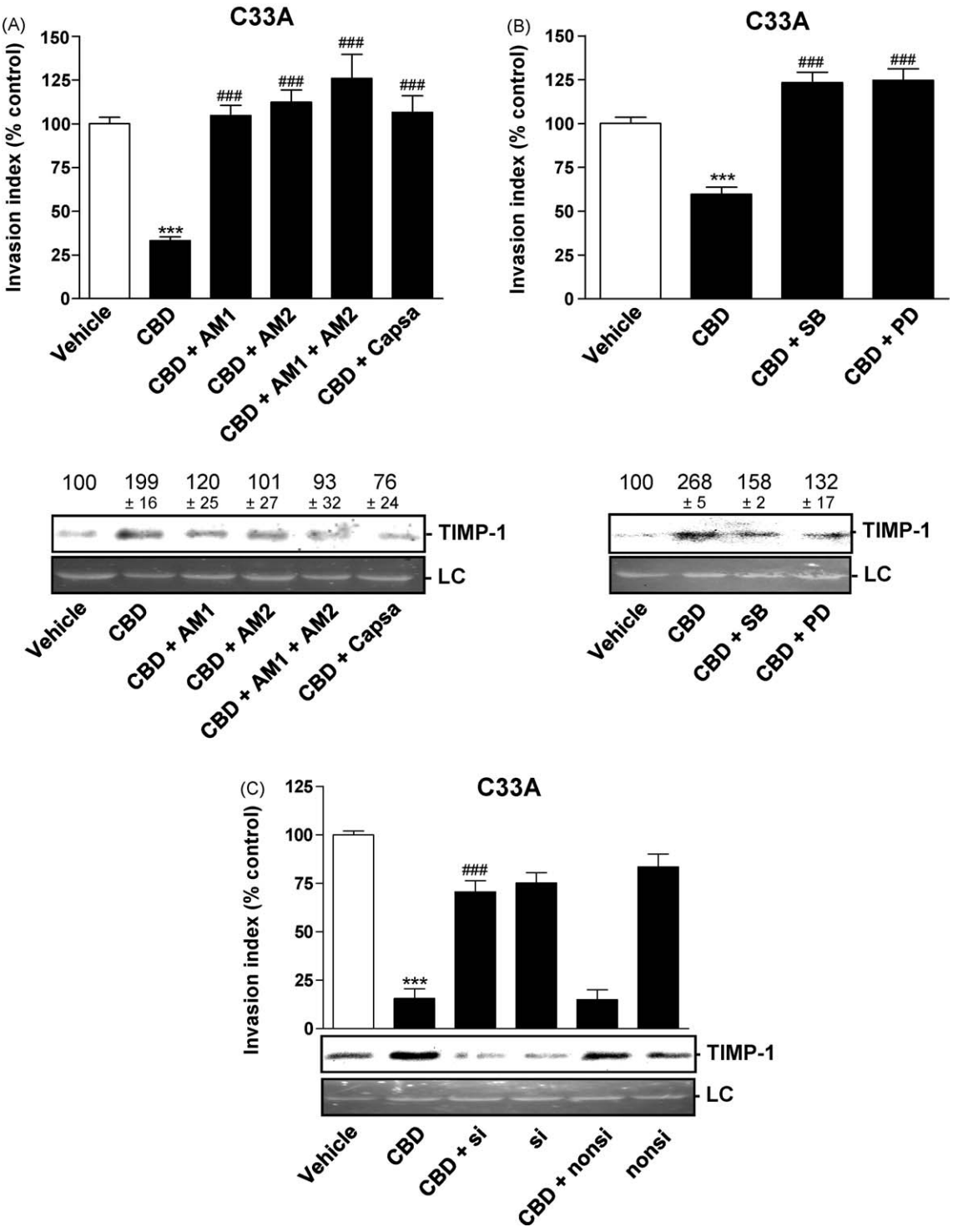

Fig. 6. Involvement of cannabinoid receptors, TRPV1, p38 and p42/44 MAPKs in the TIMP-1-dependent anti-invasive action of cannabidiol (CBD) on C33A cells. (A) Effect of a 1-h pretreatment of cells with AM-251 (AM1; CB_1 antagonist; 1 μM), AM-630 (AM2; CB_2 antagonist; 1 μM) and capsazepine (capsa; TRPV1 antagonist; 1 μM) on the anti-invasive (A, upper panel) and TIMP-1-inducing action of 10 μM cannabidiol (A, lower panel) after a 72-h incubation period. (B) Effect of a 1-h pretreatment with 10 μM SB203580 (SB; inhibitor of p38 MAPK activity) and PD98059 (PD; inhibitor of p42/44 MAPK activation) on the anti-invasive action of 10 μM cannabidiol (B, upper panel) and on TIMP-1 protein levels (B, lower panel) after a 72-h incubation period. (C) Effect of TIMP-1 siRNA (0.25 μg/ml) on cannabidiol's action on C33A cell invasion (C, upper panel) and TIMP-1 expression (C, lower panel) after a 72-h incubation period. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance of $n = 4$ experiments. $^{***}P < 0.001$, vs. corresponding vehicle control; $^{###}P < 0.001$, vs. cannabidiol (Student's t-test). Values above the representative TIMP-1 blots are means ± SEM obtained from densitometric analysis of $n = 5$ (A) or $n = 3$ (B) blots and represent percent control in comparison with vehicle-treated cells (100%) in the absence of test substance. Protein staining of supernatants is shown as loading control (LC).

most convincing, posttranscriptional silencing of cannabidiol-induced TIMP-1 was associated with a removal of its anti-invasive action. A TIMP-1-dependent anti-invasive effect involving activation of cannabinoid receptors, TRPV1 and MAPKs was also proven in another human cervical cancer (C33A) as well as in a human lung cancer cell line (A549), implying that the observed mechanism is of general importance and not restricted to one cell line. Additionally and in line with its anti-invasive action, in

