

Inhibition of Cancer Cell Invasion by Cannabinoids via Increased Expression of Tissue Inhibitor of Matrix Metalloproteinases-1

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- Background** Cannabinoids, in addition to having palliative benefits in cancer therapy, have been associated with anti-carcinogenic effects. Although the antiproliferative activities of cannabinoids have been intensively investigated, little is known about their effects on tumor invasion.
- Methods** Matrigel-coated and uncoated Boyden chambers were used to quantify invasiveness and migration, respectively, of human cervical cancer (HeLa) cells that had been treated with cannabinoids (the stable anandamide analog R(+)-methanandamide [MA] and the phytocannabinoid Δ^9 -tetrahydrocannabinol [THC]) in the presence or absence of antagonists of the CB₁ or CB₂ cannabinoid receptors or of transient receptor potential vanilloid 1 (TRPV1) or inhibitors of p38 or p42/44 mitogen-activated protein kinase (MAPK) pathways. Reverse transcriptase-polymerase chain reaction (RT-PCR) and immunoblotting were used to assess the influence of cannabinoids on the expression of matrix metalloproteinases (MMPs) and endogenous tissue inhibitors of MMPs (TIMPs). The role of TIMP-1 in the anti-invasive action of cannabinoids was analyzed by transfecting HeLa, human cervical carcinoma (C33A), or human lung carcinoma cells (A549) cells with siRNA targeting TIMP-1. All statistical tests were two-sided.
- Results** Without modifying migration, MA and THC caused a time- and concentration-dependent suppression of HeLa cell invasion through Matrigel that was accompanied by increased expression of TIMP-1. At the lowest concentrations tested, MA (0.1 μ M) and THC (0.01 μ M) led to a decrease in invasion (normalized to that observed with vehicle-treated cells) of 61.5% (95% CI = 38.7% to 84.3%, $P < .001$) and 68.1% (95% CI = 31.5% to 104.8%, $P = .0039$), respectively. The stimulation of TIMP-1 expression and suppression of cell invasion were reversed by pretreatment of cells with antagonists to CB₁ or CB₂ receptors, with inhibitors of MAPKs, or, in the case of MA, with an antagonist to TRPV1. Knockdown of cannabinoid-induced TIMP-1 expression by siRNA led to a reversal of the cannabinoid-elicited decrease in tumor cell invasiveness in HeLa, A549, and C33A cells.
- Conclusion** Increased expression of TIMP-1 mediates an anti-invasive effect of cannabinoids. Cannabinoids may therefore offer a therapeutic option in the treatment of highly invasive cancers.

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Although cannabinoids are currently used to palliate wasting, emesis, and pain in cancer patients, there is increasing evidence to suggest that these compounds may be useful for the inhibition of tumor cell growth through their modulation of several cell survival pathways [for review see Bifulco et al. (1)]. For example, in animals, cannabinoid administration has been shown to induce regression of lung adenocarcinomas (2), gliomas (3,4), thyroid epitheliomas (5), lymphomas (6), and skin carcinomas (7). Furthermore, several *in vitro* studies have confirmed proapoptotic and antiproliferative effects of cannabinoids on cancer cells by mechanisms that involve *de novo* synthesis of ceramide (3,8) and/or activation of mitogen-activated protein kinases (MAPKs) (3,9). Moreover, antiangiogenic effects such as cannabinoid-attenuated expression of vascular endothelial growth factor have been described (10). The majority of these and other cannabinoid effects are mediated by two G_{i/o} protein-coupled receptors, CB₁ and CB₂. There are also experimental data that suggest a stimulatory effect of the endocannabinoid anan-

damide on transient receptor potential vanilloid 1 (TRPV1), a non-selective cation channel (11,12). However, several cannabinoid effects, including induction of apoptosis and cell death in several cell types (8,13,14), release of arachidonic acid and intracellular calcium (15), stimulation of MAPKs (16,17), and inhibition of interleukin 2 release (18) have been associated with molecular events that are independent of either CB₁/CB₂ or TRPV1 activation.

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CONTEXT AND CAVEATS

Prior knowledge

Treatment with cannabinoids had been shown to reduce the invasiveness of cancer cells, but the cellular mechanisms underlying this effect were unclear.

Study design

Cancer cells treated with combinations of cannabinoids, antagonists of cannabinoid receptors, and siRNA to tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) were assessed for invasiveness, protein expression, and activation of signal transduction pathways.

Contribution

The expression of TIMP-1 was shown to be stimulated by cannabinoid receptor activation and to mediate the anti-invasive effect of cannabinoids.

Implications

Clarification of the mechanism of cannabinoid action may help investigators to explore their therapeutic benefit.

Limitations

The relevance of the findings to the behavior of tumor cells *in vivo* remains to be determined.

Although the mechanisms underlying the proapoptotic and antiproliferative actions of cannabinoids have been studied extensively, there are only a few reports of anti-invasive properties of these compounds (7,19,20), and the mechanism that leads to decreased invasiveness of cancer cells exposed to cannabinoids has not been clarified. Cancer cell invasion is one of the crucial events in local spreading, growth, and metastasis of tumors. Matrix metalloproteinases (MMPs) have emerged as a group of enzymes that exert an important function during tumor invasion, that is, degradation of extracellular matrix components such as collagens and proteoglycans (21,22). Tissue inhibitors of MMPs (TIMPs) have been shown to inhibit the proteolytic activity of tumor tissues by binding noncovalently with 1:1 stoichiometry to the active forms of these enzymes and thereby inhibiting proteolytic activity. Among the four distinct members of the TIMP family, the 28.5-kDa glycoprotein TIMP-1 has been demonstrated to be a potent MMP inhibitor that suppresses vascular tumor growth and angiogenesis in xenographic animal models (23). Furthermore, several studies have demonstrated a correlation between high cancer invasiveness and decreased TIMP-1 expression (24,25). Consistent with this finding, the anti-invasive action of several anticarcinogenic substances has been associated with elevated TIMP-1 levels (26–30).

To better understand the mechanism by which cannabinoids exert inhibitory effects on cancer progression, we studied the effect of the hydrolysis-stable endocannabinoid analog R(+)-methanandamide (MA) and the plant-derived cannabinoid Δ^9 -tetrahydrocannabinol (THC) on the expression of TIMP-1 and cancer cell invasiveness. In view of recent studies demonstrating p38 and p42/44 MAPK activation as intracellular signaling events that lead to induction of TIMP-1 (31,32) and findings showing cannabinoid receptor-dependent activation of MAPKs (3,9,33), we also assessed a possible role of both MAPKs in cannabinoid-modulated invasion and TIMP-1 expression.

Materials and Methods

Materials

MA was purchased from Calbiochem (Bad Soden, Germany), AM-251, AM-630, capsazepine, PD98059, and SB203580 from Alexis Deutschland GmbH (Grünberg, Germany), and THC from Sigma (Steinheim, 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.

Cell Culture

The highly invasive cervical cancer cell line HeLa (12,34) as well as additional human cervical (C33A) and lung carcinoma (A549) cell lines were used to study the anti-invasive action of cannabinoids. HeLa, C33A, and A549 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 MA and THC) or 0.1% (v/v) dimethyl sulfoxide (DMSO) (for AM-251, AM-630, capsazepine, PD98059, and SB203580).

