

ORIGINAL ARTICLE

Δ^9 -Tetrahydrocannabinol inhibits epithelial growth factor-induced lung cancer cell migration *in vitro* as well as its growth and metastasis *in vivo*

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Δ^9 -Tetrahydrocannabinol (THC) is the primary cannabinoid of marijuana and has been shown to either potentiate or inhibit tumor growth, depending on the type of cancer and its pathogenesis. Little is known about the activity of cannabinoids like THC on epidermal growth factor receptor-overexpressing lung cancers, which are often highly aggressive and resistant to chemotherapy. In this study, we characterized the effects of THC on the EGF-induced growth and metastasis of human non-small cell lung cancer using the cell lines A549 and SW-1573 as *in vitro* models. We found that these cells express the cannabinoid receptors CB₁ and CB₂, known targets for THC action, and that THC inhibited EGF-induced growth, chemotaxis and chemoinvasion. Moreover, signaling studies indicated that THC may act by inhibiting the EGF-induced phosphorylation of ERK1/2, JNK1/2 and AKT. THC also induced the phosphorylation of focal adhesion kinase at tyrosine 397. Additionally, in *in vivo* studies in severe combined immunodeficient mice, there was significant inhibition of the subcutaneous tumor growth and lung metastasis of A549 cells in THC-treated animals as compared to vehicle-treated controls. Tumor samples from THC-treated animals revealed antiproliferative and antiangiogenic effects of THC. Our study suggests that cannabinoids like THC should be explored as novel therapeutic molecules in controlling the growth and metastasis of certain lung cancers.

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Introduction

Lung cancer is the leading cause of cancer death for both men and women in the United States (Jemal *et al.*,

2006). The high case:fatality ratio observed in lung cancer is attributed to a poor response to therapy and the aggressive biological nature of the disease. High expression of the epidermal growth factor receptor (EGFR) and/or its ligands is common in non-small cell lung cancer (NSCLC), and correlates with a more aggressive disease, resistance to chemotherapy and poor prognosis (Salomon *et al.*, 1995). A series of targets and therapeutic strategies for the treatment of lung cancer are currently being investigated (Li *et al.*, 2005; Adjei, 2006; Erler *et al.*, 2006; Molina *et al.*, 2006). Recent studies on cannabinoids suggest their potential application in the inhibition of tumor cell growth by modulating key survival signaling pathways (Casanova *et al.*, 2003; Carracedo *et al.*, 2006). In the present investigation, we studied the effects of the cannabinoid Δ^9 -tetrahydrocannabinol (THC) on lung cancer growth and metastasis.

Δ^9 -Tetrahydrocannabinol, the active component of *Cannabis sativa* (marijuana), is considered the most important of the 60 known cannabinoids and is known to exert a wide spectrum of effects on the central nervous system as well as peripheral sites (Di Marzo and Petrocellis, 2006). THC exhibits equal affinity toward CB₁ and CB₂, the two G protein-coupled cannabinoid receptors characterized and cloned from mammalian tissues (Matsuda *et al.*, 1990; Munro *et al.*, 1993). THC exerts its various biological activities after engaging these cannabinoid receptors (Hauck *et al.*, 2001) and has had variable antitumor effects in animal and clinical studies (Munson *et al.*, 1975; Kogan, 2005; Guzmán *et al.*, 2006), yet little is known about its impact on the invasion and growth of NSCLC. In this investigation, we focused our studies on NSCLC, which constitutes the majority of the lung cancer cases.

Since EGFR activation is known to regulate cell proliferation, motility, survival and differentiation as well as angiogenesis (Schlessinger, 2000; Yarden and Sliwkowski, 2001), we analysed the effects of THC on various EGF-mediated functions and signaling pathways in lung cancer.

We demonstrate for the first time that THC treatment inhibited the EGF-induced migration and invasion of the NSCLC cell lines, A549 and SW-1573. THC also inhibited the EGF-induced proliferation of these cells. Furthermore, we show that these inhibitory effects of THC were correlated with reductions in the EGF-induced

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phosphorylation of AKT and mitogen-activated protein (MAP) kinases (ERK1/2 and JNK1/2). Both AKT and MAP kinase pathways are known to play an important role in cancer cell migration and invasion (Williams *et al.*, 1993; Bost *et al.*, 1997; Clarke *et al.*, 1998). We then confirmed the inhibitory effects of THC on tumor growth and metastasis *in vivo* (Guzmán *et al.*, 2006). These results provide the basis for studying cannabinoids in the treatment of lung cancer.

Results

Human lung cancer cell lines express cannabinoid receptors

We first examined the expression of cannabinoid receptors in human NSCLC cell lines. We observed that both the NSCLC cell lines A549 and SW-1573 express the CB₁ and CB₂ receptors, as demonstrated by western blotting (Figure 1a) and reverse transcription (RT)-PCR (Figure 1b).

