

Full-length article

Tetrahydrocurcumin inhibits HT1080 cell migration and invasion via downregulation of MMPs and uPA¹

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Key words

tetrahydrocurcumin; invasion; matrix metalloproteinases; urokinase plasminogen activator

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Abstract

Aim: Tetrahydrocurcumin (THC) is an active metabolite of curcumin. It has been reported to have similar pharmacological activity to curcumin. The proteases that participate in extracellular matrix (ECM) degradation are involved in cancer cell metastasis. The present study investigates the effect of an ultimate metabolite of curcumin, THC, on the invasion and motility of highly-metastatic HT1080 human fibrosarcoma cells. **Methods:** The effect of THC on HT1080 cell invasion and migration was determined using Boyden chamber assay. Cell-adhesion assay was used for examining the binding of cells to ECM molecules. Zymography assay was used to analyze the effect of THC on matrix metalloproteinase (MMP)-2, MMP-9, and urokinase plasminogen activator (uPA) secretion from HT1080 cells. Tissue inhibitor of metalloproteinase (TIMP)-2 and membrane-type 1 matrix metalloproteinase (MT1-MMP) proteins levels were analyzed by Western blotting. **Results:** Treatment with THC reduced HT1080 cell invasion and migration in a dose-dependent manner. THC also decreased the cell adhesion to Matrigel and laminin-coated plates. Analysis by zymography demonstrated that treatment with THC reduced the levels of MMP-2, MMP-9, and uPA. THC also inhibited the levels of MT1-MMP and TIMP-2 proteins detected by Western blot analysis. **Conclusion:** Our findings revealed that THC reduced HT1080 cell invasion and migration. The inhibition of cancer cell invasion is associated with the downregulation of ECM degradation enzymes and the inhibition of cell adhesion to ECM proteins.

Introduction

Cancer metastasis consists of a complex cascade of events. The invasion of cancer cells through the basement membrane occurs via the initial adhesion of cancer cells to the extracellular matrix (ECM) components followed by degradation of the ECM by proteolytic enzymes^[1]. The key proteases that are involved in the degradation of the ECM are the serine proteases (plasmins), the urokinase plasminogen activator (uPA), cysteine proteases, such as cathepsin B and L, and matrix metalloproteinases (MMP)^[2]. Among these enzymes, MMP play a key role in cancer cell invasion and metastasis, which can degrade most components of the ECM^[3]. Most MMP are produced in zymogen form (pro-

MMP), requiring activation for catalytic activity. All activated MMP are specifically inhibited by the endogenous inhibitor, tissue inhibitor of metalloproteinase (TIMP)^[4]. Membrane-type 1 matrix metalloproteinase (MT1-MMP) has been shown to be a key enzyme in tumor metastasis. The role of MT1-MMP in pericellular proteolysis can play a direct role in ECM turnover^[5]. Moreover, it was identified as the first physiological activator of pro-MMP-2^[6]. Therefore, the inhibition of ECM degradation enzymes and cell adhesion to ECM molecules could be considered as a preventive approach for cancer metastasis.

Tetrahydrocurcumin (THC) is an active metabolite of curcumin *in vivo*. After absorption, curcumin undergoes

metabolism to its sulfate, glucuronide, and sulfated-glucuronide conjugate^[7]. In the liver, curcumin is reduced by endogenous reductase systems to hexahydrocurcumin, tetrahydrocurcumin, and hexahydrocurcuminol^[8], and THC has been demonstrated to be the major curcumin metabolite *in vivo*^[9]. THC has been widely studied due to its potential antioxidant, anti-inflammatory, and anticarcinogenic activities, as well as for its ability to modulate multidrug-resistant proteins^[10-13]. Unlike curcumin, THC is stable in phosphate buffer and in saline at various pH values; furthermore, it is easily absorbed through the gastrointestinal tract. THC might also play a crucial role in curcumin-induced biological effects.

