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Cannabinoid Receptors, CB1 and CB2, as Novel Targets for Inhibition of Non-Small Cell Lung Cancer Growth and Metastasis

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Abstract

Non-small cell lung cancer (NSCLC) is the leading cause of cancer deaths worldwide; however, only limited therapeutic treatments are available. Hence, we investigated the role of cannabinoid receptors, CB1 and CB2, as novel therapeutic targets against NSCLC. We observed expression of CB1 (24%) and CB2 (55%) in NSCLC patients. Furthermore, we have shown that the treatment of NSCLC cell lines (A549 and SW-1573) with CB1/CB2 and CB2-specific agonists Win55,212-2 and JWH-015, respectively, significantly attenuated random as well as growth factor-directed *in vitro* chemotaxis and chemoinvasion in these cells. We also observed significant reduction in focal adhesion complex, which plays an important role in migration, upon treatment with both JWH-015 and Win55,212-2. In addition, pre-treatment with CB1/CB2 selective antagonists AM251 and AM630, prior to JWH-015 and Win55,212-2 treatments, attenuated the agonist-mediated inhibition of *in vitro* chemotaxis and chemoinvasion. Additionally, both CB1 and CB2 agonists Win55,212-2 and JWH-133, respectively, significantly inhibited *in vivo* tumor growth and lung metastasis (~50%). These effects were receptor-mediated as pre-treatment with CB1/CB2 antagonists abrogated CB1/CB2 agonist-mediated effects on tumor growth and metastasis. Reduced proliferation and vascularization along with increased apoptosis was observed in tumors obtained from animals treated with JWH-133 and Win55,212-2. Upon further elucidation into the molecular mechanism, we observed that both CB1 and CB2 agonists inhibited phosphorylation of AKT, a key signaling molecule controlling cell survival, migration and apoptosis, and reduced MMP-9 expression and activity. These results suggest that CB1 and CB2 could be used as novel therapeutic targets against NSCLC.

Introduction

Non-small cell lung cancer (NSCLC), particularly metastatic lung cancer which accounts for approximately 85% of lung cancer cases, and is the leading cause of cancer-related mortality in the United States (1). Fewer than 15% of patients survive beyond 5 years after diagnosis. Overexpression of epidermal growth factor receptor (EGFR) is associated with a majority of NSCLC and has been implicated in the process of malignant transformation by promoting cell proliferation, cell survival and motility (2,3). A series of targets and therapeutic

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strategies for the treatment of lung cancer are currently being investigated. All patients ultimately develop resistance against these agents, including chemotherapy, possibly due to abnormal signal transduction and high EGFR expression levels (4,5). Hence, abrogation of EGFR action is considered a promising strategy for anticancer therapy (6). However, recent experimental evidence suggests that cancer cells may escape from growth inhibition by using alternative growth pathways or by constitutive activation of downstream signaling effectors in presence of direct EGFR inhibitors (7). Consequently, there is a need for alternate therapy where other receptors specifically expressed on tumor cells can be targeted to abrogate EGFR-mediated signaling events directly or indirectly. In the present study, therefore, we analyzed the role of cannabinoid receptors CB1 and CB2 in NSCLC growth and metastasis.

There are three general types of cannabinoids: phytocannabinoids, THC and carbinodiol, are derived from plants; endogenous cannabinoids, 2AG and AEA, which are produced inside the body; and synthetic cannabinoids, JWH-133/JWH-015, CP-55 and Win55,212-2. These cannabinoids bind to two different cell surface G-protein coupled receptors, CB1 and CB2. CB1 receptor is predominantly expressed in the central nervous system (8,9), whereas CB2 receptor is expressed by immune cells (10). Recently, CB1 and CB2 have been shown to be overexpressed on tumor cells compared to normal cells in various types of cancers, such as breast (11) and liver (12), and therefore could be used as novel targets for cancer. In addition, several cannabinoids, including THC and cannabidiol, synthetic cannabinoid-agonists JWH-133, Win55,212-2, were shown to inhibit tumor growth and progression of several types of cancers, including glioma, glioblastoma multiforme, breast, prostate, colon carcinomas, leukemia and lymphoid tumors(13,14). In addition, synthetic cannabinoids were shown to inhibit breast tumor growth in MMTV-neu and polyoma middle T oncoprotein (PyMT) breast cancer transgenic mouse models (11,15). However, not much is known about the role of synthetic cannabinoids in lung cancer growth and metastasis.

The use of cannabinoid related drugs for medicinal purposes can be limited due to concerns of their psychotropic effects. However, synthetic cannabinoids have been shown to possess limited psychotropic effects. Also, cannabinoids have been shown to possess minimal adverse effects and are currently being used for medical purposes in various countries. Another important feature of synthetic cannabinoids is that they have been shown to be well-tolerated by *in vivo* studies and do not present any generalized toxic effects, as observed in most conventional chemotherapeutic agents. They have been used for the treatment of various disease conditions, such as wasting and vomiting associated with cancer chemotherapy, AIDS, Multiple Sclerosis and Parkinson's disease (16).

In the present study, we analyzed the expression of CB1 and CB2 receptors in human lung cancer patient samples over their normal counterparts. We further analyzed the activity of CB1 and CB2 synthetic agonists on NSCLC migration *in vitro* as well as *in vivo* growth and metastasis. We also determined the CB1/CB2 mechanisms that regulate tumor growth and migration of NSCLC. This study provides novel insights about the role of cannabinoid receptors CB1 and CB2 in NSCLC growth and metastasis.

Materials and Methods

Cell culture

Human NSCLC cells, A549 cells and SW-1573 cells were obtained from American Type Culture Collection (ATCC). The cell lines have been characterized by ATCC and we have not done any further characterization. Cells were cultured and maintained in DMEM or RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5 units/mL penicillin, and 5 mg/mL streptomycin.

Cell viability and Proliferation

Cells grown for 24 h at a density of 1×10^4 cells per 100 μ l of complete medium in 96-well plate were incubated with JWH-015/Win55,212-2 (1–20 μ M) (Tocris Cookson, Ellisville, MO) for 24 h. Cell viability was determined by the CellTiter 96 Aqueous One Solution Reagent (MTS, Promega, Madison, WI), according to the manufacturer's instructions.