THC treatment inhibits EGF-induced cell motility

Recent studies have reported on the cannabinoid-mediated inhibition of cell migration (Vaccani *et al.*, 2005; Ghosh *et al.*, 2006). In addition, EGF and EGFR are known to play important roles in cell migration (Beckmann *et al.*, 2001). Thus, we investigated the ability of THC to modulate EGF-induced cell motility. We observed that THC induced cell rounding and led to a failure of the cells to produce characteristic protrusions on EGF stimulation in A549 and SW-1573 cells (data not shown). No significant effect of THC (1–20 μ M) on the viability of the cells was observed over a 24 h time period. However, THC was found to induce apoptosis and inhibit proliferation in these cells over 72 h of treatment (data not shown).

As illustrated in Figure 2, THC significantly decreased EGF-stimulated cell migration as a function of the percent colonization of the wound areas in both A549 and SW-1573 cells when compared to vehicle-treated EGF-stimulated wounds, based on the scratch wound assay. These results demonstrate the mitigating effects of THC on the EGF-induced migration of NSCLC cells.

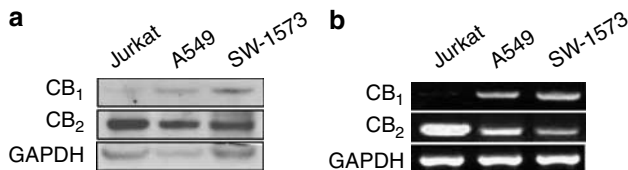


Figure 1 NSCLC cell lines express cannabinoid receptors. (a) A549 and SW-1573 cell lysates were subjected to immunoblot analysis using anti-CB₁, anti-CB₂ or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific antibodies. (b) Total RNA was isolated from the cells and RT-PCR was performed using selective primers for human CB₁, CB₂, or GAPDH. Data shown are representative of two experiments. HMEC-1 (data not shown) and Jurkat cells were taken as positive controls for CB₁ and CB₂ expression.

Furthermore, we found that THC inhibited the EGF-induced transwell migration of both cell lines in a dose-dependent manner, as represented by the A549 cells (Figure 3a). Maximum inhibition of the EGF-induced migration (~50% for A549 and 40% for SW-1573 cells) was observed with 10 μ M of THC. However, THC alone did not induce migration at the concentrations used in this study (data not shown).

THC treatment inhibits the EGF-induced Matrigel invasion of NSCLC cell lines

After confirming *in vitro* the EGF-induced chemoinvasiveness of the two adenocarcinoma cell lines using Matrigel-coated Boyden chambers, the effect of THC on cell invasiveness was evaluated. As shown, THC significantly inhibited the EGF-induced invasion in a dose-dependent manner (Figure 3b). Maximum inhibition of the EGF-induced invasion (~60% for A549 cells (Figure 3b) and 50% for SW-1573 cells (data not shown) was observed with the THC-treated cells when compared to the vehicle-treated cells. The concentrations of THC used did not have any significant effect on the viability of the cell lines, as confirmed by trypan blue staining of the cells in the upper chambers of the transwell inserts (data not shown).

THC treatment inhibits EGF-induced downstream signaling events

To elucidate the underlying mechanisms of THC-mediated attenuation of chemotaxis and chemoinvasion induced by EGF, we analysed the effects of THC on the expression and activation of EGFR. We observed that THC had no significant effect on EGFR expression or phosphorylation after EGF treatment as shown by western blot analysis (Figure 4). While THC-mediated transactivation of EGFR has been reported earlier (Hart *et al.*, 2004), THC alone had no activating effects on EGFR phosphorylation in our studies of these NSCLC cells. EGFR activation following ligand binding is known to induce a series of downstream signaling events involving focal adhesion kinase (FAK), PI3-kinase and MAP kinase (Ullrich and Schlessinger, 1990; Yarden, 2001). We observed that THC enhanced the EGF-induced phosphorylation of FAK at tyrosine 397 (Figure 4), whereas it inhibited AKT phosphorylation (ser-473) in both the A549 (Figure 5a) and SW-1573 (Figure 5b) cells. Moreover, THC treatment also inhibited the EGF-induced phosphorylation of MAP kinases ERK1/2 (p44/p42) and JNK1/2 in these lung cancer cells (Figure 5). We also observed a significant reduction in the concentrations of vascular endothelial growth factor (VEGF) in supernatants obtained from THC-treated cells (Figures 5c and d).

THC treatment suppresses the metastatic spread of lung cancer and subcutaneous tumor growth in SCID mice

We further investigated the inhibitory potential of THC on tumor cell growth and metastasis *in vivo* in a mouse model. For the metastatic studies, A549 cells were injected intravenously through the lateral tail vein of