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 μ m pore size) were coated with 28.4 μ g Matrigel per insert in 24-well plates. Trypsinized and pelleted cells were suspended to a final concentration of 5×10^5 cells in 500 μ L serum-free DMEM in each insert and treated with MA and THC or ethanol vehicle for various times. To address the role of cannabinoid receptors, TRPV1, and MAPKs p38 and p42/44, specific antagonists (AM-251, AM-630, capsazepine, PD98059, SB203580) were tested vs DMSO vehicles. DMEM containing 10% FCS was used as a chemoattractant in the companion plate. Following incubation in a humidified incubator at 37°C and 5% CO₂ for the indicated times, the noninvading 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, HeLa cells that had invaded through Matrigel-coated membranes were fixed and stained with Diff-Quick (Medion Diagnostics GmbH, Dürdingen, Switzerland) and visualized using a microscope at ×200 magnification.

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

Quantitative Reverse Transcriptase–Polymerase Chain Reaction Analysis

HeLa cells were seeded into 48-well plates at a density of 5×10^5 cells per well. Following incubation of cells with the respective test compounds or their vehicles for the indicated times, supernatants were removed, and cells were lysed as previously described (17) for subsequent RNA isolation using the RNeasy total RNA Kit (Qiagen GmbH, Hilden, Germany). β -actin (internal standard) and TIMP-1 mRNA levels were determined by quantitative real-time RT-PCR as described (35). Primers and probe for human TIMP-1 was an Assay-on-demand product (Applied Biosystems, Darmstadt, Germany).

Western Blot Analysis

For determination of TIMP-1, MMP-2, TIMP-2, and MMP-9 protein levels, cells grown to confluence in 6-well plates were incubated with test substances or vehicles for the indicated times. Afterward, cell culture supernatants were centrifuged and concentrated using Microcon YM-10 centrifugal filter units (Millipore GmbH, Schwalbach, Germany) with a 10-kDa cutoff as described (35). In some instances, TIMP-1 was determined in supernatants collected from the upper Boyden chambers. Total protein was measured using the bicinchoninic acid assay (Pierce, Rockford, IL).

For Western blot analysis of p38, phospho-p38, p42/44, and phospho-p42/44, cells that had been grown to confluence in 6-well plates were incubated with test substance or 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 phenylmethylsulfonyl fluoride, 1 μ g/mL leupeptin, and 10 μ g/mL aprotinin), homogenized by sonication, and centrifuged at $10000 \times g$ for 5 minutes. Supernatants were used for Western blot analysis.

All proteins were separated on a 12% sodium dodecyl sulfate-polyacrylamide 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, TIMP-2, MMP-9 (diluted with 1% milk powder to 1:1000 for TIMP-1, MMP-2, TIMP-2 and 1:500 for MMP-9; all antibodies from Oncogene Research Products, San Diego, CA) or p38, phospho-p38, p42/44, and phospho-p42/44 (diluted with 5% milk powder to 1:500 for p38 and phospho-p38 and 1:1000 for p42/44 and phospho-p42/44; all antibodies from New England BioLabs

GmbH, Frankfurt, Germany). Membranes were probed with horseradish peroxidase-conjugated Fab-specific anti-mouse IgG for detection of TIMPs and MMPs (diluted with 1% milk powder to 1:1000) or anti-rabbit IgG for analysis of MAPKs (diluted with 5% milk powder to 1:1000; both antibodies from New England BioLabs GmbH). Antibody binding was visualized by enhanced chemiluminescence western blotting detection reagents (Amersham Biosciences, Freiburg, Germany).

To ensure that equal amounts of protein in cell culture supernatants used for protein analysis of TIMPs 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). As a corresponding standard, a band with a size of about 65 kDa that appeared unregulated was chosen as a loading control for protein analysis of supernatants. Nonphosphorylated MAPK bands were chosen as loading control for MAPK activation. Vehicle controls were defined as 100% for evaluation of changes in protein expression. Densitometric analysis of all protein band intensities (normalized to respective loading controls) was performed using an optical scanner and the Multi-Analyst program, version 1.1 (Bio-Rad Laboratories, Hercules, CA).

For determination of cellular levels of cannabinoid receptor and TRPV1, membrane fractions of proteins were obtained as described previously (17). The blots were probed with antibodies raised to the CB₁ receptor (Becton Dickinson GmbH, Heidelberg, Germany), CB₂ receptor (Calbiochem), or TRPV1 (Chemicon International, Temecula, CA) (all diluted with 1% milk powder to 1:1000). Subsequently, membranes were probed with anti-rabbit IgG (diluted 1:1000 with 1% milk powder).