In recent years, it has also been reported that curcumin induces a reduction in cancer cell invasion *in vitro* and *in vivo* by downregulating the level of the ECM degradation enzymes, MMP-2, MMP-9, MMP-3, and MT1-MMP^[14-17]. However, the effect of the curcumin metabolite, THC, on various aspects of the anti-invasion phenomenon has not been investigated. Thus we explore in the present study the effect of THC on the regulation of the ECM degradation enzymes as well as the invasion of HT1080 cells.

Materials and methods

Materials Dulbecco's modified Eagle's medium (DMEM) with or without phenol red, penicillin-streptomycin, and trypsin-EDTA were purchased from GIBCO-BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Collagen type IV, laminin, plasminogen, and casein were purchased from Sigma-Aldrich (St Louis, MO, USA). Fibronectin and the antibody against MT1-MMP were purchased from Chemicon (Chemicon International, Euromedex, France) and the antibody against TIMP-2 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Matrigel was purchased from Becton Dickinson (Bedford, MA, USA).

Cell lines HT1080 human fibrosarcoma and NIH3T3 fibroblasts cells were grown in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated FBS. Cultures were maintained at 37 °C in a 5% CO₂/95% air atmosphere.

Preparation of THC THC was prepared as described previously^[13]. Briefly, curcumin was converted to THC by hydrogenation with 10% PtO₂. After hydrogenation, the crude THC was purified by preparative thin layer chromatography (TLC) (5% MeOH in CHCl₃, R_f=0.86). The identity and purity of THC were confirmed by using mass spectrometer (MS), infrared (IR) spectroscopy, and nuclear magnet resonance (NMR) data.

MTT assay for cell viability Cell viability was measured

by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described previously^[18]. Briefly, the HT1080 cells were inoculated at a density of 3×10³ cells/well in 96-well plates for 24 h in 200 µL DMEM with 10% FBS; subsequently, the culture supernatant was removed, and DMEM containing various concentrations of THC was added and incubated for 24 h. MTT dye (15 µL, 5 mg/mL) was added and the plate was incubated for an additional 4 h. The MTT-formazan was dissolved with DMSO, and absorbance was measured using a microplate reader at 570 nm with a reference wavelength of 630nm.

Cell invasion and motility assay The invasive and migration behaviors of the HT1080 cells were tested using a modified Boyden chamber assay^[19]. Polyvinylpyrrolidone-free polycarbonate filters (Millipore, Co. Cork, Ireland) (8 µm pore size) were coated with gelatin (0.01% w/v) for the chemotaxis (cell migration) assay or with Matrigel (15 µg/filter) for the invasion assay. The medium in the lower chamber contained serum-free culture-conditioned medium of NIH3T3 fibroblast cells, which acted as chemoattractants. The HT1080 cells (1×10⁵ cells/chamber) were plated onto the upper chamber with or without various concentrations of THC and incubated for 5 h at 37 °C in 5% CO₂. After incubation, the non-invading cells were removed from the upper surface of the membrane. The invading cells on the lower surface of the membrane were fixed with methanol for 1 min and stained with 1% w/v of toluidine blue for 5 min. The cells that actively migrated to the under surface of the filter were dissolved with 20% acetic acid and indirectly quantified by measuring the absorbance at 570 nm. Control experiments were performed in the absence of the chemoattractant. The results of 3 independent experiments were averaged after background subtraction.

Cell-adhesion assay The cell-adhesion assay was performed using a slight modification of a previously-described method^[20]. In our assay, the 96-well plates were coated with 100 µL/well Matrigel (25 µg/mL), 100 µL/well fibronectin (10 µg/mL), 100 µL/well collagen type IV, or laminin (20 µg/mL), followed by incubation for 16 h at 4 °C. Non-specific binding sites were blocked with 0.1% bovine serum albumin for 1 h at 4 °C followed by washing 3 times with phosphate-buffered saline (PBS). In the meantime, the HT1080 cells were trypsinized and resuspended in serum-free DMEM solution containing various concentrations of THC; 3×10⁴ cells/well were added to each coated well. The cells were incubated at 37 °C for 1 h, and the non-adherent cells were removed by shaking the plate at 1000 r/min for 30 s and washed with DMEM 3 times. The cells were fixed with 4% paraformaldehyde.

hyde for 15 min and stained with 0.5 % w/v crystal violet in 20% methanol for 10 min. Unbound dye was removed in tap water, and the plate was dried in air. Bound dye was extracted with 20% acetic acid. The absorbance of the samples was measured at 570 nm using a microplate reader.