For proliferation assay, 2×10^5 cells/well were plated in six-well plates in the presence of different concentrations of JWH-015 or Win55,212-2 or vehicle in presence of EGF (100 ng/ml). Cells were trypsinized and counted using a haematocytometer after 24, 48 and 72 h (11), (17).

Cell Scattering assay

1×10^4 cells/well were plated in 6-well culture plates. Once they formed small colonies, they were serum starved for 24 h and pretreated with JWH-015 or Win55,212-2 for 30' prior to stimulation with EGF (10 ng/ml) and incubated for another 48 h. Cell scattering was examined by phase-contrast microscopy and photographed.

Cell migration and invasion assays

A series of assays were used to study the effect of cannabinoid receptor activation on migratory and invasive potential of NSCLC cells. In wound-healing assay, cells were plated at 70% confluence in 10% serum-DMEM. At 24 h after seeding, the monolayers were wounded by scoring with a sterile plastic 200- μ l micropipette tip, washed, and fed with serum-free DMEM (0.1% FBS). After culturing the cells in presence or absence of JWH-015 and Win55,212-2 with pertussis toxin (PTX) and EGF (10 ng/ml) for 72 h, cells were fixed with 4% paraformaldehyde in PBS for 5 min at room temperature and photographed using a low-magnification phase-contrast microscope. The extent of migration into the wound area was evaluated qualitatively.

For trans-well chamber (Corning-Costar, Lowell, MA) migration assays, 100 μ l of 1×10^6 cells were pretreated with JWH-015 or Win55,212-2 prior to antagonist (PTX, AM630 or AM251) (Tocris Cookson, Ellisville, MO) treatment and were seeded in the upper chamber of transwells in serum-free medium as described previously (10). DMEM with 10% serum or EGF (10 ng/ml) in presence of corresponding cannabinoid agonist/antagonist was added to the lower chamber. Cells that migrated into the lower chamber were counted 12 h after stimulation with EGF.

In *in vitro* invasion assay, pre-coated Matrigel 24-well invasion chambers (BD Biosciences, San Jose, CA) were used. Treatment of the cells was similar to migration assay as above. After 24 hr stimulation with EGF, cells adherent to the outer surface of the filter membrane separating upper and lower chambers were fixed and stained with Harris modified Fisher hematoxylin (Fisher, Waltham, MA) and the cells counted (11,17).

Immunoblot analysis

Extraction of proteins from cultured cells and immunoblot analysis was conducted as per standard protocols. Briefly, the cells were lysed in cell lysis buffer, extracts clarified by centrifugation at 12,000 rpm, and the supernatants subjected to immunoblot analysis. Primary antibodies directed against phospho-AKT and AKT (Cell Signaling Technology, Beverly, MA) were used in dilutions of 1:1000. After washing three times each for 10 min in 1X TBST, blots were exposed to the secondary antibody (anti-mouse or anti-rabbit IgG-HRP) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a dilution of 1:2000 and visualized using ECL chemiluminescence detection system (Amersham, Buckinghamshire, UK).

Immunofluorescence microscopy

Cells were grown on tissue-culture treated chambered slides (BD Biosciences, San Jose, CA), overnight, treated with cannabinoids and stimulated for 3 h with EGF 10 ng/ml in serum-free medium, washed and fixed with 4% paraformaldehyde, permeabilized in phosphate-buffered saline (PBS) containing 0.2% Triton, and blocked with 3% bovine serum albumin (BSA). Cells were incubated with Texas Red-labeled phalloidin (Molecular Probes, Carlsbad, CA) and FITC-labeled antivinculin (Sigma, St. Louis, MO) for 2 h at room temperature and washed. After final washes and mounting, cells were examined using a confocal microscope.

ELISA

MMP-9 levels were measured in supernatants from cells treated with different concentrations of JWH-015 and Win55,212-2 for 24 h in serum-free growth medium. Protein levels in the supernatants were assayed using a MMP-9 ELISA Kit (R&D Systems, Minneapolis, MN) following the manufacturer's instruction. Optical density was measured at 450 nm. MMP-9 concentration was calculated by comparing the data to the known standards for MMP-9 proteins.

Mouse Model Study for Tumor Xenograft Growth and Metastasis

Sub-cutaneous tumors were generated in immunodeficient SCID CB-17 mice (Charles River Laboratories Inc. Wilmington, MA) by s.c. injection of 3×10^6 viable A549 cells in PBS. To grow pulmonary tumor colonies, mice were given injections of 5×10^5 viable tumor cells through the lateral tail vein. Once the tumors reached an average palpable size in subcutaneous model and 7 days after cell injection in metastatic model, animals were assigned randomly to various groups and injected daily with different doses of cannabinoid agonists JWH-133 (1mg/kg/day) and Win55,212-2 (0.1mg/kg/day) alone and in combination with CB2 specific antagonists SR144528 (Sanofi Recherche, Montpellier, France) and CB1/CB2 non-specific antagonist AM281 (analogue of AM251 for use in animal studies) at a dose 0.1mg/kg/day peritumorally or intraperitoneally for 28 days. Tumor sizes were measured with external calipers weekly in two dimensions throughout the study and calculated as: tumor volume = length \times (width)²/ 2 (11,17).

Immunohistochemistry

Lung carcinoma tissue microarray (TMA) slides were obtained from Imgenex (San Diego, CA). Antigen retrieval was performed by Heat-Induced Antigen retrieval (HIER), where slides were placed in Dako TRS solution (pH 6.1) for 25 min at 94°C and cooled for 15 min. Slides were then placed on a Dako Autostainer, immunostaining system, for use with immunohistochemistry. Slides were incubated for 60 min in CB1 or CB2 antibodies (1:200) or their respective peptides and detected using a labeled polymer system, ImmPRESS Anti-Rabbit Ig (peroxidase) Kit (Vector Laboratories, Burlingame, CA) as per manufacturer's protocol. Staining was visualized with DAB chromogen. Slides were then counterstained in Richard Allen hematoxylin, dehydrated through graded ethanol solutions and coverslipped. Sections were examined for CB1 and CB2 expression and immunoreactivity was scored as 0 (no or weak focal expression) and 1 (strong diffuse expression).