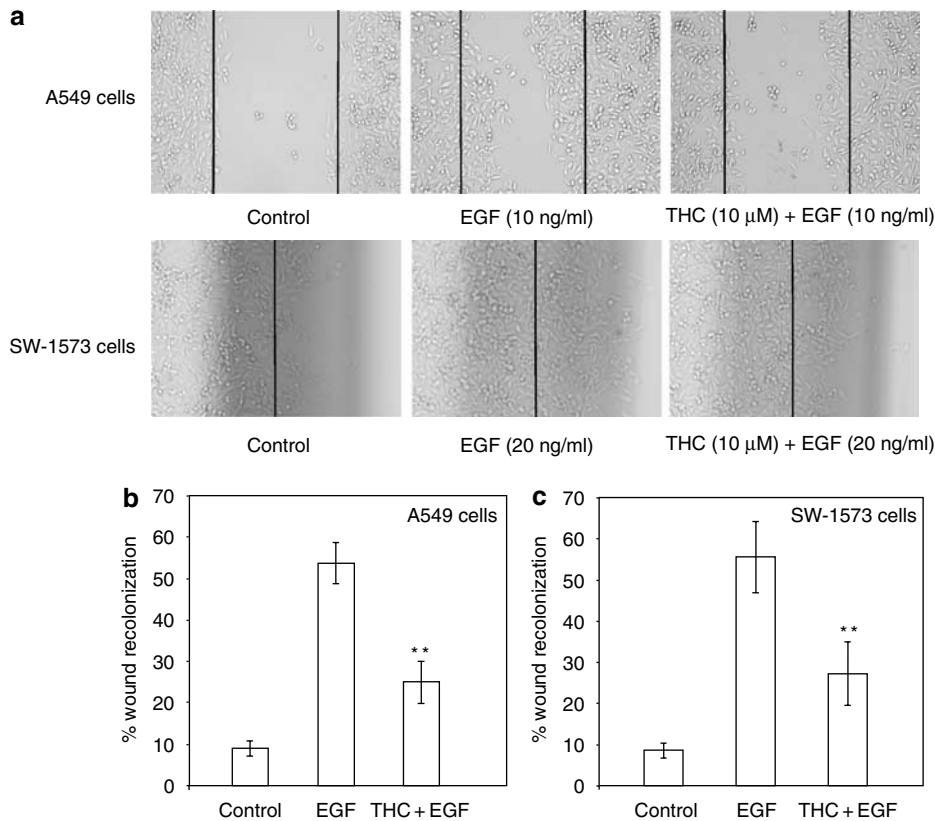


Figure 2 Δ^9 -Tetrahydrocannabinol (THC) inhibits epidermal growth factor (EGF)-induced wound healing in NSCLC cells. Confluent layers of A549 and SW-1573 cells were scratched with sterile tips to form wounds and then were cultured in the presence of EGF + vehicle or EGF + THC (72 h). Phase-contrast images were collected using a light microscope (Nikon). Total wound areas were measured using Scion Image software, and the % wound recolonization was calculated. (a) Illustration of the wound healing experiments described above. (b and c) Quantitative analysis of wound healing shows a significant inhibition of the EGF-induced chemotaxis upon THC treatment of A549 and SW-1573 cells. Data reflect the mean \pm s.d. Representative experiments ($n=2$) are shown (** $P<0.001$; compared to EGF-only stimulated cells).

severe combined immunodeficient (SCID) mice. We observed that the surface lung metastases were significantly ($P<0.001$) reduced in animals treated daily with THC (5 mg/kg body weight) for 28 days compared to animals treated with vehicle alone (Figure 6a). In addition, THC treatment significantly reduced lung weight (~50%) and the number of lesions (~60%) in tumor-bearing animals compared to the vehicle-treated animals (Figures 6b and c).

Next, we evaluated the *in vivo* effect of THC on the xenograft growth of A549 cells in SCID mice. Subcutaneous tumors were generated by inoculating the mice with A549 cells. After 14 days when the tumors had reached a palpable size, animals were injected with THC (5 mg/kg) or vehicle peritumorally daily for 21 days. As shown, tumor growth in THC-treated animals was significantly inhibited (~60%) compared to that in the vehicle-treated animals (Figure 7). Tumor samples were then analysed for tumor cell proliferation, vascularization and for the phosphorylation of important signaling molecules like FAK, ERK1/2 and AKT. THC treatment was shown to inhibit *in vivo* tumor cell proliferation and vascularization as determined by Ki67 and CD31

immunostaining (Figure 8). In addition, the phosphorylations of FAK, ERK1/2 and AKT were also found to be reduced in tumors from THC-treated animals compared to animals treated with vehicle control, as determined by western blot analysis (Figure 9). No effect on total protein was observed in these experiments. Moreover, no significant alterations in physiological parameters like body or liver weight were observed upon THC administration (data not shown).

Discussion

Previous studies have demonstrated tumor-promoting or antineoplastic effects of THC (Munson *et al.*, 1975; Kogan, 2005). However, cannabinoid effects on the EGFR-mediated growth and motility of lung cancer cells have not been characterized to our knowledge. The majority of NSCLCs overexpress EGFR, which has been correlated with a poor prognosis and resistance to chemotherapy (Yarden and Sliwkowski, 2001). Here, we report for the first time on the attenuating effects of THC on the EGF-induced migration and invasion of