Zymography The secretions of MMP-2 and MMP-9 in the culture-condition medium were assayed by gelatin zymography^[21]. The HT1080 cells (1×10^5 cells/well) were seeded into 6-well plates and maintained for 24 h in DMEM with 10% FBS. Subconfluent cell cultures were incubated for 24 h in various concentrations of THC in serum-free DMEM, and the culture supernatants were collected from equal numbers of cells. Without heating and under non-reducing conditions, the samples were subjected to electrophoresis in 0.1% w/v gelatin-containing 10% PAGE in the presence of SDS. After electrophoresis, the gel was washed 2 times for 30 min in 2.5% Triton X 100 and incubated for 18 h at 37 °C in Tris buffer (50 mmol/L Tris-HCl, 200 mmol/L NaCl, and 10 mmol/L CaCl₂, pH 7.4). The gels were stained with Coomassie Brilliant Blue R (0.1% w/v) and destained in a solution of 30% methanol and 10% acetic acid. Gelatinolytic activity appeared as a clear band on a blue background. Digestion bands were quantified by Bio-1D software (Viber Lourmat, Marne-la-Vallée, France).

The uPA secretion in the culture-conditioned medium was examined by casein-plasminogen zymography^[22]. The culture supernatant was separated by electrophoresis in 10% polyacrylamide gel (PAGE) in the presence of SDS, which was copolymerized with 1 mg/mL β -casein and 10 mg/mL human plasminogen under non-reducing conditions. After electrophoresis, the gel was washed 2 times for 30 min in 2.5% Triton X 100 and incubated for 18 h at 37 °C in Tris buffer (50 mmol/L Tris-HCl, 200 mmol/L NaCl, and 10 mmol/L CaCl₂, pH 7.4). The gels were stained and destained as described earlier.

Preparation of conditioned media and whole cell lysates

The HT1080 cells (1×10^6 cells/flask) were seeded into 75 mm³ T flasks and maintained for 24 h in DMEM with 10% FBS. Subconfluent cell cultures were incubated for 24 h in various concentrations of THC in serum-free DMEM without phenol red. After treatment, the culture supernatant was collected and concentrated with Amicon-Ultra 4 (Millipore, Ireland) for the TIMP-2 analysis, and the cells were washed twice with ice-cold PBS and scraped with a cell scraper into ice-cold PBS. The cells were centrifuged at 500×g for 10 min, the supernatant was removed, and the cell pellets were lysed with a lysis buffer containing protease inhibitor (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 10 mmol/L EDTA, 1% Triton X 100, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 µg/mL

leupeptin, and 10 µg/mL aprotinin) for 30 min on ice. The insoluble matter was removed by centrifugation at 12 000×g for 15 min at 4 °C, and the supernatant fraction was collected for MT1–MMP analysis.

Western blot analysis of TIMP-2 and MT1–MMP The expressions of the MT1–MMP and TIMP-2 proteins were determined by immunoblotting with specific antibodies. Briefly, equal amounts of lysates or concentrated-conditioned media proteins from control and treated cells were resuspended in sample buffer and separated by SDS–PAGE using 10% acrylamide gels. After electrophoresis, the proteins were electroblotted to a Hybond-C extra nitrocellulose membrane (Amersham, Little Chalfont, UK). The membrane was blocked at room temperature (RT) with 5% non-fat dry milk in PBS containing 0.3% Tween-20 (PBS-T). The membrane was washed twice with PBS-T and incubated 2 h at RT with the primary antibody, rabbit polyclonal anti-MT1–MMP (1:2000; Chemicon, France) or mouse monoclonal anti-TIMP-2 (1:200; Santa Cruz Biotechnology, USA). After washing 5 times with PBS-T, the membrane was incubated with the second antibodies at RT for 1 h in PBS-T containing 5% non-fat dry milk. After incubation, the membrane was washed with PBS-T 5 times. The second antibodies were horseradish peroxidase-conjugated antirabbit immunoglobulin G (IgG; 1:10,000; Amersham, UK) or antimouse IgG (1:10,000; Chemicon, France). After incubation, the immunoreactive material was visualized by enhanced chemiluminescence and exposed to X-ray film (Kodak, Windsor, Colorado, USA) for periods of 5–30 min.