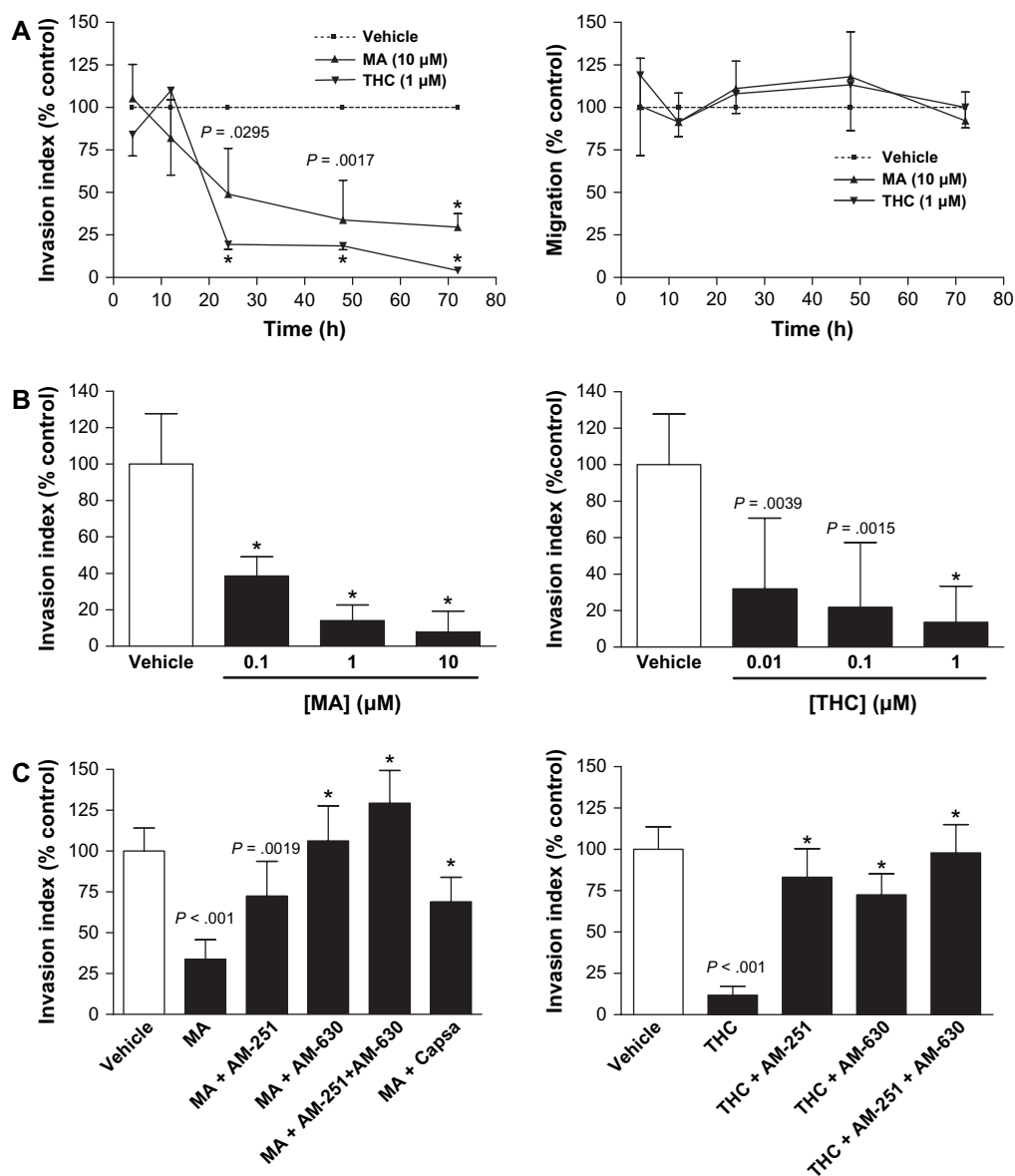
SiRNA Transfections

HeLa, C33A, and A549 cells were transfected with siRNA targeting the indicated sequences using RNAiFect as the transfection reagent (Qiagen GmbH, Hilden, Germany) or negative control RNA (Eurogentec, Seraing, Belgium; Cat. No. OR-0030-neg). The target sequences of siRNAs (Qiagen GmbH) were as follows: 5'-tcccattttttccggacaa-3' for TIMP-1, 5'-accattacacctacacaa-3' for MMP-2, and 5'-aaccttgaggcgacctcaa-3' for MMP-9. A BLAST search revealed that the sequences 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 or 1 μ g/mL siRNA or nonsilencing siRNA as negative control with an equal ratio (w/v) of RNA to transfection reagent for 24 hours in DMEM supplemented with 10% FCS. Subsequently, cells were treated with trypsin for 3 minutes at 37°C in a humidified incubator, centrifuged at $200 \times g$, resuspended to a final density of 5×10^5 cells in 500 μ L of serum-free DMEM containing the same amounts of siRNA or nonsilencing siRNA to provide constant transfection conditions, and seeded for invasion analysis as described above.

Statistical Analyses

Differences in invasion, migration, mRNA levels, protein levels, and viability between groups were analyzed with a two-sided unpaired Student's *t* test by use of GraphPad Prism 3.00 (GraphPad

Fig. 1. Influence of cannabinoids on HeLa cell invasion. Cell invasion indices were analyzed as previously described (30). Briefly, cells that were able to pass through the Matrigel-coated (invasion) or uncoated (migration) membranes toward the chemoattractant (10% FCS) in the companion plate were quantified using the WST-1 test after removal of cells at the upper part of the transwell chambers. The invasion index was 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%). Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. **A)** Time dependence of invasion (**left**) and migration (**right**) after treatment of cells with R(+)-methanandamide (MA) and Δ^9 -tetrahydrocannabinol (THC). Cells were incubated with cannabinoids (10 μ M MA or 1 μ M THC) or vehicle for the indicated times. *P* values for difference in invasion (cannabinoid-treated cells vs vehicle-treated control) were .554, .418, .030, .002, and **P* < .001 for MA after 4, 12, 24, 48, 72 hours, respectively, and .060 (after 4 hours), .704 (after 12 hours), and **P* < .001 (after 24, 48, 72 hours) for THC (Student's *t* test). *P* values for migration were .931, .174, .172, .234, .314 for MA and .253, .116, .255, .368, 1.00 for THC after 4, 12, 24, 48, 72 hours, respectively, vs corresponding vehicle control. **B)** Concentration-dependence of the anti-invasive action of MA (0.1, 1, or 10 μ M; **left graph**) and THC (0.01, 0.1, or 1 μ M; **right graph**) after a 72-hour incubation. *P* = .0039 for 0.01 μ M THC and *P* = .0015 for 0.1 μ M THC, **P* < .001, vs corresponding vehicle control (Student's *t* test). **C)** Effect of a 1-hour pretreatment with the CB₁ antagonist AM-251 (1 μ M), the CB₂ antagonist AM-630 (1 μ M), or the TRPV1 antagonist capsazepine (Capsa; 1 μ M) on invasiveness of cells that were treated with MA (10 μ M) or THC (1 μ M) for 72 hours. *P* < .001 for MA- or THC-treated vs vehicle controls, *P* = .0019 for



Software, San Diego, CA). Results were considered to be statistically significant at *P* < .05.

Results

Time Course and Concentration Dependence of the Inhibitory Effect of Cannabinoids on HeLa Cell Invasion

HeLa cells incubated with the cannabinoids MA (10 μ M) or THC (1 μ M) showed diminished invasion through a reconstituted basement membrane (Matrigel) after 24 hours, and invasiveness was diminished further after 72 hours incubation (Fig. 1, A). Moreover, MA and THC treatment led to statistically significant and concentration-dependent decreases of invasion through Matrigel even at concentrations as low as 0.1 μ M for MA

MA + AM-251 vs MA, **P* < .001 for cannabinoid and AM-251- or AM-630-treated vs cannabinoid alone (Student's *t* test). Values are means with the upper or lower 95% confidence interval (**error bar**) from *n* = 4 (A, B), *n* = 16 (C, left), or *n* = 12 (C, right) independent experiments.

(decrease in invasion index relative to that of vehicle-treated cells = 61.5%, 95% CI = 38.7% to 84.3%, *P* < .001) and 0.01 μ M for THC (decrease = 68.1%, 95% CI = 31.5% to 104.8%, *P* = .0039 [Fig. 1, B]).

Reduced invasion was not associated with decreased migration through membranes that were not coated with Matrigel (Fig. 1, A). To rule out the possibility that decreased Matrigel invasion by cells that were treated with cannabinoids was an unspecific cytotoxicity-related phenomenon, cellular viability was measured following exposure to 10 μ M MA or 1 μ M THC under experimental conditions of the invasion assays (5×10^5 cells per well of a 48-well plate in 500 μ L serum-free DMEM, 72-hour incubation). Incubation with MA or THC at these concentrations had no statistically significant effect on viability (percentage of viable

MA-treated to viable vehicle-treated cells = 104.4%, 95% CI = 94.4% to 114.5%, $P = .553$, and percentage of viable THC-treated to viable vehicle-treated cells = 105.9, 95% CI = 102.4% to 109.4%, $P = .391$).

However, toxic effects of both cannabinoids were observed when lower cell densities were used in viability assays. The percentage of viable cells after treatment with 10 μM MA to viable cells after treatment with vehicle for 72 hours was 81.1% (95% CI = 46.1% to 116.1%, $P = .117$) when 2.5×10^5 cells were seeded per well, 76.1% (95% CI = 60.2% to 92.0%, $P = .012$) for 1×10^5 cells per well, 69.4% (95% CI = 63.0% to 75.8%, $P = .002$) for 0.5×10^5 cells per well, and 17.4% (95% CI = 11.0% to 23.8%, $P < .001$) for 0.1×10^5 cells per well. Similarly, decreasing cell density was associated with increased cell death by 1 μM THC with viabilities relative to controls (100%) of 106.4% (95% CI = 93.7% to 119.1%, $P = .202$) for 2.5×10^5 cells per well, 68.2% (95% CI = 55.5% to 80.9%, $P < .001$) for 1×10^5 cells per well, 73.1% (95% CI = 57.2% to 89.0%, $P = .002$) for 0.5×10^5 cells per well, and 32.0% (95% CI = 19.3% to 44.7%, $P < .001$) for 0.1×10^5 cells per well (all groups vs vehicle [100%] at $n = 4$; Student's t test).