Samples from tumor xenografts of mice were dissected and embedded in OCT (Tissue-Tek, Torrance, CA). Standard immunohistochemistry techniques were used as per the manufacturer's recommendations (Vector Laboratories, Burlingame, CA) using the primary antibody against Ki 67 (Neomarkers, Fremont, CA) at a dilution:1/100 and CD31 (BD Pharmingen, Franklin Lakes, New Jersey) at a 1:50 dilution for 60 min at RT. Vectastain Elite ABC reagents (Vector Laboratories, Burlingame, CA) using avidin DH:biotinylated

horseradish peroxidase H complex with 3,3'-diaminobenzidine (Polysciences, Warrington, PA) and Mayer's hematoxylin (Fisher Scientific, NJ) was used for detection of the bound antibodies. The nuclei for Ki67 staining and CD31 expression in vessels was quantitated in 5 random microscopic ($\times 10$) fields per tumor. For TUNEL assay, frozen tumor sections from treated mice were analyzed by the terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using the In Situ Cell Death Detection Kit following the instructions of the manufacturer (Roche, Indianapolis, IN). The number of apoptotic nuclei per area was counted in 20 visual fields per section under a fluorescence microscope. The results were expressed as the percent change with vehicle-treated samples normalized to 100% (11).

Statistical Analysis

Results were analyzed using a two-tailed Student's *t* test to assess statistical significance. Values of $P < 0.05$ were considered statistically significant.

Results

Cannabinoid receptors are expressed in human lung cancers

Cannabinoid receptors have been shown to be over-expressed in different cancers such as skin and breast (18,19). However, to the best of our knowledge, their expression in lung carcinomas is not documented in the literature. Therefore, we first investigated cannabinoid receptor expression in a set of non-small cell lung carcinomas and showed that CB1 expression was seen in 24% (7/29) while CB2 was expressed in 55% (16/29) of cases. To ascertain the specificity of the cannabinoid receptor antibodies used for detection of the two receptors, antigen pre-absorption experiments were carried out with the corresponding blocking peptides. The peptides blocked anti-CB1 and anti-CB2 antibody binding, both in cancerous as well as normal tissue samples (Fig. 1).

Synthetic cannabinoids inhibit proliferation of NSCLC cell lines *in vitro*

EGFR over-expression and over-activation is reportedly associated with resistance of lung cancer to conventional chemotherapy. Hence, the effect of CB1/CB2 and CB2 specific cannabinoids Win55,212-2 and JWH-015, respectively, was analyzed on direct EGF-induced as well as serum mediated proliferation in NSCLC cell lines, A549 and SW-1573 cells. CB1 and CB2 are receptors that belong to the class of G-protein coupled receptors (GPCRs). Reports suggest cross-talks between GPCRs and EGFR, where GPCRs are shown to transactivate the EGF receptors (20). In the present study, we observed dose-dependent inhibition of proliferation of NSCLC cell line, A549 cells, with both Win55,212-2 as well as JWH-015. The CB1/CB2 agonist Win55,212-2 significantly attenuated the proliferation of A549 in response to serum and EGF by 70% and 60%, respectively (Fig. 2A). A corresponding dose of the CB2-specific agonist JWH-015 also reduced the number of viable cells significantly by 40% and 50% in a dose dependent manner in serum and EGF stimulated A549 cells (Fig. 2A). Analogous inhibitory tendencies were observed on the proliferation of SW-1573 cell line (data not shown).

Synthetic cannabinoids inhibit cell migration and invasion of NSCLC

The ability of tumor cells to migrate from the site of the primary tumor and to invade surrounding tissues is a prerequisite for metastasis, which is the major cause of cancer related mortality. Hence, to evaluate the therapeutic potential of cannabinoid receptors as possible targets, the effects of CB1 and CB2 agonists on EGF-induced cell migration and invasiveness were investigated. It was observed that cannabinoid agonists pretreatment impaired the cells to generate initial protrusions and scatter in response to EGF resulting in a

rounded morphology (Fig. 2B). Furthermore, different *in vitro* methods such as scratch wound assay, trans-well migration and invasion assays were employed to study the effect of cannabinoid treatment on EGF-induced migration of NSCLC cell lines. As shown in Fig. 2C, Win55,212-2 and JWH-015 significantly decreased EGF-induced colonization of the wound areas. The expansion of cell population upon EGF stimulation was quantified by calculating the percentage re-colonization of the scratched wound surface over 72 h. Since CB1 and CB2 belong to the class of G-protein coupled receptors (GPCRs) (8,9), we pretreated cells with pertussis toxin (PTX), which inhibits GPCR signaling. As shown in Fig 2C, PTX pretreatment abrogated Win55,212-2 and JWH-015-mediated inhibitory effects on EGF-induced wound healing. These results implicate the involvement of cannabinoid receptors CB1 and CB2 in the inhibition of EGF-induced wound healing.

To understand the mechanism of CB1 and CB2 receptor mediated inhibition of migration, we analyzed the effect of cannabinoid receptor agonists on formation of focal adhesions induced with EGF. Focal adhesions are sites of tight adhesion and provide a structural link between the actin cytoskeleton and the extracellular matrix. Focal adhesions were visualized through immunofluorescent staining for Vinculin, a membrane-cytoskeletal protein in focal adhesion plaques. Control and experimental cells exhibit focal adhesions at the periphery upon stimulation with EGF (Fig. 3). However, treatment of the cells with JWH-015 and Win55,212-2 prior to stimulation with EGF caused significant reduction in the number of focal adhesions of the cells. Furthermore, the polymerization state of actin, visualized as stress fibers stained with phalloidin actin, another important component related to focal adhesion formation and cell migration, was also found to be decreased in the cannabinoid treated cells.