Statistical analysis Statistical analyses were performed using one-way ANOVA. $P < 0.01$ was considered statistically significant. All statistical analyses were performed using SPSS 10.0 software (SPSS, Chicago, IL, USA).

Results

Effect of THC on the cytotoxicity of HT1080 cells The effect of various concentrations of THC (0–100 µmol/L) on the viability of HT1080 cells was assayed by MTT. Treatment with high concentrations of THC (up to 100 µmol/L), for 24 h did not affect the cytotoxicity to HT1080 cells (Figure 1). This concentration range was used in all subsequent experiments.

THC inhibited the invasion and migration of HT1080 cells The effect of THC on the invasiveness and motility of HT1080 cells was determined by Boyden chamber assay. THC reduced the invasion of HT1080 through Matrigel (reconstitutive basement membrane) in a dose-dependent manner, with an inhibitory concentration at 50 % of control (IC_{50}) value of >100 µmol/L (Figure 2A). THC also inhibited

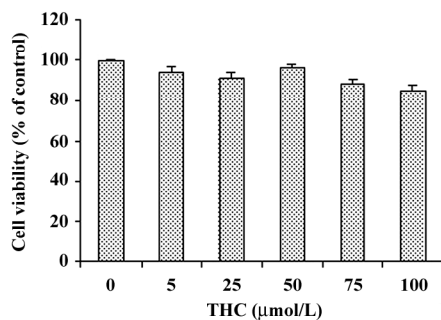


Figure 1. Effect of THC on the cytotoxicity of HT1080 cells. Cells were treated with the indicated concentrations of THC for 24 h before being subjected to MTT assay. Data represent the mean \pm SD of 3 independent experiments.

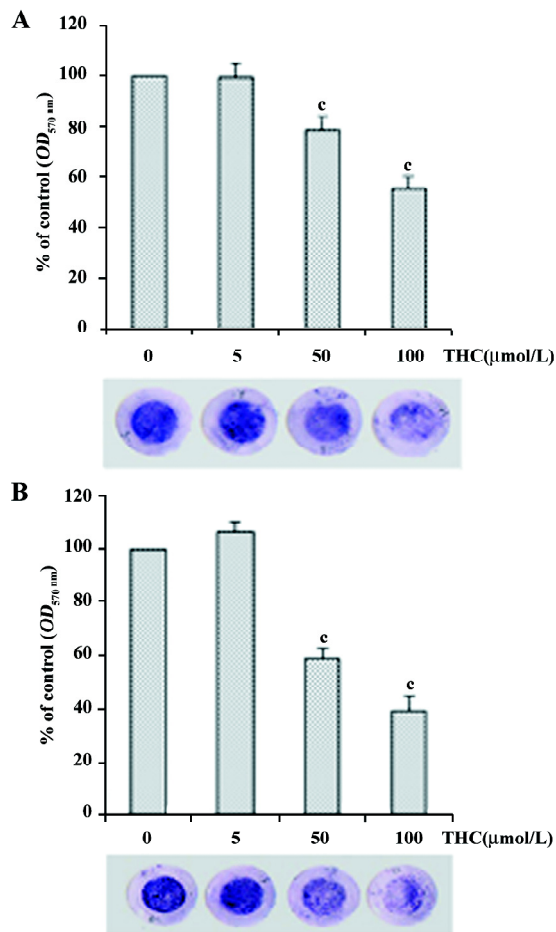


Figure 2. Effect of THC on HT1080 cell invasion (A) and migration (B). HT1080 cells were seeded onto a filter coated with Matrigel (A) or gelatin (B) containing THC at the indicated concentrations (0–100 μ mol/L) and then incubated for 5 h at 37 $^{\circ}$ C. Cells that actively migrated to the lower surface of the filter were quantified. Invasion and migration are expressed as a percentage of the untreated control. Data represent the mean \pm SD of 3 independent experiments. ^c P <0.01.

the migration of the cells through the gelatin-coated filters in a dose-dependent manner, with an IC_{50} value of 75 μ mol/L (Figure 2B).