Involvement of Cannabinoid Receptors and TRPV1 in Anti-invasive Action of Cannabinoids

To investigate whether cannabinoid receptors and TRPV1 are involved in cannabinoid-mediated reduction of HeLa cell invasiveness, the effect of antagonists of the CB₁ receptor (AM-251), the CB₂ receptor (AM-630), and TRPV1 (capsazepine) on cannabinoid action was tested. These inhibitors were all used at a concentration of 1 μM , which is within the range of concentrations of these substances that have been reported to inhibit responses of cells to activation of the cognate receptors (36,37). MA-induced inhibition of cancer cell invasion was completely prevented by 1-hour incubation with the CB₂ antagonist (invasion indices [relative to control] of MA- and MA + AM-630-treated cells = 33.9% and 106.2%, respectively; difference = 72.3%, 95% CI = 48.8% to 95.9%, $P < .001$ [Fig. 1, C]). Preincubation of cells for 1 hour with antagonists to both CB₁ and CB₂ further increased the invasion index of cells treated with MA (33.9% vs 129.3%; difference = 95.4%, 95% CI = 73.2% to 117.7%, $P < .001$). Preincubation of cells with the CB₁ antagonist AM-251 led to a partial reconstitution of invasion (invasion index for MA and MA + AM-251 = 33.9% and 72.5%, respectively; difference = 38.6%, 95% CI = 15.5% to 61.8%, $P = .0019$). Preincubation of cells for 1 hour with the TRPV1 antagonist capsazepine also restored invasiveness in the presence of MA (invasion indices for MA and MA + capsazepine = 33.9% and 68.9%, respectively; difference = 35.0%, 95% CI = 16.7% to 53.3%, $P < .001$ [Fig. 1, C]), suggesting that TRPV1 activity contributes to the anti-invasive action of MA.

THC-induced inhibition of cancer cell invasion was also prevented by 1-hour incubation with the CB₁ antagonist (invasion indices [relative to control] of THC- and THC + AM-251-treated cells = 11.8% and 83.2%, respectively; difference = 71.4%, 95% CI = 54.2% to 88.6%, $P < .001$ [Fig. 1, C]). Preincubation of cells with the CB₂ antagonist AM-630 led to a reconstitution of invasion (invasion indices [relative to control] for THC and THC +

AM-630 = 11.8% and 72.6%, respectively; difference = 60.8%, 95% CI = 47.5% to 74.1%, $P < .001$). As noted for cells treated with MA preincubation of THC-treated cells for 1 hour with antagonists to both CB₁ and CB₂ further increased the invasion index of cells treated with THC (invasion indices [relative to control] of THC- and THC + AM-251 + AM-630-treated cells = 11.8% vs 97.9%; difference = 86.1%, 95% CI = 69.0% to 103.3%, $P < .001$ [Fig. 1, C]).

Effect of Cannabinoids on the Expression of TIMP-1

To investigate a causal link between modulation of invasiveness and cannabinoid-induced release of proteolytic enzymes into the cell culture microenvironment, supernatants and lysates of HeLa cells were analyzed for changes in MMP and TIMP expression after stimulation with cannabinoids (Fig. 2, B). Treatment of cells with 10 μM MA or 1 μM THC led to induction of TIMP-1 mRNA and protein expression after a 12-hour incubation period (mean mRNA expression in cells treated with 10 μM MA or 1 μM THC as a percentage of that of vehicle-treated cells = 181%, 95% CI = 163% to 199%, $P = .0033$ and 119%, 95% CI = 109% to 129%, $P = .0499$, respectively; mean TIMP-1 protein expression in cells treated with 10 μM MA or 1 μM THC as a percentage of that of vehicle-treated cells = 119%, 95% CI = 89% to 149%, or 140%, 95% CI = 69% to 211%, respectively [Fig. 2, A]).

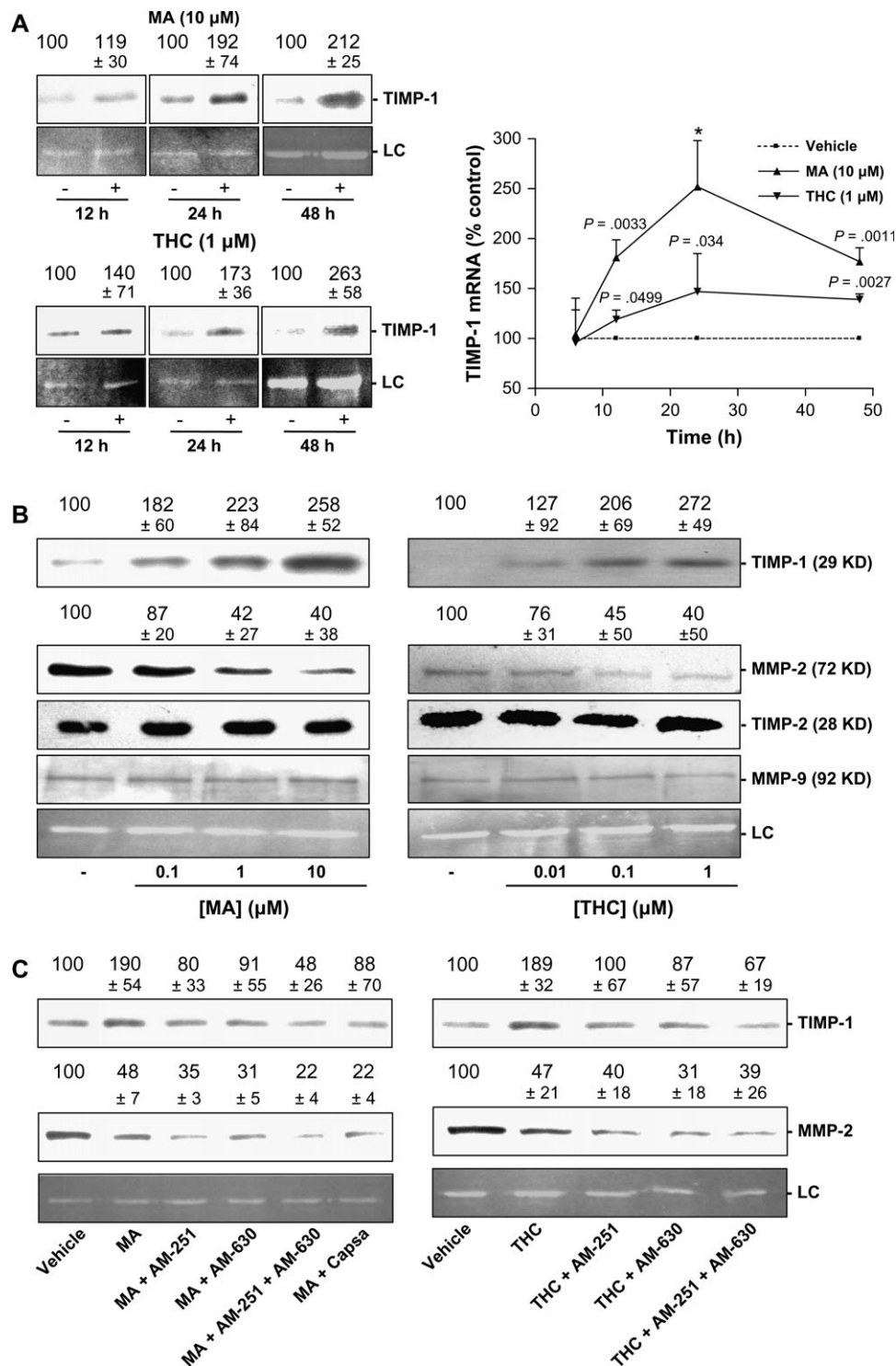
Increased TIMP-1 levels in cannabinoid-treated HeLa cells were detected at concentrations as low as 1 μM for MA (mean expression relative to vehicle = 223%, 95% CI = 139% to 307%) and 0.1 μM for THC (mean expression relative to vehicle = 206%, 95% CI = 137% to 275% [Fig. 2, B]).