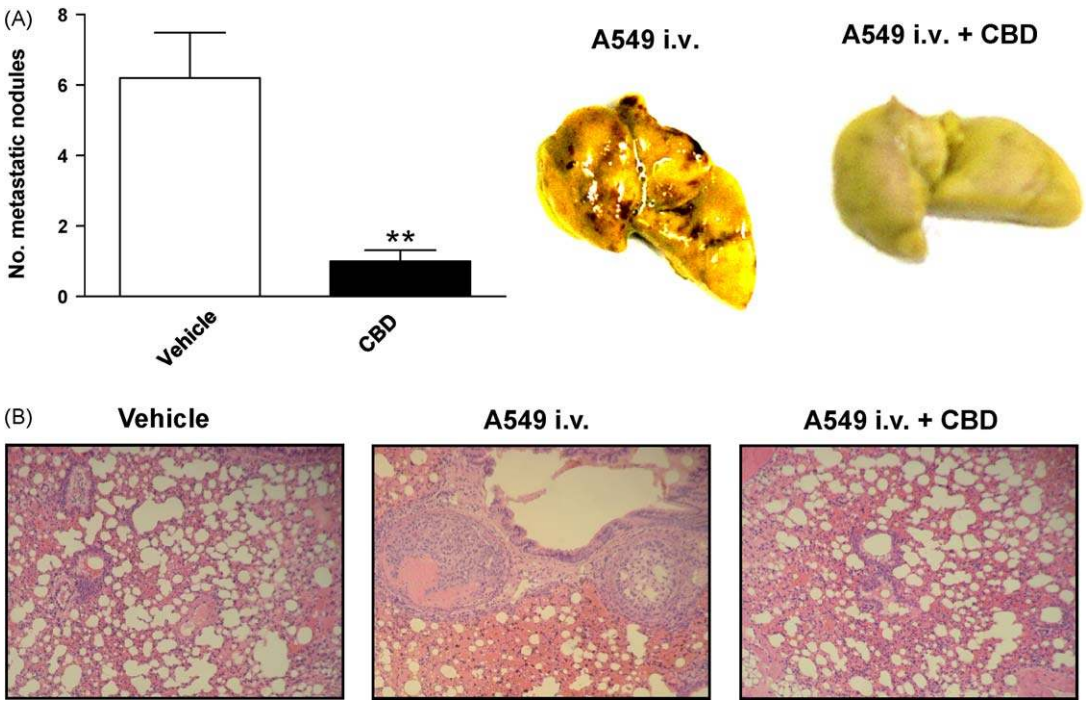

Fig. 7. In vivo action of cannabidiol (CBD) on tumor metastasis. A549 cells were injected intravenously in athymic nude mice. Mice were given cannabidiol (5 mg/kg body weight) all 72 h for 28 days starting 24 h after injection of the cells. At the end of the experiment, the animals were sacrificed and one lung per animal was evaluated for metastatic lesions. (A) Left panel: number of metastatic nodules in vehicle- and cannabidiol-treated mice. Data are means ± SEM obtained from $n = 5$ mice per group. Right panel: illustration of metastatic lesions in murine lungs from different experimental groups as indicated above. (B) Illustration of metastatic lesions in hematoxylin and eosin stained paraffine sections in murine lungs from vehicle- and cannabidiol-treated mice.

vivo studies in thymic-aplastic nude mice revealed a significant inhibition of A549 lung metastasis in cannabidiol-treated animals as compared to vehicle-treated controls. This finding is in agreement with a previous report in which cannabidiol treatment inhibited breast cancer metastasis [17].

An invasion-associated effect that is mainly determined by the ability of cancer cells to secrete matrix-degrading enzymes or to modulate their activity rather than on cellular motility was substantiated by the finding that reduced Matrigel invasion was not associated with a decrease of transmigration through uncoated Boyden chambers after cannabidiol treatment.

To rule out the possibility that decreased invasion by cannabidiol was an unspecific cytotoxicity-related phenomenon, control experiments were also performed to analyse cellular viability which revealed no significant cytotoxicity by cannabidiol under conditions of the experimental setup of invasion monitoring. In line with recent data obtained with THC and R(+)-methanandamide [23], cannabidiol showed a significant and progressive toxicity only when the density of HeLa, A549 and C33A cells was reduced. Such "inoculum effect" has been described for a number of chemotherapeutic drugs such as tamoxifen [40], doxorubicin and vincristine [41]. Kobayashi et al. [41] demonstrated that at high cell densities, a decreased cellular uptake of chemotherapeutics results in attenuated availability of drug molecules at its intracellular binding sites. Finally, the anti-invasive effect of cannabidiol was confirmed to be comparable in another experimental setting, where HeLa cells treated with cannabidiol or vehicle for 48 h were readjusted to equal numbers of cells prior to the 24-h incubation in Matrigel inserts.