Effect of THC on HT1080 cell adhesion to Matrigel, fibronectin, laminin, and collagen type IV Assuming that the adhesion of cancer cells to ECM molecules is the first step of tumor cell invasion, we further determined the effect of THC on the adhesion of HT1080 cells to Matrigel, fibronectin, laminin, and collagen type IV substrates. HT1080 cells were treated with THC at concentrations ranging from 0 to 100 μ mol/L. THC inhibited the adhesion of the cells to Matrigel and laminin in a dose-dependent manner, with an IC_{50} value of >100 and 90 μ mol/L, respectively (Figure 3). In contrast, THC did not affect the cell adhesion to fibronectin and collagen type IV-coated plates.

THC reduced the secretion of MMP-2, MMP-9, and uPA from HT1080 cells To investigate whether THC can inhibit the secretion of MMP-2, MMP-9, and uPA from HT1080 cells, the cells were treated with various concentrations of THC (0–100 μ mol/L) in serum-free medium for 24 h. The levels of MMP-2, MMP-9, and uPA were analyzed by zymography. The levels of total MMP-2 (pro and active form) and Pro-MMP-9 were reduced by THC in a dose-dependent manner, with an IC_{50} value of 85 μ mol/L and >100 μ mol/L, respectively (Figure 4A). The uPA (pro and active form) level was also inhibited by THC in a dose-dependent manner, with an IC_{50} value of 80 μ mol/L (Figure 4B).

Effect of THC on MT1-MMP and TIMP-2 expressions in HT1080 cells To examine the influence of THC on the modulation of pro-MMP-2 activation, we assessed the TIMP-2 and MT1-MMP protein expressions in THC-treated cells. After the cells were treated with various concentrations of THC, the culture supernatant was used for determination of the TIMP-2 level, and the cell lysates were used for determination of the MT1-MMP protein level. As shown in Figure 5A, THC decreased the TIMP-2 protein level in a dose-dependent manner, with an IC_{50} value in the range of 75 μ mol/L. Total MT1-MMP protein levels were also significantly reduced by treatment with THC in a dose-dependent manner, with an IC_{50} value in the range of 100 μ mol/L (Figure 5B), demonstrating that THC reduced the protein levels of both TIMP-2 and MT1-MMP.

Discussion

In recent years, it has been reported that curcumin exhibits its antimetastatic properties *in vivo* and *in vitro*^[14,15]. The pharmacokinetic features of curcumin in several species have indicated poor systemic bioavailability, which may be related to its inadequate absorption and avid metabolism^[8,9,23,24].