We observed no alteration of TIMP-2 and MMP-9 levels in cells treated with MA or THC (Fig. 2, B). By contrast, concentrations of MMP-2 were decreased upon treatment of cells with increasing concentrations of MA or THC (mean MMP-2 expression of cells treated with 1 μM MA or 0.1 μM THC as a percent of expression in vehicle-treated cells = 42%, 95% CI = 15% to 69% or 45%, 95% CI = -5% to 95% [Fig. 2, B]).

Effect of Cannabinoid Receptor and TRPV1 Antagonists on Cannabinoid-Elicited TIMP-1 Induction

Cells were preincubated with cannabinoid receptor and TRPV1 antagonists to determine the receptors of MA and THC that mediated changes in TIMP-1 and MMP-2 expression. The TIMP-1 expression that was observed upon induction with MA was substantially reduced by treatment of cells with 1 μM AM-251, an antagonist of the CB₁ receptor (decrease = 110%, 95% CI = 57% to 162%, $P = .0013$), or 1 μM AM-630, an antagonist of the CB₂ receptor (decrease = 99%, 95% CI = 35% to 163%, $P = .0073$), or treatment with both antagonists at a 1 μM concentration (decrease = 142%, 95% CI = 92% to 192%, $P < .001$ [Fig. 2, C]). At the same concentrations, these antagonists exerted similar effects on THC-stimulated TIMP-1 expression. Furthermore, MA-induced TIMP-1 expression was prevented by treatment with 1 μM capsazepine (decrease = 103%, 95% CI = 29% to 176%, $P = .0123$ [Fig. 2, C]). By contrast, the decrease in MMP-2 levels in response to MA or THC treatment was not antagonized by cannabinoid receptor antagonists or capsazepine (Fig. 2, C).

Fig. 2. Time- and concentration-dependent action of cannabinoids on the expression of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) and other matrix metalloproteinases (MMPs). **A)** Time course of TIMP-1 mRNA (right graph) and protein expression (left panel) after treatment of cells with cannabinoids. Cells treated with cannabinoids (10 μ M R(+)-methanandamide [MA] or 1 μ M Δ^9 -tetrahydrocannabinol [THC] or vehicle for the indicated times were solubilized, and total RNA was used to determine TIMP-1 mRNA levels by quantitative real-time reverse transcriptase-polymerase chain reaction. Proteins separated from the cell culture supernatant were transferred to nitrocellulose membranes and probed with an antibody to TIMP-1. Bound secondary antibodies were visualized by chemiluminescence. Densitometric analyses were obtained from $n = 3$ blots. For mRNA values, means and upper 95% confidence intervals of $n = 4$ independent experiments are shown. P values were .003 for MA and .0499 for THC vs corresponding vehicle after 12 hours, $*P < .001$ for MA and .034 for THC vs corresponding vehicle after 24 hours, .001 for MA and .003 for THC vs corresponding vehicle after 48 hours (Student's t test). **B)** Concentration-dependent effect of MA and THC on expression of the TIMP-1, MMP-2, TIMP-2 and MMP-9 proteins. Cells were treated with the indicated concentrations of MA or THC for 48 hours. **C)** Effect of a 1-hour pretreatment with AM-251 (1 μ M), AM-630 (1 μ M), or capsazepine (Capsa; 1 μ M) on TIMP-1 and MMP-2 levels of cells incubated with 10 μ M MA or 1 μ M THC for 48 hours. Densitometric analyses were obtained from $n = 4-5$ (B) or $n = 4-6$ blots (C). A band corresponding to a protein of about 65 kDa whose expression was not affected by treatment with cannabinoids or receptor antagonists was used as the loading control (LC) and internal standard for densitometric analyses.

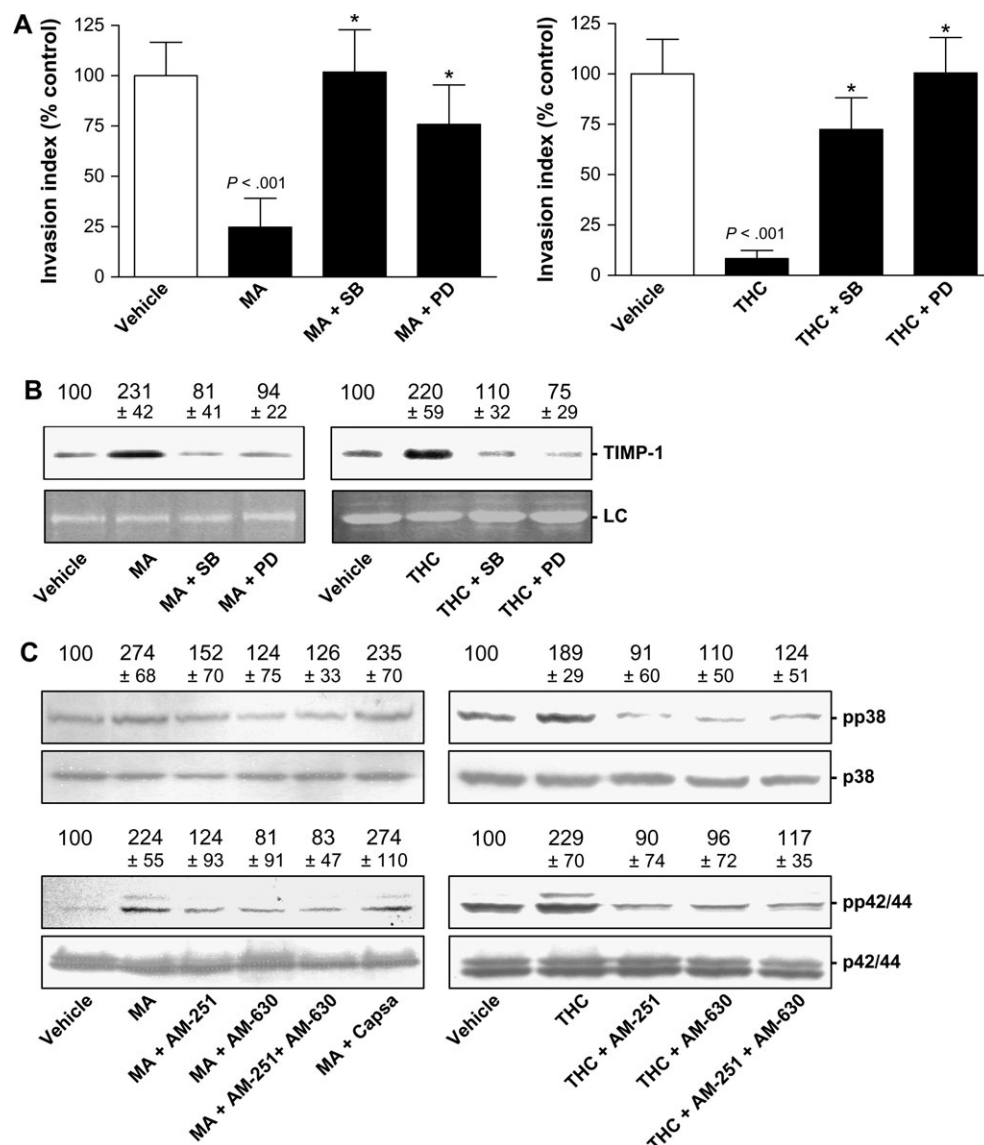


Involvement of p38 and p42/44 MAPK Pathways in Cannabinoid Effects on HeLa Cell Invasion and TIMP-1 Induction