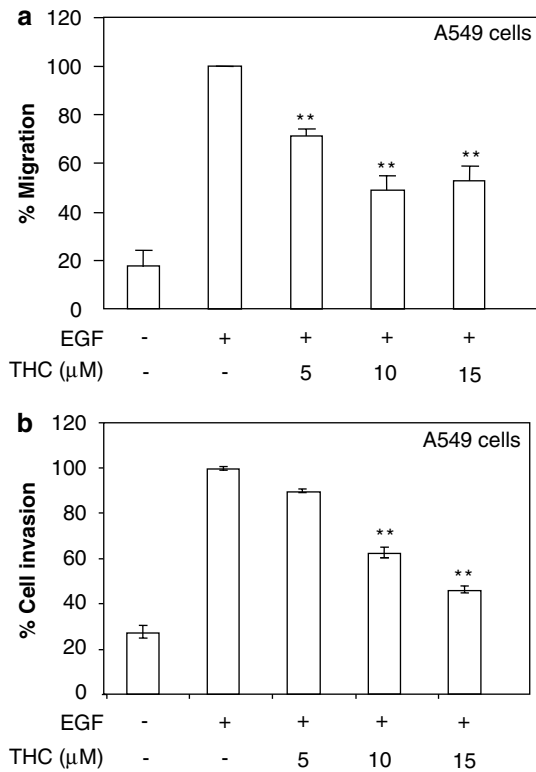


Figure 3 Δ^9 -Tetrahydrocannabinol (THC) inhibits the epidermal growth factor (EGF)-induced migration and Matrigel invasion of NSCLC cells. A549 cells were treated with different concentrations of THC or with vehicle alone (ethanol) before being subjected to EGF-induced transwell migration (a) and invasion (b) assays. Cells that migrated in response to EGF were normalized as 100%. Data represent the mean \pm s.e.m. from three independent experiments (** $P < 0.001$; compared to EGF-only stimulated cells).

NSCLC cell lines *in vitro*. Furthermore, we have shown that THC inhibits lung cancer growth and metastasis in an *in vivo* murine model.

THC is known to act through the cannabinoid receptors, CB₁ and CB₂. Although expression of the CB₂ receptor in A549 cells has been questioned (Sancho *et al.*, 2003), we confirm CB₁ and CB₂ expression by western blotting and RT-PCR in both A549 and SW-1573 NSCLC cell lines.

In our investigation, we observed that THC treatment attenuated EGF-induced morphological changes like cell elongation and generation of protrusions leading to the rounding and reduced motility of NSCLC cells. Other studies have reported similar morphological alterations, including retraction of neurites and cell rounding in neuroblastoma cells with THC (Cabral *et al.*, 1987). Moreover, THC was also found to significantly inhibit the EGF-stimulated transwell migration and invasion of NSCLC cells. Similar antimigratory effects of THC have been reported in some malignant lymphoma cell lines (Bifulco *et al.*, 2006).

The molecular mechanisms involved in the THC-mediated inhibition of chemotaxis induced by EGF are not well characterized. Some reports have suggested that THC mediates the transactivation of EGFR (Hart *et al.*,

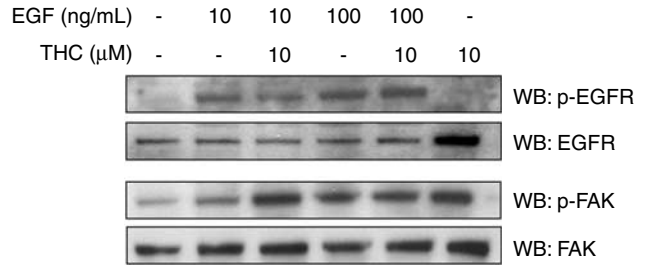


Figure 4 Effect of Δ^9 -tetrahydrocannabinol (THC) on the epidermal growth factor (EGF)-induced phosphorylation of both epidermal growth factor receptor (EGFR) and focal adhesion kinase (FAK) in A549 cells. A549 cells were incubated overnight with 10 μ M of THC in serum-free medium supplemented with 0.1% FBS and then stimulated with EGF (10 ng/ml; 100 ng/ml) or vehicle for 10 min. The blots were analysed for EGFR phosphorylation and FAK phosphorylation by western blotting with anti-p-EGFR (Tyr-1173) antibody and anti-p-FAK (Tyr-397) antibody, respectively. Total levels of EGFR and FAK protein in each lane are shown.

2004). However, we did not observe modulation of EGFR expression or phosphorylation with THC. We observed that THC enhanced the EGF-induced phosphorylation of FAK at tyrosine residue 397. THC has been previously reported to induce FAK phosphorylation at tyrosine 397 in brain hippocampal slices (Derkinderen *et al.*, 2001). Although enhanced FAK phosphorylation has been associated with increased cell migration, elevation in FAK tyrosine phosphorylation with genetically inactive protein tyrosine phosphatases has also been shown to reduce the migratory potential of cells (Lu *et al.*, 2001; McLean *et al.*, 2005).

EGFR-mediated activation of MAP kinases (ERK1/2, JNK1/2) has been reported to regulate EGF-induced cell migration and invasion (Ullrich and Schlessinger, 1990; Hauck *et al.*, 2001; Yarden, 2001). In our studies, we observed a reduction in EGF-induced ERK1/2 and JNK1/2 phosphorylation in THC-pretreated cells. This contradicts earlier reports in which THC has been shown to induce ERK1/2 activation, possibly through transactivation of EGFR (Hart *et al.*, 2004). THC-mediated inhibition of EGF-stimulated ERK/JNK activation may be responsible for the reduced migration and invasion observed in our study. A correlation between reduced ERK/JNK activation and inhibition of growth factor-stimulated cell migration has been shown with inhibitors and upon genetic inactivation of ERK1/2 and JNK1/2 (Yujiri *et al.*, 2000; Yarden, 2001).