To confirm the CB1 and CB2 receptor mediated inhibitory effects on EGF-induced migration and invasion, transwell migration studies were conducted with NSCLC cells pretreated with CB1 and CB2 specific antagonists, prior to synthetic cannabinoid agonist's exposure. We observed that synthetic cannabinoid agonists inhibited EGF-induced migration and invasion of NSCLC lung cancer cells in a dose-responsive manner (Fig. 4A). Both the agonists, Win55,212-2 and JWH-015 significantly inhibited EGF-induced transwell migration as well invasion by ~50% in nano-molar doses in comparison to the corresponding vehicle treated cells. Furthermore, pretreatment with CB1 and CB2 specific antagonist AM281 and AM630, respectively, prior to the agonists treatment resulted in abrogation of the cannabinoid induced inhibitory effect on EGF-stimulated migration and invasion (Fig. 4C & 4D). The concentrations of cannabinoid receptor agonists and antagonists used in this study did not have any significant effect on the viability of the cell lines as was confirmed by trypan blue staining of the cells in upper chambers of the transwell inserts as well as by viability (MTS) assay (data not shown). In view of previous reports where cannabinoids have been reported to induce migration in different cell lines (21–23), we investigated the induction of chemotaxis in A549 and SW-1573 cells by JWH-015 and Win 55-212,2 and found that there was no increased migration over the spontaneous migration observed in control unstimulated cells (data not shown).

Synthetic cannabinoids inhibit NSCLC tumor growth and metastasis *in vivo*

Since regression in experimental animal tumor models represents an important endpoint of clinical relevance, we evaluated the ability of cannabinoids to inhibit tumor growth and lung metastasis *in vivo*. To analyze the anti-tumor effects of cannabinoids *in vivo*, tumors generated by sub-cutaneous inoculation of the highly malignant NSCLC cell line (A549) were treated with vehicle or CB2 selective agonist JWH-133 (analogue of JWH-015 used for animal studies) and CB1/CB2 agonist Win55,212-2. Furthermore, to characterize the role of cannabinoid receptors in this inhibition of growth, CB1 selective antagonist AM281 (analogue of AM251 used for animal studies) and CB2 selective antagonist SR144528 were

administered in different combinations along with the agonist. Cannabinoid administration blocked the subcutaneous growth of tumor cells *in vivo* by ~70% with JWH-133 (1mg/kg/day) and ~50% with Win55,212-2 (0.1mg/kg/day) (Fig. 5A). The attenuation of tumor growth with the cannabinoids was abrogated when the cannabinoids were administered in presence of antagonists, suggesting the direct implication of the cannabinoid receptors in this growth inhibitory effect. As the tumor growth is directly correlated to the proliferation of cells, which depends on adequate vascularization required for oxygen and nutrient supply to the dividing cells, we analyzed important cellular parameters like proliferation, angiogenesis and apoptosis within the tumor tissues. In tumors from animals treated with both CB1/CB2 and CB2 specific agonists, we observed a significant reduction in Ki67 immunostaining (marker for proliferation), as well as CD31 immunostaining (marker for angiogenesis). Besides this, an increase in apoptosis was observed, as analyzed with tunnel staining shown as percent changes with respect to the untreated control animals (Fig. 5B).

Given the inhibition of migration and invasion of NSCLC cells *in vitro*, we evaluated the effect of cannabinoid agonist treatment on *in vivo* lung metastasis in SCID mice. We observed significant surface lung metastasis resulting in increased lung weight in animals inoculated with A549 cells via lateral tail vein injection (Fig. 5C). Both CB1/CB2 agonists Win55,212-2 (~60%) and CB2 JWH-133 (~50%) treatment attenuated lung metastasis in experimental animals over control animals. Furthermore, co-administration of CB1 and CB2 selective antagonists along with agonists abrogated this inhibitory effect, as evident by the surface lung metastases and reversal of lung weight in comparison to vehicle treated animal (Fig. 5C). Of interest, no significant alterations in physiological parameters like body weight and liver weight could be ascribed to cannabinoid receptor agonist or antagonist administration, except for non-significant reduction in body weight with JWH-133 (data not shown).

Synthetic cannabinoids inhibit AKT/PKB phosphorylation and secretion of MMP9 in human NSCLC cell lines

We next sought to determine CB1/CB2-mediated signaling mechanisms that inhibit tumor growth. We observed reduction in the tyrosine phosphorylation of AKT in cells pretreated with Win55,212-2 and JWH-015 prior to EGF-stimulations (Fig. 6A, B). However, cannabinoids pretreatment did not change AKT protein level. AKT is the key survival molecule which is essential for EGF-induced cell migration, invasion and proliferation (17, 24, 25).

In our study, we also observed that both Win55,212-2 and JWH-015 significantly inhibited the secretion and activity of MMP-9 in PMA-stimulated NSCLC cell lines, A549 measured through ELISA (Fig. 6C) and zymography (data not shown). Of importance, the reduction observed in the secretion of MMP-9 was significantly inhibited when the treatment was carried out in the presence of PTX, an inhibitor for GPCRs, directly implicating the involvement of CB1 and CB2 receptors. Matrix metalloproteinase are important proteins that play critical role in cancer growth and metastasis. A significant upregulation and co-expression of MMP-9 is observed in different cancers (26–28).

DISCUSSION

Lung cancer is the leading cause of cancer-related mortality for both men and women in United States and is estimated to remain the most fatal cancer-related malignancy (1). Little improvement in the efficacy of chemotherapy has been made in the last 20 years usually attributed to the over-expression and over-activity of EGFR in NSCLC (4,29–31). However, the use of selective EGFR tyrosine kinase inhibitors (gefitinib) and monoclonal antibodies against EGFR towards the treatment of lung cancer has continuously failed (32,33).

Therefore, additional alternative therapies with low toxicity and increase efficacy should be explored. Although recent studies suggest that non-psychoactive synthetic cannabinoids possess anti-tumor effects against various tumors including breast, not much is known about the effects of synthetic CB1/CB2 agonists on NSCLC growth and metastasis. In the present study, we analyzed the anti-tumorigenic and anti-metastasis effects of CB1/CB2 agonists Win55,212-2 and CB2 agonists JWH-015 or JWH-133 on NSCLC cells. We report for the first time that CB1/CB2 synthetic agonists significantly inhibited NSCLC migration and growth *in vitro* and *in vivo*. In addition, we have shown that these effects are abrogated by CB1/CB2 antagonists indicating the direct role of CB1/CB2 receptors in regulating NSCLC growth and metastasis.