TIMP-1 has been described as a target of p38 and p42/44 MAPKs (31,32). To investigate the possible role of p38 and p42/44 MAPKs in cannabinoid-mediated suppression of invasion, HeLa cells were pretreated with 10 μ M SB203580 or PD98059, which are inhibitors of p38 and p42/44 MAPK activity, respectively. Treatment of

HeLa cells with these inhibitors prevented the effects of MA and THC on invasiveness to the extent that levels of invasiveness were indistinguishable from those of cells treated with vehicle alone (Fig. 3, A). Consistent with these data, SB203580 and PD98059 decreased TIMP-1 protein expression in cells treated with MA to levels observed when cells were treated with vehicle alone [Fig. 3, B]). Similar effects of these inhibitors on TIMP-1 expression were observed in cells that had been treated with THC.

Fig. 3. Role of p38 and p42/44 mitogen-activated protein kinase (MAPK) pathways in the effects of cannabinoids on HeLa cell invasion and tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) expression. **A)** Effect of a 1-hour pretreatment with 10 μ M SB203580 (SB; inhibitor of p38 MAPK activity) and 10 μ M PD98059 (PD; inhibitor of p42/44 MAPK activation) on the anti-invasive action of R(+)-methanandamide (MA; 10 μ M) and Δ^9 -tetrahydrocannabinol (THC; 1 μ M) after a 72-hour incubation period. Values are means + the upper 95% confidence interval (error bar) of $n = 8$ independent experiments. $P < .001$ vs vehicle control, $*P < .001$ vs MA or THC (Student's t test). **B)** Effect of SB203580 and PD98059 on TIMP-1 protein levels in cell culture supernatants after a 48-hour incubation with both MA and THC. Densitometric analyses were performed on 4–5 experiments standardized to loading control (LC), a band corresponding to a protein of about 65 kDa whose expression was not affected by treatment with cannabinoids or MAPK inhibitors. **C)** Effect of a 1-hour pretreatment with AM-251 (1 μ M), AM-630 (1 μ M), and capsazepine (Capsa; 1 μ M) on phosphorylation of p38 and p42/44 MAPKs after a 48-hour incubation with 10 μ M MA or 1 μ M THC. To analyze MAPK phosphorylations, immunoblots were probed with antibodies directed against the phosphorylated form of p38 or p42/44. Equal loading of lysates is ensured by probing membranes with antibodies against the nonphosphorylated forms of p38 and p42/44 MAPKs. Densitometric values were obtained from $n = 3$ experiments and were standardized to the nonphosphorylated forms of p38 or p42/44.



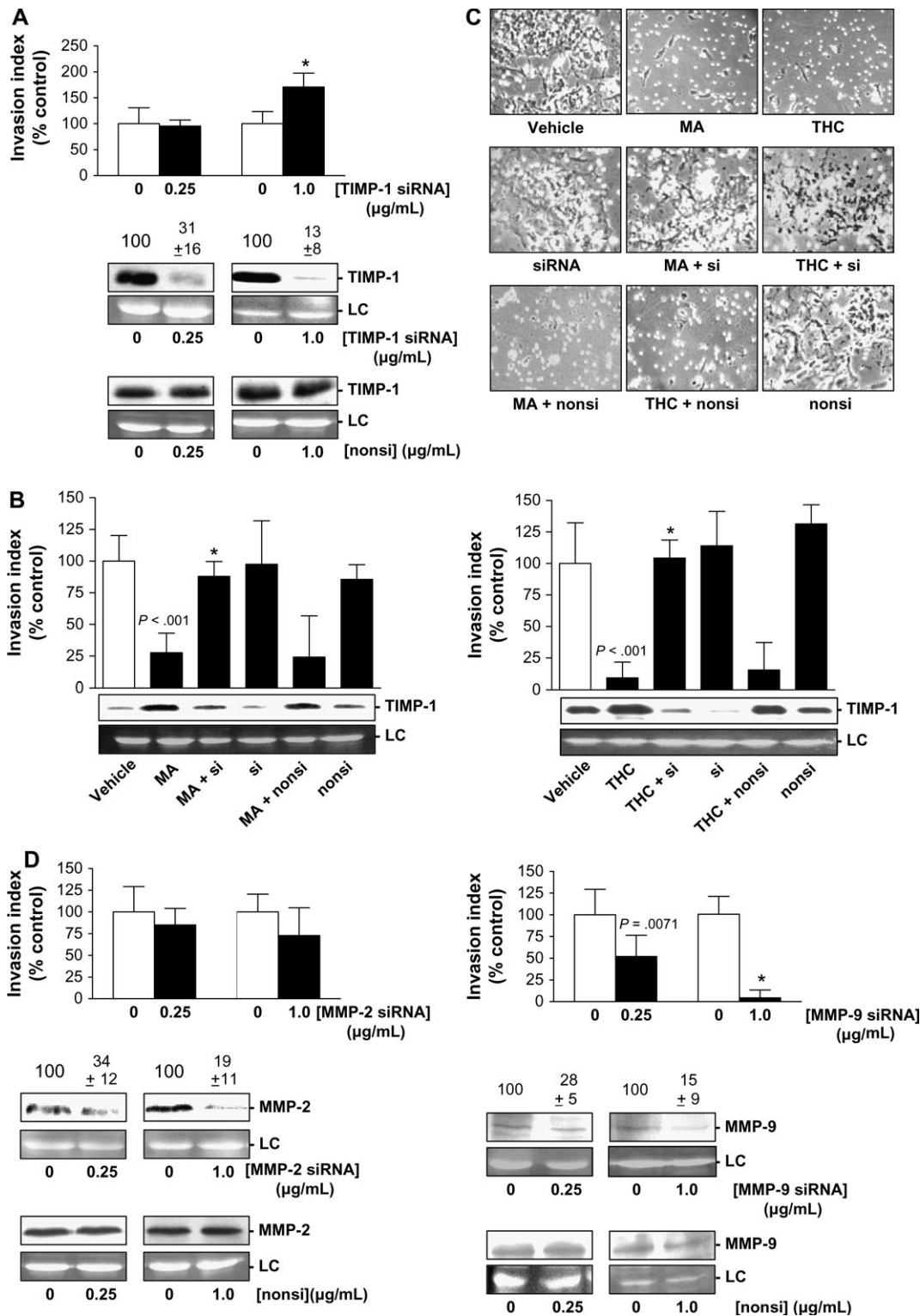
We assessed the relationship between receptor and MAPK activation as detected with antibodies to the phosphorylated (active) forms of the kinases as previously described (17). MA-induced activation of p38 (mean increase in phosphorylated protein = 274%, 95% CI = 206% to 342%) was decreased by CB₁ and CB₂ antagonists AM-251 and AM-630 (decrease = 122%, 95% CI = 59% to 185%, $P = .006$, and 150%, 95% CI = 85% to 215%, $P = .0031$, respectively), whereas blockade of TRPV1 with 1 μ M capsazepine left phosphorylation of p38 virtually unaltered (Fig. 3, C). Similar effects of AM-251 and AM-630 were observed on p42/44 phosphorylation (activation) when cells were treated with MA (decrease = 100%, 95% CI = 30% to 170%, $P = .0167$, and 143%, 95% CI = 74% to 211%, $P = .0044$, respectively). Simultaneous treatment with both cannabinoid receptor antagonists did not further decrease MAPK activation compared with treatment with a single antagonist. Capsazepine treatment caused a small decrease in p44/42 phosphorylation that was not statistically significant. Similar effects of the receptor antagonists on MAPK activation were observed in cells treated with THC (Fig. 3, B).