Increased AKT phosphorylation is well documented as one of the signaling pathways involved in EGF-induced cell growth and migration (Yarden and Sliwkowski, 2001). Other investigators have reported THC-induced activation of AKT/PKB in Chinese hamster ovary cells stably transfected with the CB₁ receptor (Díaz-Laviada and Ruiz-Llorente, 2005). However, we found that THC reduced the AKT phosphorylation induced by EGF in both the A549 and SW-1573 NSCLC cell lines. This reduced phosphorylation of AKT may be due to the different cell types used in our study.

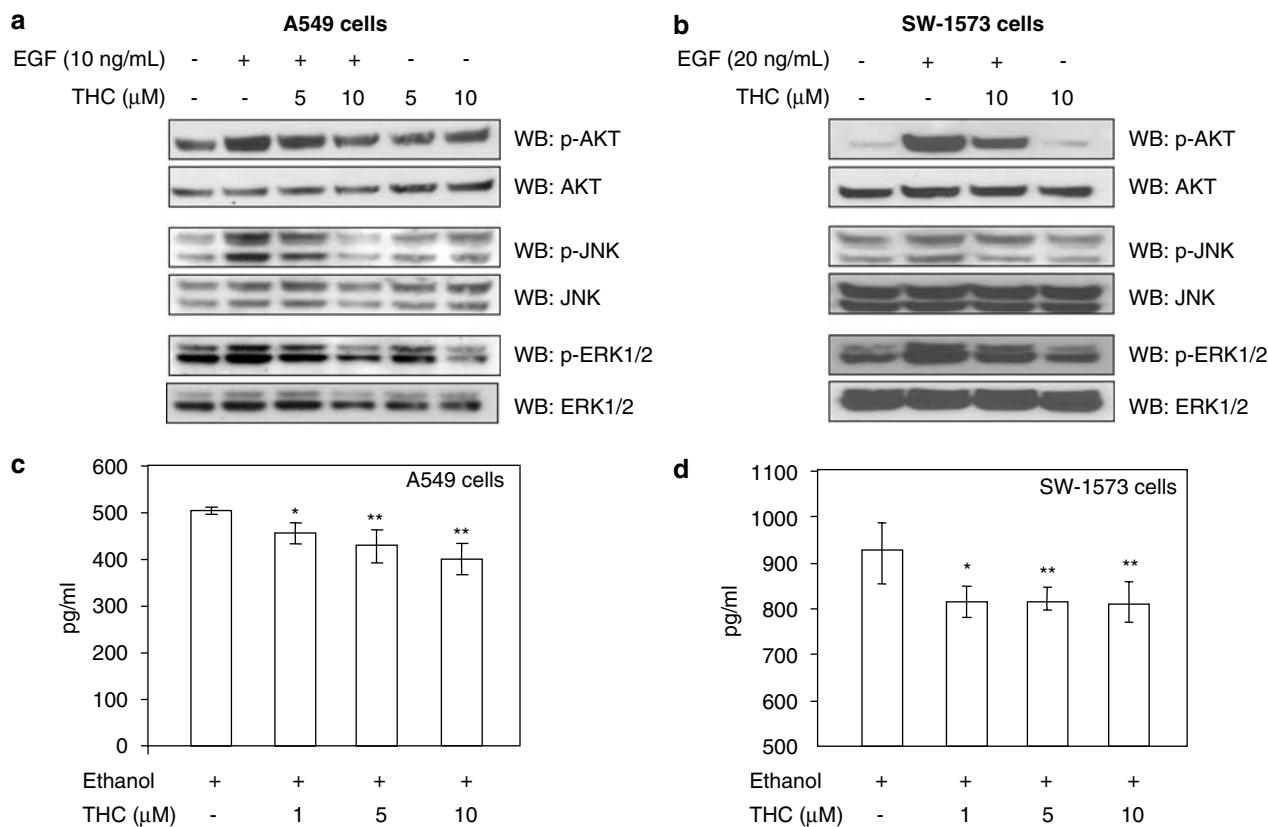


Figure 5 Δ^9 -Tetrahydrocannabinol (THC) inhibits the epidermal growth factor (EGF)-induced phosphorylations of ERK1/2, JNK1/2 and AKT as well as VEGF production in NSCLC cells. A549 (a) and SW-1573 (b) NSCLC cells were incubated overnight with the indicated concentrations of THC in serum-free medium supplemented with 0.1% FBS, and then were stimulated with EGF for 10 min. The phosphorylations of ERK1/2, JNK, and AKT were analysed in the cells by western blot analysis using the indicated phospho-specific antibodies. WB, western blot. Representative blots from three stimulations are shown. VEGF production was inhibited in A549 cells (c) and SW-1573 cells (d) on treatment with different concentrations of THC for 24 h (* P <0.05; ** P <0.001; compared to vehicle-treated cells).