Synthetic cannabinoids exert a wide variety of biological effects by mimicking endogenous compounds (endocannabinoids). However, synthetic cannabinoids do not possess psychotropic activity compared to endocannabinoids. Furthermore, endocannabinoids are metabolically unstable as they are degraded by endogenous enzymes such as FAAH (Fatty Acid Amide hydrolase). Synthetic cannabinoids have also been shown to possess minimal adverse effects and are currently being used for medical purposes in various countries.

Synthetic cannabinoids have been shown to exert their effects through two cannabinoid-specific G-protein-coupled receptors, CB1 and CB2. Synthetic cannabinoids, especially JWH, has been shown to specifically bind to CB2 receptor while endocannabinoids bind to CB1/CB2 and other G-protein coupled receptors, especially transient receptor potential channels (TRPV1). In this study we have shown for the first time overexpression of CB1 and CB2 in primary human lung tumor samples by TMA. The expression of CB1 (~24%) and CB2 (~55%) receptors was higher in NSCLC compared to normal lung tissue. Recently CB1 and CB2 also have been reported to be overexpressed in various cancers, such as prostate, skin and hepatocellular carcinoma (12,14,19).

In the present investigation, we show for the first time that synthetic cannabinoids which do not possess psycho-activity significantly inhibit *in vitro* as well as *in vivo* growth and metastasis of NSCLC cells. Pretreatment of NSCLC cells with CB2 and CB1/CB2 agonists, JWH-015 and Win55,212-2 respectively, prior to stimulation with epidermal growth factor (EGF) significantly inhibited EGF-induced proliferation, migration and invasion in NSCLC cell lines. Although conflicting data is available regarding modulation of migration with cannabinoids based on the cell type, not much is known about the role of synthetic cannabinoids in blocking EGF-induced migration (17,34). The *in vitro* inhibitory effects were further confirmed *in vivo*, in murine models of xenograft tumor growth and lung metastasis where SCID mice were injected with human lung adenocarcinoma cell line A549. Treatment of animals with JWH-133 and Win55-212, 2 resulted in significant inhibition of tumor growth and metastases in comparison to animals treated with vehicle alone. Win55,212-2 showed similar results with doses 0.1mg/kg/day and 1mg/kg/day, whereas JWH-133 was more effective at a higher dose of 1 mg/kg/day rather than 0.1mg/kg/day (data not shown). This may be attributed to the fact that Win55,212-2 acts through both CB1 and CB2 receptors whereas JWH-133 being CB2 specific ligand acts only through CB2 receptors. A simultaneous delivery of these agonists along with the CB1 and CB2 specific antagonists, AM281 and SR144528 respectively, abrogated the growth and metastasis inhibitory effects observed in the synthetic agonist only treated groups. This suggests that the growth inhibitory effects of synthetic agonists are mediated by CB1 and CB2 receptors. Direct role of CB1 and CB2 receptors in inhibiting growth of different cancers by cannabinoids have been reported (19,35,36).

The molecular mechanism of cannabinoid-mediated inhibition of tumor growth and metastasis is not well-known. Although Δ^9 -tetrahydrocannabinol downregulates RAS-

MAPK/ERK and phosphatidylinositol 3-kinase (PI3K)-AKT cell survival pathways leading to induction of apoptosis in various types of cancers (17,37), not much is known about the role of synthetic cannabinoids in lung cancer. We observed that CB1/CB2 and CB2 synthetic agonists significantly reduced the phosphorylation of the key signaling molecule AKT. AKT phosphorylation is known to play an important role in migration by regulating actin-myosin contractility and the focal adhesions formation (38,39). Reduction in phosphorylation of AKT observed with both the cannabinoids JWH-015 and Win55,212-2 might be the plausible cause for reduced migration and metastases observed in *in vivo* studies. Previous studies have shown that inhibition of AKT phosphorylation results in a global negative effect on cell motility (40,41). We also observed an inhibition in focal adhesion formation in response to EGF and serum stimulation in NSCLC cells along with a decrease in stress fibers. Focal adhesions are the primary sub-cellular macromolecules, the closest contacts between cells and the extracellular matrix (ECM). Focal adhesion component-mediated signaling has been shown to regulate growth and migration. During cell migration, focal adhesions are formed toward the leading edge of cells, while disassembly of adhesions occurs in the trailing edge of cells (22). This cycle of assembly and disassembly of focal adhesions is part of the process that allows the cell to move forward. These are important sites for the signaling events involved in regulation of different steps of cancer metastasis, including detachment, migration, invasion, extravasation, and proliferation of the cancer cells (42). Moreover, the transformation of epithelial cells into invasive carcinomas also depends on re-organization of the actin cytoskeleton that leads to stress fiber assembly (43). Our results suggest that Win55,212-2 and JWH-015-mediated effects on AKT, focal adhesion and stress fiber may lead to reduced growth of NSCLC during metastasis. Furthermore, we believe that synthetic cannabinoids do not inhibit lung metastasis by inhibiting the attachment of the cells to lung tissue as mice were treated one week after injection of cells. Therefore, synthetic cannabinoids might be inhibiting growth of these cells in lung tissue by inhibiting focal adhesions or signaling through Akt. CB1-mediated inhibition of stress fiber and focal adhesion formation in different cell types has previously been reported (44,45).

Cannabinoid-treated tumors show a significantly reduced number of proliferating cells, impaired tumor vascularization and an increase in apoptosis-mediated cell death. The anti-proliferative and anti-angiogenic effect of different cannabinoids has been observed previously in *in vitro* as well as *in vivo* studies (19,46,47). We have also shown that synthetic cannabinoids inhibit MMP-9 activity. MMP-9 has been shown to play an important role in growth and metastasis of various cancers by degrading extracellular matrix (26–28).

We show for the first time that CB1 and CB2 receptors are overexpressed in NSCLC lung patient tissue samples. Furthermore, non-psychoactive small molecular weight synthetic cannabinoids inhibited growth, migration and invasion of NSCLC cells in *in vitro* as well as *in vivo* in a mouse model. In addition, molecular mechanism of inhibition reveals synthetic cannabinoid agonists may inhibit tumor growth and metastasis by inhibiting AKT phosphorylation and formation of focal adhesion structures. Our results suggest the use of non-psychoactive synthetic cannabinoid ligands as a promising strategy for inhibiting growth and metastasis of highly resistant NSCLC. Overall, our results indicate a novel role for cannabinoid receptors CB1 and CB2 in NSCLC growth and metastasis.