Effect of TIMP-1 Knockdown on the Anti-invasive Action of Cannabinoids

To confirm a causal link between cannabinoid-mediated TIMP-1 induction and decreased invasion, the expression of TIMP-1 was blocked by transfecting cells with TIMP-1 siRNA. siRNA to TIMP-1 at concentrations of 0.25 and 1.0 μ g/mL decreased TIMP-1 expression in vehicle-treated cells by 69% and 87%, respectively (Fig. 4, A). Because the higher siRNA concentration interfered with the basal level of invasion (Fig. 4, A, upper panel), the lower concentration was used.

Knockdown of TIMP-1 expression with 0.25 μ g/mL TIMP-1 siRNA led to a statistically significant abrogation of the MA- and THC-mediated decrease of invasion, restoring the invasion index to that observed in vehicle-treated cells (Fig. 4, B). A nonsilencing siRNA had no effect on the invasiveness of vehicle-, MA-, or THC-treated cells. Monitoring of TIMP-1 secretion into the culture medium of the upper Boyden chamber confirmed an inhibition of cannabinoid-induced TIMP-1 expression to control levels in samples transfected with TIMP-1 siRNA (Fig. 4, B). The effect

Fig. 4. Involvement of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) in cannabinoid-modulated invasiveness and role of MMP-2 and MMP-9 in basal HeLa cell invasion. **A**) Effect of TIMP-1 siRNA (0.25 and 1 $\mu\text{g}/\text{mL}$) on basal HeLa cell invasion. HeLa cells were transfected with TIMP-1 siRNA (si) or nonsilencing siRNA (nonsi) for 24 hours. Subsequently, cells were placed into invasion chambers, retransfected with the indicated siRNA or suspension buffer for an additional 72 hours before determination of the invasion index and preparation of cell culture supernatants from the upper Boyden chambers for Western blot analyses of TIMP-1. Invasion index values are means + the upper 95% confidence interval (CI) (error bar) of $n = 4$ independent experiments. $P = .671$ for vehicle vs 0.25 $\mu\text{g}/\text{mL}$ TIMP-1 siRNA and $*P < .001$ vs vehicle (Student's t test). **B**) Effect of TIMP-1 siRNA (0.25 $\mu\text{g}/\text{mL}$) and cannabinoids on cell invasion. In addition to transfection with 0.25 $\mu\text{g}/\text{mL}$ TIMP-1 siRNA (A), HeLa cells were subsequently coincubated with vehicle, 10 μM R(+)-methanandamide (MA) or 1 μM Δ^9 -tetrahydrocannabinol (THC) for 72 hours. $P < .001$ vs vehicle; $*P < .001$ vs MA or THC for $n = 4$ independent experiments. **C**) Microscopy of HeLa cells from the different treatment groups in B that invaded through Matrigel-coated membranes. Cells were stained with Diff-Quick[®] (Medion Diagnostics GmbH, Bűdingen, CH). Magnification of photomicrographs is $\times 200$. **D**) Effect of MMP-2 or MMP-9 siRNA (each at 0.25 or 1 $\mu\text{g}/\text{mL}$) on basal HeLa cell invasion. $P = .226$ for 0.25- $\mu\text{g}/\text{mL}$ MMP-2 siRNA vs vehicle, $P = .063$ for 1.0- $\mu\text{g}/\text{mL}$ MMP-2 siRNA vs vehicle, and $P = .007$ for 0.25- $\mu\text{g}/\text{mL}$ MMP-9 siRNA vs vehicle, $*P < .001$ vs vehicle. TIMP-1, MMP-2, and MMP-9 protein levels were determined in supernatants collected from the upper Boyden chamber. Invasion index values are means + the upper 95% CI (error bar) of $n = 4$ independent experiments. Densitometric analysis were obtained from $n = 6-7$ (A) and $n = 4$ (D) blots standardized to loading control (LC), a band corresponding to a protein of about 65 kDa whose expression was not affected by treatment with siRNA, transfection agent, or cannabinoids.



of TIMP-1 siRNA transfection on tumor cell invasion was also confirmed by staining cells that had invaded through Matrigel-coated membranes at the lower surface. A lower number of cells invaded in the presence of cannabinoids, and this effect was not

seen when cells were also treated with 0.25 $\mu\text{g}/\text{mL}$ TIMP-1 siRNA (Fig. 4, C). Knockdown of TIMP-1 also led to an inhibition of the effect of cannabinoids on invasion in A549 and C33A cells (Supplementary Fig. 1, available online).

Requirement of MMP-2 and MMP-9 for HeLa Cell Invasion

TIMP-1 is known to form inhibitory complexes with MMP-2 and MMP-9 (23). To determine whether MMP-2 and MMP-9 are essential for HeLa cell invasion, cells were transfected with 0.25 and 1.0 $\mu\text{g/mL}$ of siRNA corresponding to the genes encoding these proteins (Fig. 4, D). As measured by densitometric analysis, MMP-2 protein levels in cells treated with 0.25 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$ siRNA relative to that observed when cells were treated with vehicle alone were 34% (95% CI = 22% to 46%) and 19% (95% CI = 8% to 30%), respectively. Similarly, MMP-9 levels decreased to 28% (95% CI = 23% to 33%) and 15% (95% CI = 6% to 24%) of control level after transfection with 0.25 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$ MMP-9 siRNA, respectively. Inhibition of MMP-9 expression by 0.25 $\mu\text{g/mL}$ or 1.0 $\mu\text{g/mL}$ MMP-9 siRNA decreased invasion of HeLa cells through Matrigel (decrease = 48.0%, 95% CI = 18.6% to 77.4%, $P = .0071$ or 95.7%, 95% CI = 78.5% to 112.9%, $P < .001$, respectively), whereas MMP-2 silencing caused a decrease in invasion of only 15% that was not statistically significant. Thus, MMP-9, but not MMP-2, is essential for HeLa cell invasion in our experimental system.