Our *in vivo* extension of the *in vitro* findings further confirmed the antitumorigenic and antimetastatic properties of THC. We observed that THC significantly reduced the subcutaneous tumor growth and metastasis of NSCLC cells. These findings are in agreement with previous reports in which THC showed antiproliferative properties in glioblastomas (Caffarel *et al.*, 2006; Guzmán *et al.*, 2006). The reduction in angiogenesis (CD31 immunostaining), proliferation (Ki67 immunostaining) and phosphorylation of FAK, ERK1/2 and AKT in tumors from THC-treated animals may be responsible for the reduced tumor growth. Tumor progression has been shown to be associated with FAK, AKT and MAPK activity (Mitra *et al.*, 2006). These results correlate with the previously observed antiangiogenic effects of cannabinoids (Blazquez *et al.*, 2003).

In summary, this is the first study to report the antitumorigenic and antimetastatic properties of THC against human NSCLC. We demonstrated that THC significantly inhibited the EGF-induced growth, migration and invasion of NSCLC cell lines. Furthermore, we have shown that THC inhibited the EGF-induced phosphorylation of ERK1/2, JNK1/2 and AKT. Although THC exhibits psychoactive effects mediated

by neuronal CB₁ receptors (Piomelli, 2003), the adverse side effects tend to wane with continuous use (Kogan, 2005). Our findings suggest a therapeutic use of cannabinoids like THC in the treatment of EGFR-overexpressing, aggressive and chemotherapy-resistant lung cancers.

Materials and methods

Cell culture and treatment

A549 and SW-1573 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640, respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5 units/ml penicillin and 5 μ g/ml streptomycin under standard cell culture conditions at 37°C and 5% CO₂ in a humid environment. For the dose-response studies, the cells were treated overnight with two concentrations of THC (5 and 10 μ M in 0.1% cell medium) before EGF stimulation (10 ng/ml: A549 cells and 20 ng/ml: SW-1573 cells). Control cells were treated and stimulated with vehicle (ethanol) alone.

RT-PCR analysis for CB₁ and CB₂ expression

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA, USA). cDNA was obtained using Reverse

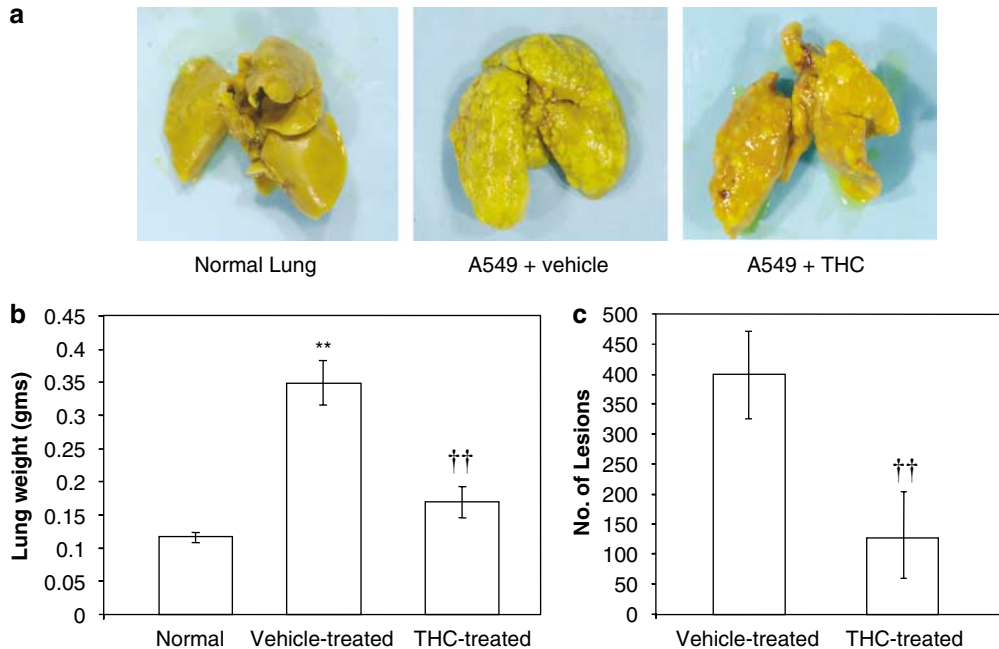


Figure 6 Δ^9 -Tetrahydrocannabinol (THC) treatment inhibits the metastases of lung tumors in SCID mice. A549 cells (1×10^6) were injected intravenously into immunodeficient SCID mice. Experimental mice were given THC (5 mg/kg body weight) daily for 28 days starting 24 h after injection of the cells. At the end of the experiment, the animals were killed and lungs were harvested and weighed before evaluation of the metastatic lesions. (a) An illustration of metastatic lesions in murine lungs from different experimental groups. THC-treated mice showed both significantly reduced lung weight (b) and less metastatic lesions (c) in comparison to the vehicle-treated animals ($n=6$ for each experimental group) (**†† $P<0.001$; compared to vehicle-treated and normal animals, respectively).