Abbreviations

EGFR	epithelial growth factor receptor
CBRs	cannabinoid receptors

NSCLC Non-small cell lung cancer

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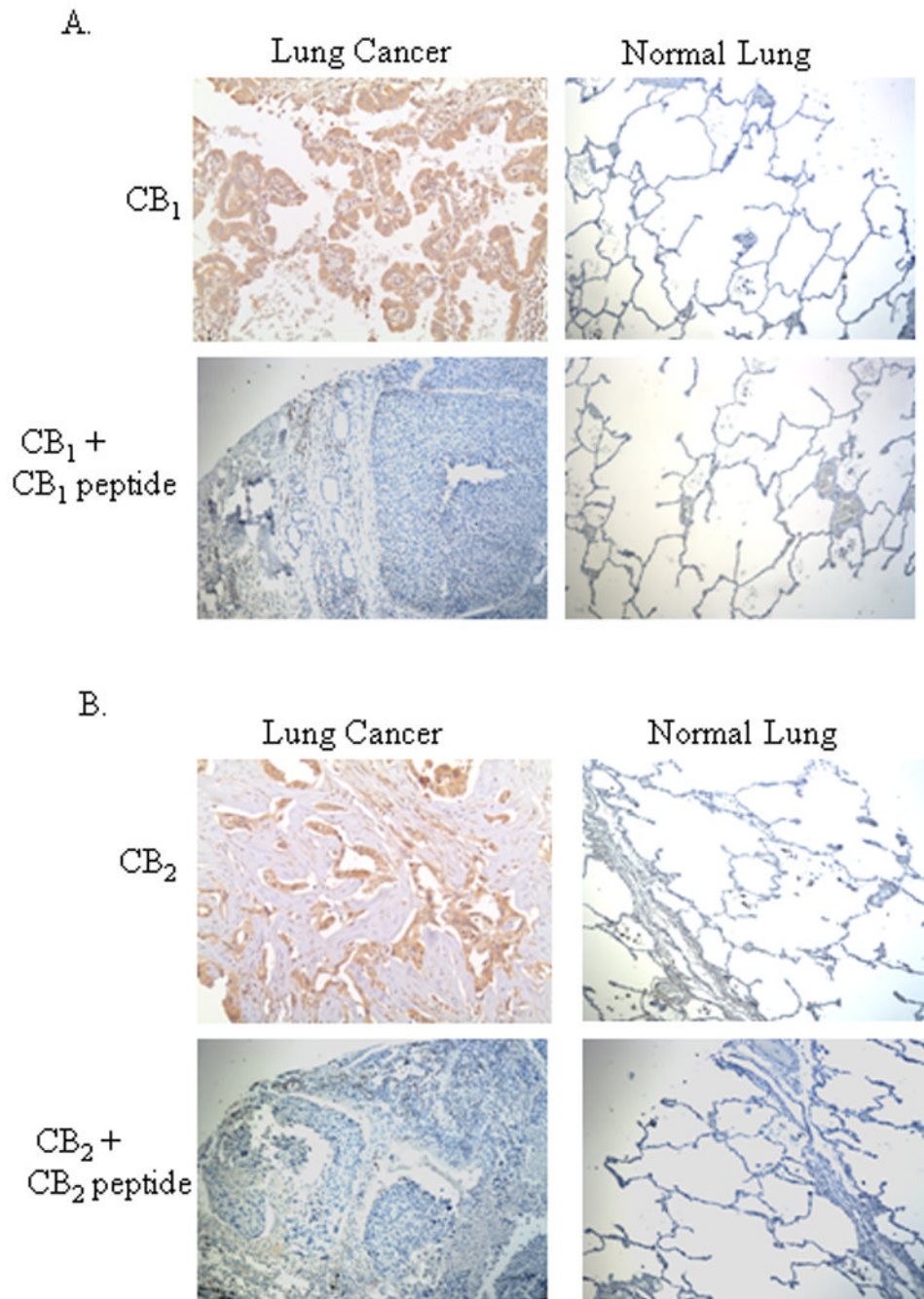


Figure 1. Expression of cannabinoid receptors in human lung adenocarcinomas and normal lung Immunohistochemical staining for cannabinoid receptor, CB1 (A) and CB2 (B) expression in human pulmonary adenocarcinoma and normal lungs. Lower panels in both (A) and (B) show controls with the Ab-blocking peptides for CB1 and CB2, respectively.