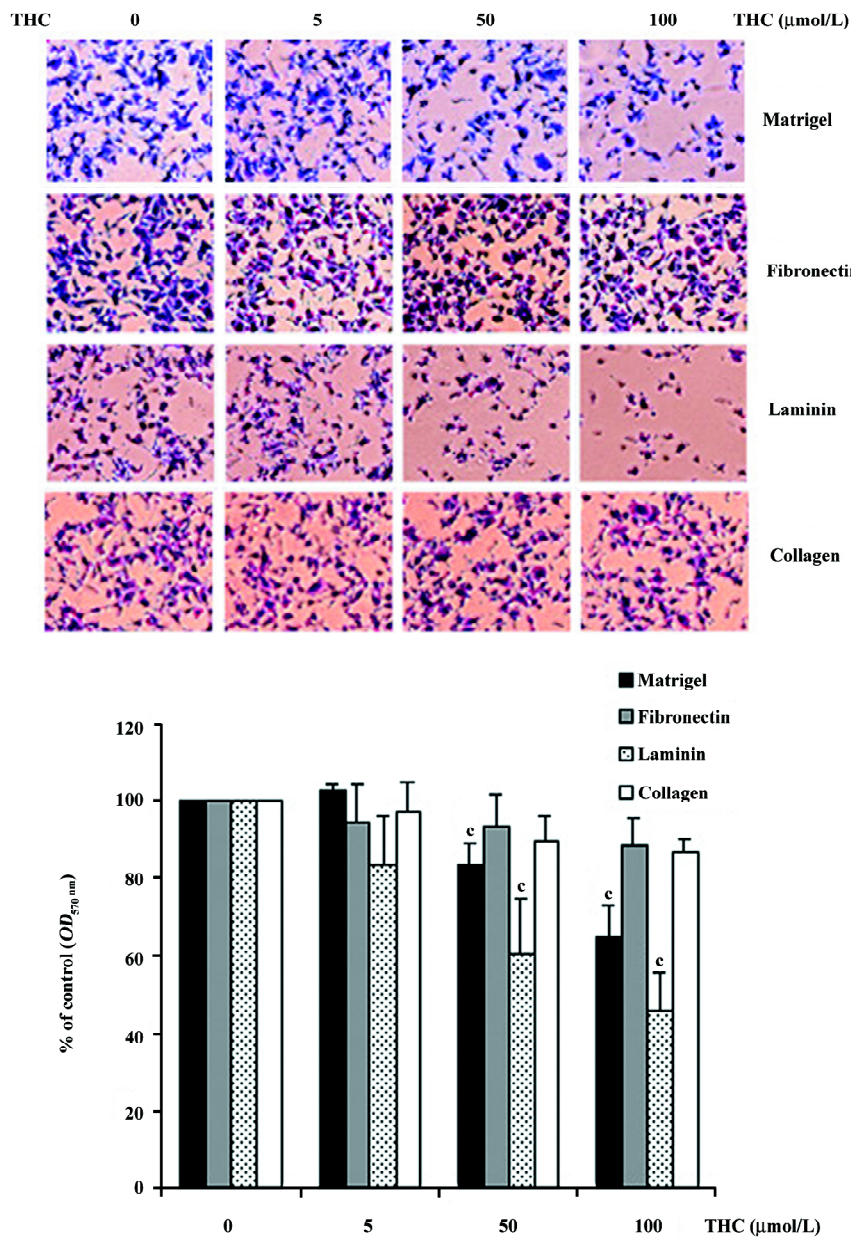


Figure 3. Effect of THC on the adhesion of HT1080 cells to the ECM molecules. Cancer cells were added to wells precoated with Matrigel, fibronectin, laminin, and collagen type IV in the presence or absence of the indicated concentration of THC. After 1 h of incubation, non-adherent cells were removed, and adherent cells were stained using crystal violet staining. After extensive washing, the stained cells were lysed with 10% acetic acid, and the absorbance was measured at 570 nm. Cancer cell adhesion is expressed as a percentage of the untreated control. Data represent the mean±SD of 3 independent experiments. ^c*P*<0.01.

After oral administration, curcumin is rapidly metabolized by enzymes in the intestine and liver. THC has been demonstrated as one of the major metabolites of curcumin *in vivo*^[25].

It is well known that tumor cell metastasis is a complex cascade of events. The process involves multiple steps, such as cell adhesion, ECM component degradation, and tumor cell migration. Hence, interruption of 1 or more of these steps is one approach for antimetastatic therapy. To

date, there is no evidence to show that THC exerts an effect on cancer cell metastasis. The present study is the first to demonstrate that THC at non-cytotoxic doses significantly inhibits HT1080 cell invasion through the basement membrane.