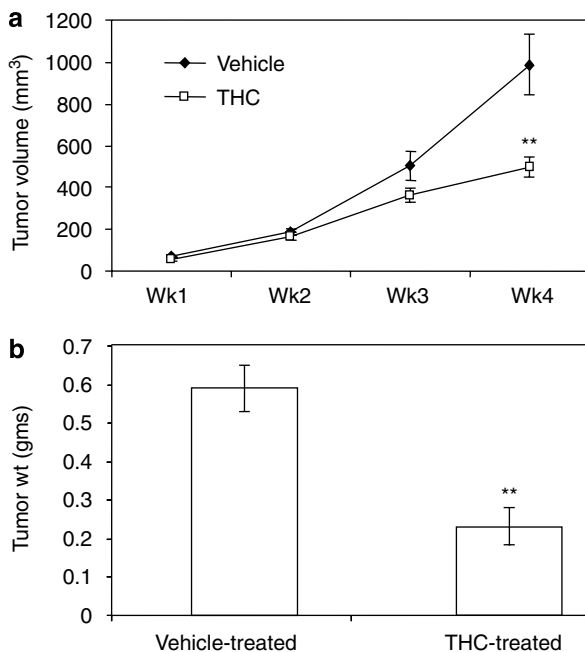


Figure 7 Δ^9 -Tetrahydrocannabinol (THC) treatment inhibits the xenograft growth of lung tumors in SCID mice. A549 cells (3×10^6) were injected subcutaneously into the right flank of immunodeficient SCID mice. THC was given at a dose of 5 mg/kg/day peritumorally for 21 days ($n=6$ for each experimental group) after palpable tumors were observed. Tumor size was measured every week. After 3 weeks, significant decreases in tumor volume (a) and tumor weight (b) were observed in the THC-treated animals compared to the vehicle-treated animals (** $P<0.001$; compared to vehicle-treated animals).

Transcriptase (Roche, Applied Sciences, Germany). Primer sequences were CB₁ (sense), 5'-GCCTGGCGGTGGCAG ACCTCC-3'; CB₁ (antisense), 5'-GCAGCACGGCGATCAC AATGG-3'; CB₂ (sense), 5'-CATGGAGGAATGCTGGGT GAC-3'; CB₂ (antisense), 5'-GAGGAAGGCGATGAACAG GAG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense), 5'-GGGAAGCTCACTGGCATGGCCTTCC-3' and GAPDH (antisense), 5'-CATGTGGGCCATGAGGTCCAC CAC-3'.

Transwell migration and invasion assays

Transwell migration and invasion assays were conducted using modifications of the method described by the manufacturer (BD Biosciences, San Jose, CA, USA). Briefly, the cells were stimulated with EGF in serum-free medium in the presence of THC or vehicle for 6 h (migration studies) and 24 h (invasion studies). The top chambers of the transwells coated with fibronectin (25 μ g/ml; Upstate Biotechnology, Charlottesville, VA, USA) and Matrigel-precoated 24-well invasion chambers (BD Biosciences) were loaded with cells (100 μ l of 1×10^6 cells/ml for migration and 0.5 ml of 1×10^5 cells/ml for invasion, respectively), which were pretreated for 30 min with vehicle or THC in serum-free medium (DMEM + 0.1% fetal calf serum (FCS) for A549, RPMI + 0.5% FCS for SW-1573). The bottom chambers had 600 μ l serum-free medium containing EGF (10 ng/ml for A549 and 20 ng/ml for SW-1573 cells). Cells adherent to the outer surface of the transwell membrane were stained and counted in five fields per well ($\times 20$ magnification). Experiments were done in triplicates and repeated thrice.

Wound healing assay

The cells, grown to form a 100% confluent monolayer on six-well plates, were scratched to produce a 'wound' using sterile