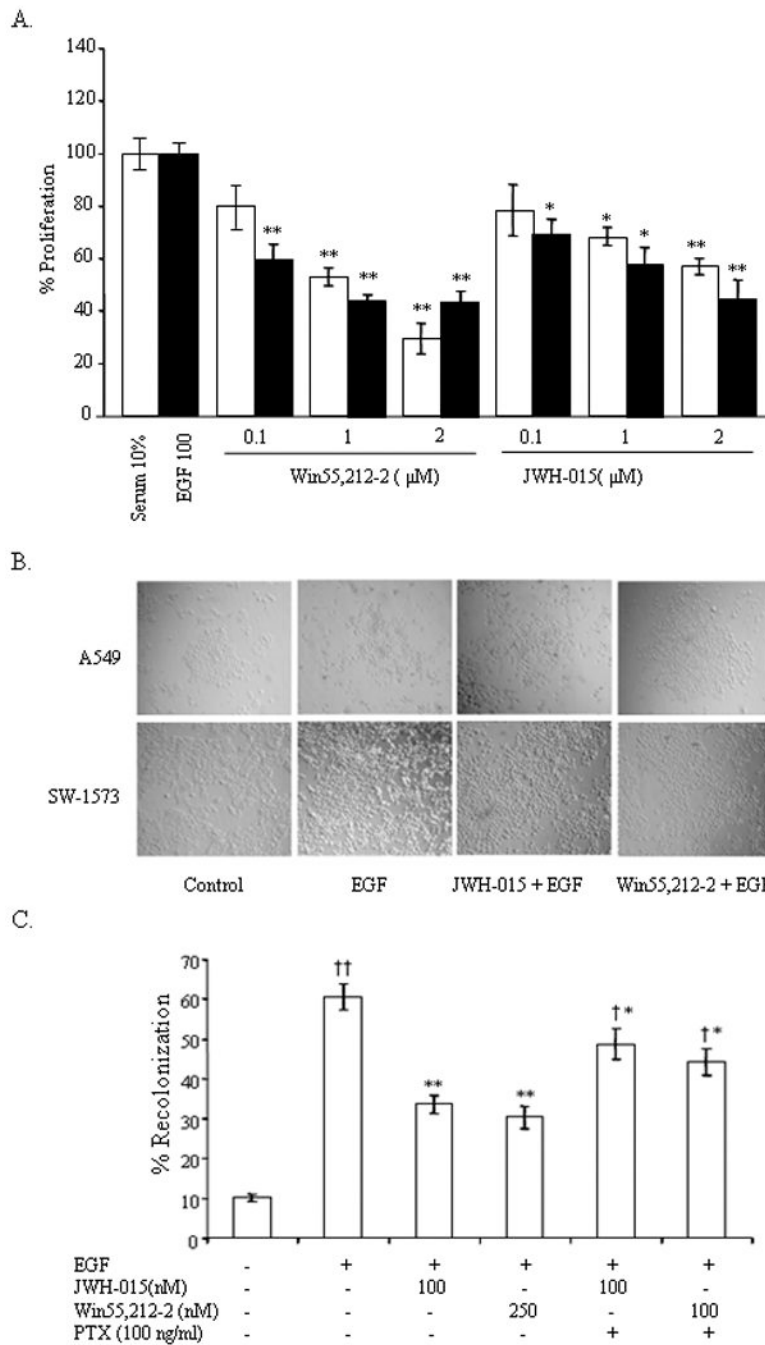


Figure 2. Inhibition of proliferation and migration in A549 cells with CB1 and CB2 specific cannabinoids

A. A549 cells were incubated in culture medium with 100ng/ml EGF (solid bars) and 10% serum (empty bars) for 72 h in the presence of different concentrations of Win55,212-2 and JWH-015 or vehicle control and then analyzed for proliferation by MTT assay. B. A549 and SW-1573 grown in 6-well culture plates were serum starved for 24 h, pretreated with JWH-015 or Win55,212-2 for 30' prior to stimulation with EGF (10 ng/ml) and incubated for 48 h. Cell scattering was examined by phase-contrast microscopy and photographed. C. Confluent layers of A549 cells were scratched with sterile tips to form wounds and were cultured in the presence of EGF+vehicle or EGF+cannabinoids in presence or absence of

GPCR inhibitor Pertussis toxin (PTX). Quantitative analysis of % wound recolonization shows a significant GPCR-mediated inhibition of the EGF-induced chemotaxis by cannabinoids treatment in the A549 cells. Data represent the mean \pm SD, representative experiments (n=3) are shown. (*, $P < 0.05$; **, $P < 0.001$; from EGF or serum stimulated, \dagger , $P < 0.05$; $\dagger\dagger$, $P < 0.001$; from cannabinoid treated)

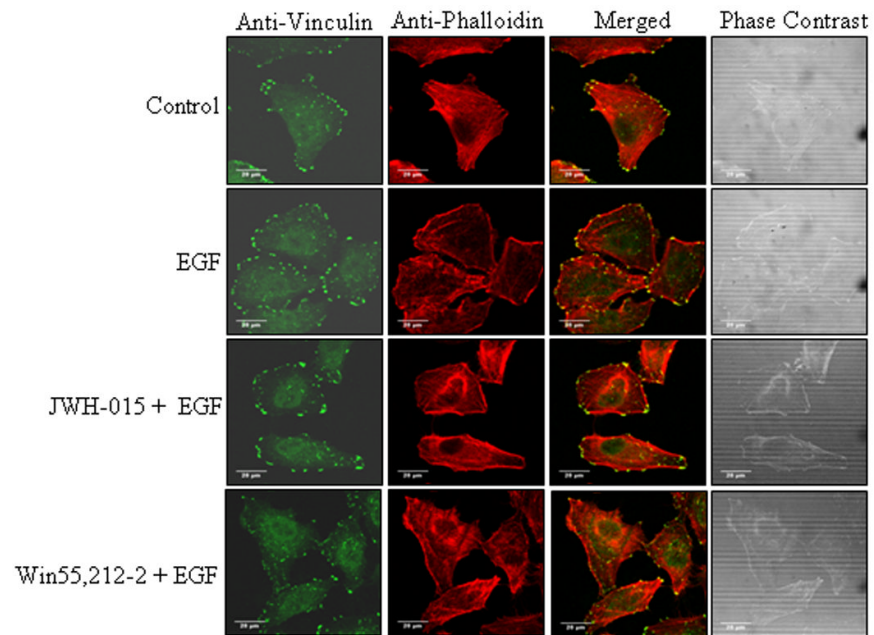


Figure 3. CB1 and CB2 receptor mediates inhibition of focal adhesions formation

Confocal microscopic visualization of JWH-015 and Win55,212-2 pretreated A549 cells stimulated with EGF (100ng/ml) for generation of focal adhesions (stained for Vinculin, green) and stress fibers (stained for phalloidin, red). JWH-015 and Win55,212-2 inhibit formation of focal adhesions and stress fibers in NSCLC cells.