Role of CB₁, CB₂, and TRPV1 in Cannabinoids' Anti-invasive Action and TIMP-1 Induction in Other Tumor Cell Lines

To exclude the possibility that the observed cannabinoid effects are restricted to HeLa cells, experiments were also performed in another human cervical carcinoma cell line (C33A) as well as in human lung carcinoma cells (A549). Like HeLa cells, both cell lines express CB₁ and CB₂ receptors as well as TRPV1, with C33A having a lower concentration of the latter protein as compared with HeLa and A549 cells (Supplementary Fig. 2 A, available online). Addition of cannabinoids to these cells resulted in a statistically significant inhibition of invasion through Matrigel accompanied by increased TIMP-1 secretion, and both events were suppressed by antagonists of CB₁ and CB₂ receptors and, in the case of MA, by a TRPV1 antagonist (Supplementary Fig. 2 B,C). Knockdown of TIMP-1 led to an inhibition of the effect of cannabinoids on invasion in A549 cells and C33A cells (Supplementary Fig. 1).

Discussion

There is considerable evidence to suggest an important role for cannabinoids in conferring anticarcinogenic activities. In this study, we identified TIMP-1 as a mediator of the anti-invasive actions of MA, a hydrolysis-stable analog of the endocannabinoid anandamide, and THC, a plant-derived cannabinoid.

Both cannabinoids decreased HeLa cell invasion in a time- and concentration-dependent manner. Following a 72-hour incubation, the decrease of invasiveness by MA and THC was statistically significant at concentrations as low as 0.1 μM and 0.01 μM (at these concentrations we observed 61.5% inhibition of invasion by MA and 68.1% inhibition by THC). In humans, average peak plasma concentrations of THC of 0.03 μM and 0.045 μM could be achieved with oral doses of 15 and 20 mg, respectively (38) and were associated with a statistically significant reduction of cancer pain (39,40). Thus, effects of THC on cell invasion occurred at therapeutically relevant concentrations.

The possibility that decreased invasion by cannabinoids was an unspecific cytotoxicity-related phenomenon was ruled out by an analysis of cellular viability that revealed no statistically significant cytotoxicity by either MA or THC under experimental conditions very similar to those used for invasion assays. However, MA and THC did lead to increasing and statistically significant toxicity when cellular density was decreased. To our knowledge, cell density-dependent toxicity has not been described for cannabinoids before but is well documented as the "inoculum effect" for several chemotherapeutics, including tamoxifen (41), doxorubicin, and vincristine (42). In the study of vincristine toxicity, measurements of cellular drug levels revealed that at high densities, cells accumulate much smaller amounts of chemotherapeutics, resulting in impaired availability of the drug at its intracellular binding sites (42). A similar pattern may occur when cells are exposed to MA or THC given that both cannabinoids cause receptor-independent apoptosis (8). In the case of MA, this effect probably involves a lipid raft-dependent intracellular uptake of the compound (43). However, high cell densities do allow considerable binding of cannabinoids to their extracellular membrane receptors, as revealed by the profound receptor-dependent anti-invasive action observed here.

Our finding that reduced invasion was not associated with decreased cellular motility suggested that the reduction in invasiveness that was observed when cells were treated with cannabinoids was a specific effect that was dependent on the modulation of matrix-degrading enzymes. Although this result rules out a decisive role of migration in mediating the anti-invasive action of cannabinoids in our system, others have reported antimigrative properties of cannabinoids that suggest that these substances affect migration in a cell type-specific and/or chemoattractant-dependent manner. For example, in human breast cancer cells, cannabinoid treatment inhibits adhesion and migration on type IV collagen, possibly via decreased tyrosine phosphorylation of focal adhesion kinase (44). Furthermore, a cannabinoid receptor-independent mechanism was proposed to underlie the antimigrative action of cannabidiol on human glioma cells (45).

Because the role of TIMP-1 in reducing invasiveness is well established (24–30), we assessed the role of this endogenous MMP inhibitor in the context of the anti-invasive effects of cannabinoids on HeLa cells. Our results suggest a causal link between cannabinoid receptor activation, TIMP-1 induction, and decreased invasiveness of HeLa cells. Consistent with the hypothesis that TIMP-1 is a mediator of the effects of cannabinoids on cell invasion, TIMP-1 induction first became evident after a 12-hour incubation period with both cannabinoids and a decrease in invasion appeared between 12 and 24 hours after cannabinoid exposure. Furthermore, inhibitors of the cannabinoid receptors CB₁ and CB₂ and TRPV1 that caused a profound reduction of cannabinoid-induced TIMP-1 expression reversed cannabinoid-mediated effects on invasion. The most convincing evidence for a crucial role of TIMP-1 in cannabinoid-mediated decreased invasion was our finding that transfection of cells with siRNA targeting TIMP-1 markedly suppressed cannabinoid-mediated decreases in invasion. We confirmed a TIMP-1-dependent anti-invasive effect of cannabinoid treatment in another human cervical cell line (C33A) as well as in human lung carcinoma cells

(A549), suggesting that increased expression of TIMP-1 is part of a general anti-invasive mechanism of cannabinoids.

Our results also suggest that MAPKs are targets of cannabinoid receptor signaling that are upstream of TIMP-1. In support of this idea, preincubation of cannabinoid-treated cells with the inhibitor of p38 MAPK activity SB203580 and the inhibitor of p42/44 MAPK activation PD98059 led to impaired induction of expression of TIMP-1 by MA and THC. Moreover, inhibitor experiments with AM-251 and AM-630 revealed a CB₁ and CB₂ receptor-mediated MAPK activation consistent with previous studies that demonstrated a mediating role of MAPKs in cannabinoid receptor-elicited effects (3,9,33). These results suggest that both p38 and p42/44 MAPKs are mediators of cannabinoid receptor activation and subsequent TIMP-1 regulation. Treatment of cells with the TRPV1 antagonist capsazepine had virtually no effect on MA-mediated activation of p38 and p42/44 MAPKs, suggesting that TRPV1 activation increases TIMP-1 expression by a mechanism that bypasses MAPKs.

Specific inhibition of MMP expression by siRNA suggested a decisive role of MMP-9 but not MMP-2 in basal HeLa cell invasion. Thus, the decreased expression of MMP-2 mediated by cannabinoids we observed does not appear to contribute to their anti-invasive action in our experimental system. The lack of a role for MMP-2 in modulating the anti-invasive effects of cannabinoids is further supported by the finding that the suppressive effect of cannabinoids on MMP-2 expression was not mediated by CB₁ or CB₂. Thus, if lowering MMP-2 was responsible for the inhibitory effect of cannabinoids on tumor cell invasion, treatment of cells with receptor antagonists should be expected to elicit only partial suppression of invasiveness. However, the anti-invasive action of cannabinoids was of fully reversed when both CB₁ and CB₂ receptors were blocked.

Our study has some limitations. First, it is not known to what extent the principal finding of this study can be generalized to cell types other than those examined in this study. Moreover, further studies will be required to examine the relevance of our findings to in vivo tumors. Finally, we did not identify the mechanism underlying receptor-independent decreased expression of MMP-2. It remains to be determined whether lipid raft microdomains that have been recently proposed to be an initial target of the endocannabinoid analog MA in eliciting receptor-independent induction of ceramide synthesis, MAPK activation, and cyclo-oxygenase-2 expression (43) are involved in this response.

In conclusion, our results suggest that there exists a signaling pathway by which the binding of cannabinoids to specific receptors leads via intracellular MAPK activation to induction of TIMP-1 expression and subsequent inhibition of tumor cell invasion. To our knowledge, this is the first report of TIMP-1-dependent anti-invasive effects of cannabinoids. This signaling pathway may play an important role in the antimetastatic action of cannabinoids, whose potential therapeutic benefit in the treatment of highly invasive cancers should be addressed in clinical trials.

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Notes

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