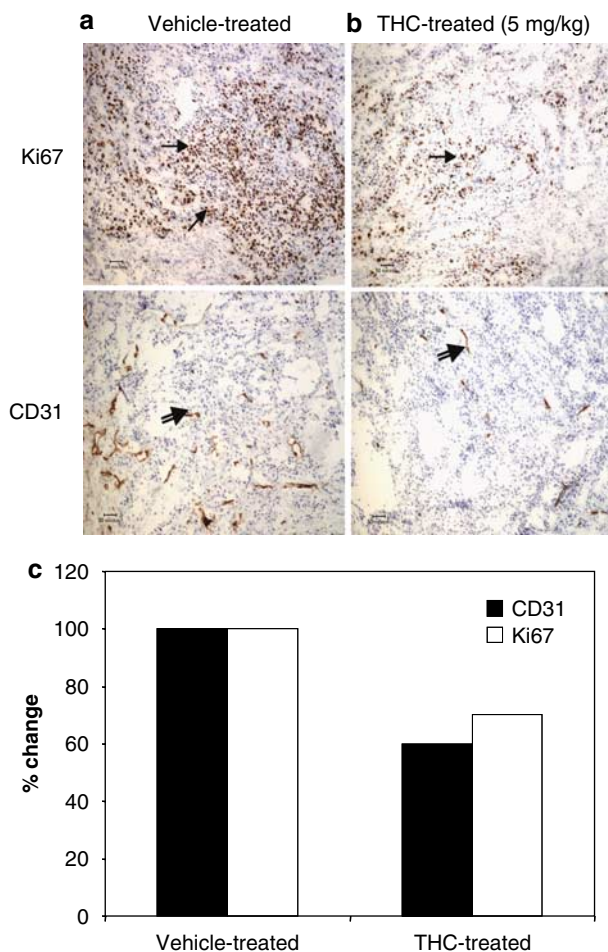


Figure 8 Δ^9 -Tetrahydrocannabinol (THC) treatment inhibits cell proliferation and vascularization in xenograft tumors in SCID mice. Representative photomicrographs of tumors showing immunohistochemical staining for Ki67 (a proliferation marker, solid arrow) and for CD31 (a marker for vascularization, open arrow) in vehicle-treated control (a) and THC-treated (b) animals. The inhibitory effects of THC are shown as the percent change in THC-treated animals with respect to the vehicle-treated controls, which were taken as 100% (c).

200 μ l pipette tips. Debris was removed from the culture by washing with serum-free medium: DMEM (0.1% FBS) for A549 and RPMI (0.5% FBS) for SW-1573 cells. The cells were then cultured in the presence or absence of THC in serum-free medium along with EGF (10 ng/ml for A549 and 20 ng/ml for SW-1573 cells) for 72 h. The images were recorded using a photomicroscope (Nikon) and cell migration was quantitated with reference to the control using Scion Image software (Alpha 4.0.3.2).

Protein isolation and western immunoblotting

Cells were lysed and processed for western blotting as described previously (Ghosh *et al.*, 2006). Tumor samples were homogenized in cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA). Primary antibodies directed against EGFR, phospho-EGFR, ERK1/2, phospho-ERK1/2, AKT, JNK1/2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-CB₁ and anti-CB₂ (Affinity Bioreagents, Golden, CO, USA), phospho-FAK(pY397) (Biosource, Camarillo, CA,

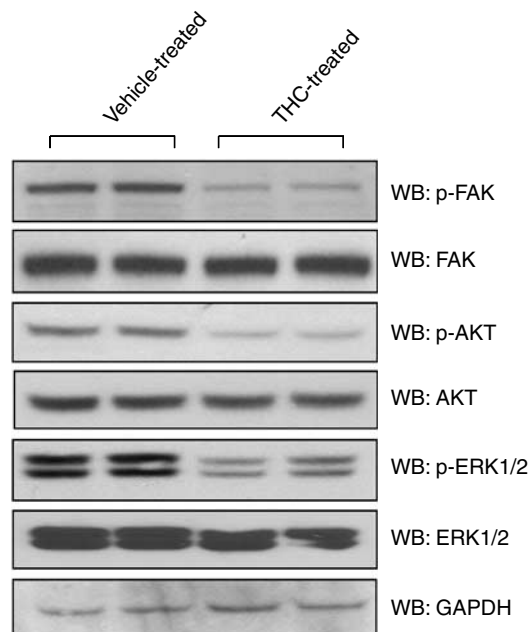


Figure 9 Δ^9 -Tetrahydrocannabinol (THC) treatment inhibits the phosphorylations of focal adhesion kinase (FAK), ERK1/2 and AKT in tumors. Western blot analysis of tumors from vehicle-treated or THC-treated (5 mg/kg body weight for 21 days) animals was performed using phospho-specific antibodies, as indicated. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

USA), phospho-AKT (ser-473) (Cell Signaling Technology) and phospho-JNK1/2 (Promega, Madison, WI, USA) were used in dilutions of 1:1000.

Mouse model of tumor xenograft growth and metastasis

Tumors were induced in immunodeficient SCID CB-17 mice (Charles River Laboratories Inc., Wilmington, MA, USA) by subcutaneous injection of 3×10^6 viable A549 cells in phosphate-buffered saline (PBS). When tumors reached an average palpable size, animals were divided into THC-treated and vehicle-treated groups ($n=6$) and were injected peritumorally with THC (5 mg/kg/day) or with vehicle in 100 μ l saline for 21 days. Tumor size was measured with calipers weekly in two dimensions throughout the study. Tumor volume was calculated as: tumor volume = length \times (width)²/2.

To grow pulmonary tumor colonies, mice were given injections of A549 cells ($1 \times 10^6/100 \mu$ l in PBS) through the lateral tail vein and, after 24 h, were treated with THC (5 mg/kg/day) or with vehicle intraperitoneally for 28 days.

Immunohistochemistry

Snap-frozen tissue sections were processed for immunological investigation. For the Ki67 immunohistochemistry, an anti-Ki67 antibody (Lab Vision, Fremont, CA, USA) was used to detect proliferating cells. Anti-CD31 (BD Biosciences) antibody was used for staining the blood vessels. Expression of the proteins was detected using standard immunoperoxidation/immunohistochemical techniques per the manufacturer's recommendations (Vector Laboratories, Burlingame, CA, USA). Cells and vessels staining positive for Ki67 and CD31, respectively, were counted in five fields per tumor

($\times 20$ and $\times 4$, respectively) and quantitated as the percent change, with vehicle-treated samples normalized to 100%.

Statistical analysis

Results were analysed using the two-tailed Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

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