The initial invasive action of metastatic cells involves the interaction of tumor cells with the ECM molecules through the process of cell matrix adhesion^[26-28]. The result from our cell-adhesion assay shows that THC significantly reduced

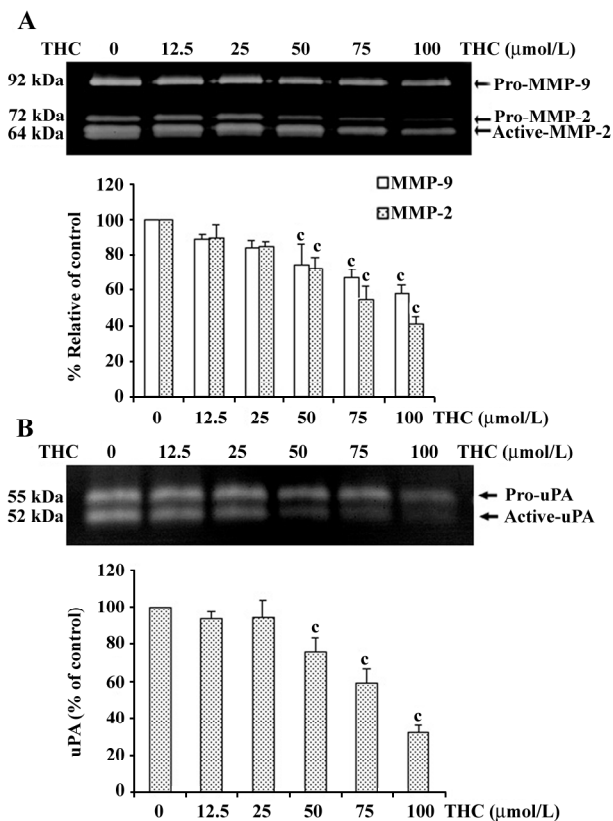


Figure 4. THC inhibits the secretion level of MMP-2, MMP-9, and uPA from HT1080 cells. After the HT1080 cells were treated with THC at the indicated concentrations (0–100 $\mu\text{mol/L}$) for 24 h in serum-free medium, an equal amount of proteins was loaded (5 $\mu\text{g}/\text{lane}$). Gelatin zymography was performed for MMP-2 and MMP-9 activities in culture-conditioned medium (A). UPA activity was analyzed by casein-plasminogen zymography (B). Levels of MMP-2 (pro and active form), MMP-9 (pro form), and uPA (pro and active form) activities were quantified by densitometric analysis with that of the untreated group being 100% as shown just below the gel. Data represent the mean \pm SD of 3 independent experiments. $^{\circ}P<0.01$.

HT1080 cell adhesion to Matrigel (reconstituted basement membrane). This result led us to investigate further which ECM molecules in Matrigel were blocked by THC. Our results demonstrate that THC reduced HT1080 cell adhesion to laminin, but not to collagen type IV and fibronectin. The above data indicate that THC blocked the binding between cancer cells and laminin, which led to the observed inhibition of cancer cell adhesion to Matrigel.

The process of tumor cell metastasis requires the degradation of ECM molecules in the basement membrane, which is the largest barrier between cancer cells and the bloodstream; it must disintegrate before cancer cells can enter the circulatory system. The key proteases that are involved in ECM degradation are MMP and serine proteases, such as uPA.