There are several issues raised in this study that have to be addressed in future experiments. First, the effect of cannabidiol on TIMP-1 was specific in that the expression of other regulable components of this system, i.e. MMP-2 and MMP-9, were not influenced by cannabidiol on the level of protein expression. This finding stands in contrast to a recent study from our laboratory reporting a cannabinoid receptor-independent down-regulation of MMP-2 by THC and R(+)-methanandamide [23]. Second, in view of the low affinity of cannabidiol to either CB_1 and CB_2 receptors [10], the involvement of cannabinoid receptors in the anti-invasive and TIMP-1-stimulatory action of cannabidiol was rather surprising. However, in line with our data cannabidiol has been reported to exert several of its effects, including modulation of cytokine release and macrophage chemotaxis [11] as well as antiproliferative [12] and proapoptotic properties [13] in a cannabinoid receptor-dependent manner. One possible explanation for the discrepancy between the low receptor affinity and the apparent involvement of cannabinoid receptors in cannabidiol-mediated effects may be given by cannabidiol's inhibitory action on fatty acid amidohydrolase activity that confers release of anandamide and increased receptor-mediated signalling by this endocannabinoid [16–18]. Additionally, the non-selective cation channel TRPV1 seems to be involved in cannabidiol's anti-invasive effect which is in line with recent findings demonstrating antihyperalgesic [19] and antiproliferative effects [17] of cannabidiol to be mediated via TRPV1. Third, more research is needed to understand the complete mode of action underlying the anti-invasive effect of cannabidiol. Thus, besides TIMP-1, other components such as a modulation of the urokinase plasminogen activator system (Ramer and Hinz, unpublished data) may contribute to the anti-invasive action as well.

Collectively, this is the first report on a cannabinoid receptor- and TRPV1-dependent anti-invasive action of cannabidiol that adresses the aspect of modulation of the matrix metalloproteinase system. Induction of TIMP-1 expression and subsequent reduction of invasiveness may play an important role in the antitumorigenic activity of cannabidiol whose therapeutical benefits in cancer treatment should be adressed in future clinical trials.

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