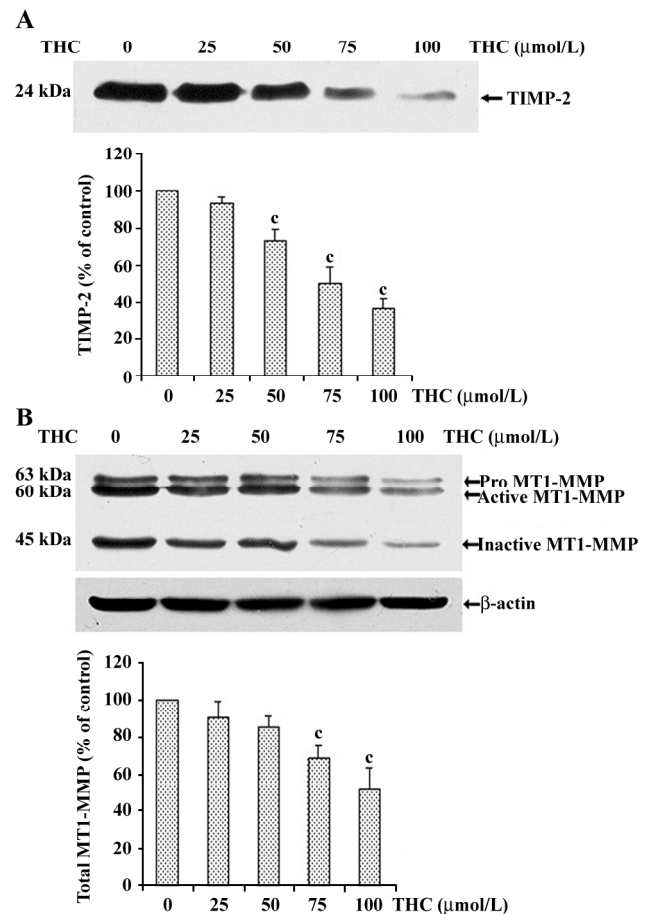


Figure 5. Analysis of the THC effect on the TIMP-2 and MT1-MMP protein expressions in HT1080 cells was performed using Western blot analysis. HT1080 cells were treated with THC at the indicated concentrations (0–100 $\mu\text{mol/L}$) for 24 h, the conditioned medium was collected and concentrated for TIMP-2 determination (A), and the cell lysate was used for MT1-MMP determination (B). Equal amounts of proteins were loaded (40 $\mu\text{g}/\text{lane}$ for TIMP-2 and 50 $\mu\text{g}/\text{lane}$ for MT1-MMP). Band intensity of TIMP-2 and total MT1-MMP were quantified by densitometry, where the untreated group represented 100%. Data represent the mean \pm SD of 3 independent experiments. $^{\circ}P<0.01$.

Although several enzymes are involved in this process, it appears that MMP-2 and MMP-9 play important roles in cell invasion because both enzymes degrade type IV collagen, a major component of the basement membrane^[29]. In the present study, we investigated the effect of THC on MMP-2, MMP-9, and uPA secretion from HT1080 cells using zymography assay. The results revealed that THC significantly reduces MMP-2, MMP-9, and uPA secretion from the cells in a dose-dependent manner. These data collectively support the inhibitory effect of THC on cancer cell invasion.

In particular, MMP-2 is constitutively expressed and secreted as a latent zymogen, pro-MMP-2, and its main activa-

tion takes place on the cell surface; this process is mediated by MT1–MMP and TIMP-2^[30-32]. After activation, MT1–MMP proteolytic processing leads to its 45 kDa inactive form^[33,34]. The appearance of this inactive MT1–MMP form has been strongly correlated with MMP-2 activation^[35]. In the present study, we found that THC significantly reduced total MT1–MMP (pro, active, and inactive form). This result suggests that THC decreases pro-MMP2 activation through a reduced MT1–MMP level. Interestingly, THC decreases the TIMP-2 level, even though TIMP-2 is considered to be inhibitor of MMP-2; however, the relationship between MMP-2 and TIMP-2 with regard to anticancer metastasis remains a topic of controversy. For example, several studies have reported that decreased levels of MMP-2 together with increased levels of TIMP-2 might lead to reduced cancer cell invasion^[36]. However, other reports argue that anticancer cell invasion correlates with decreased levels of both MMP-2 and TIMP-2^[37,38].

In conclusion, we report that THC, a major metabolite of curcumin, inhibits cancer cell invasion and motility. The inhibition was effected by decreasing the secretion of ECM degradation enzymes from invasive cells and by blocking the cell adhesion to Matrigel. However, its exact mechanism of action remains elusive. This study provides additional evidence that THC can inhibit metastasis activity in cancer cells.

Author contribution

Pornngarm LIMTRAKUL, Supachai YODKEEREE designed research; Supachai YODKEEREE performed research; Spiridione GARBISA contributed new reagents or analytic tools; Supachai YODKEEREE, Pornngarm LIMTRAKUL analyzed data; Pornngarm LIMTRAKUL wrote the paper.

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