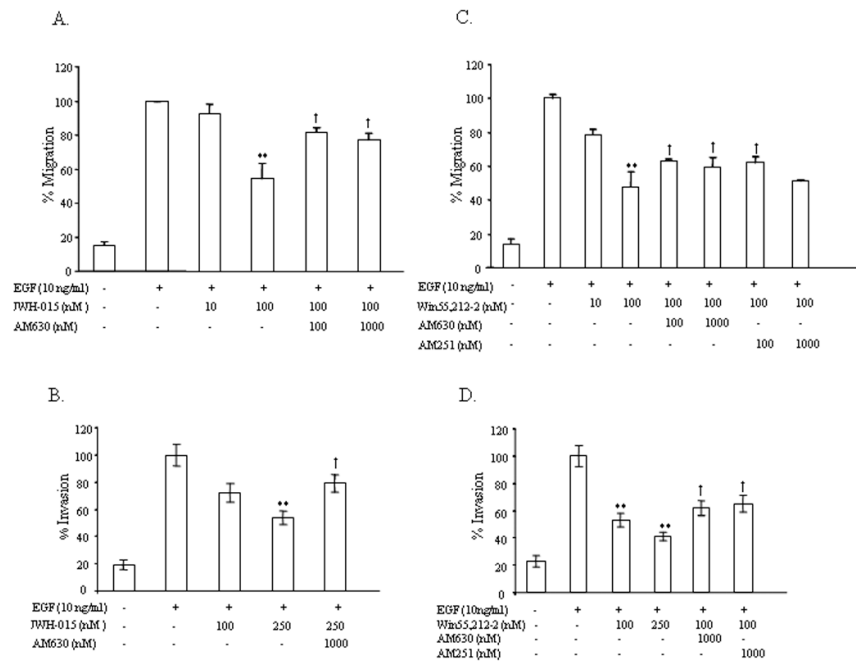


Figure 4. CB1 and CB2 receptor mediated inhibition of EGF-induced migration and invasion in NSCLC cell lines

A549 cells were treated with different concentrations of JWH-015 or Win55,212-2 or vehicle alone (ethanol) before being subjected to the EGF-induced (A, C) transwell migration and (C, D) invasion assays. Data represent the mean \pm SEM from 3 independent experiments. (*, $P < 0.05$; **, $P < 0.001$; compared to only EGF-stimulated cells, †, $P < 0.05$; ††, $P < 0.001$; to cannabinoid treated cells).

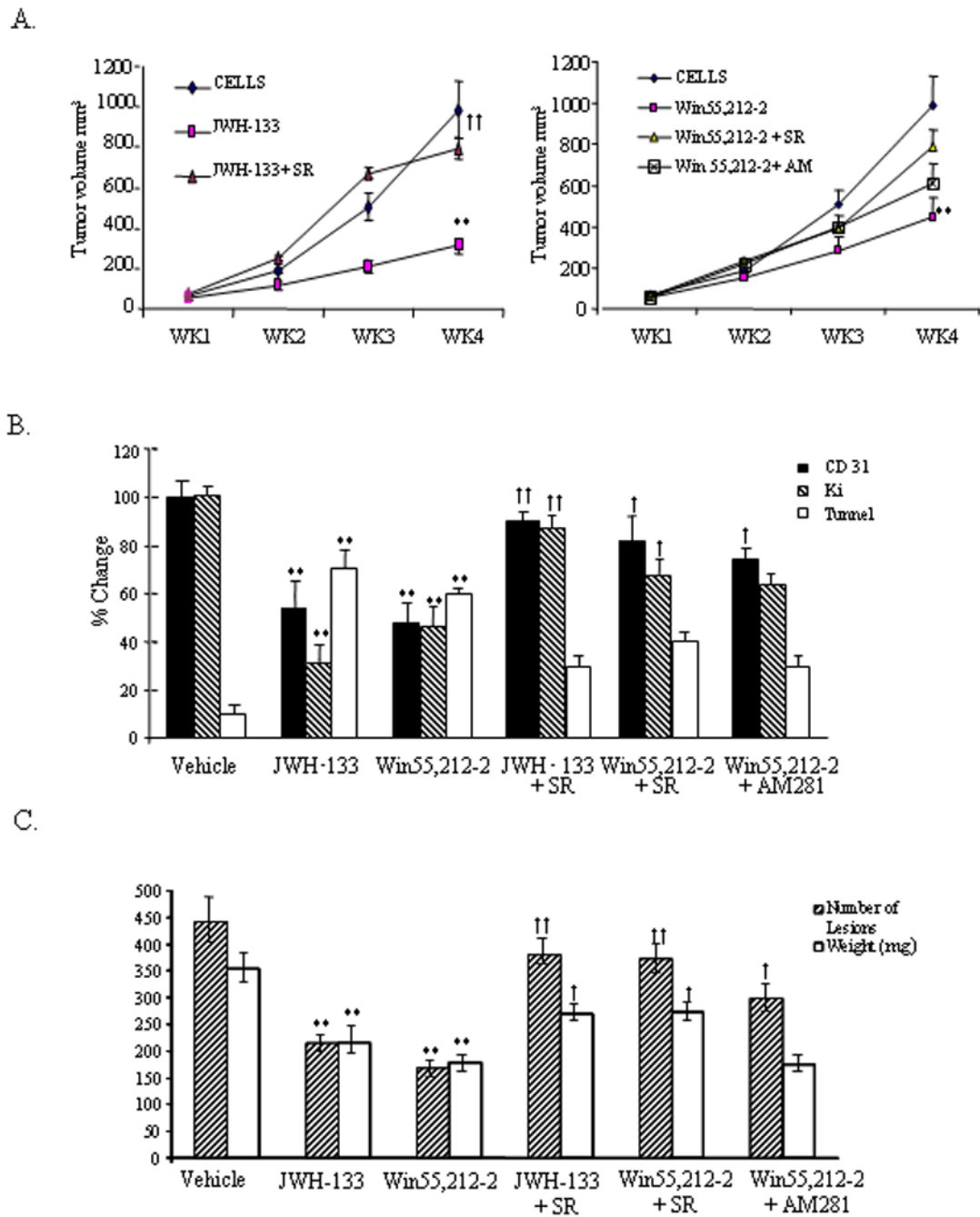


Figure 5. CB1 and CB2 receptor activation inhibits the xenograft growth and metastasis of lung tumors in SCID mice

A549 cells were injected subcutaneously (3×10^6) and intravenously (1×10^6) into immunodeficient SCID mice. Experimental mice were given JWH-133 (1 mg/kg body weight) and Win55,212-2 (0.1 mg/kg body weight) daily for 28 days starting 14 days and 1 day after injection of the cells subcutaneously and intravenously, respectively. At the end of the experiment, the animals were sacrificed; tumors and lungs harvested and weighed before evaluation of other parameters like angiogenesis, proliferation, apoptosis (in tumors) and metastatic lesions (on lungs). Receptor-mediated inhibitory effects of cannabinoids were partially reversed with animals treated with different combinations of agonist and

antagonists, AM281 (AM) for CB1 and SR (SR 144528) for CB2. A. Receptor-mediated reduction in tumor growth, B. Changes in the levels of proliferation (% staining for Ki67), vascularization (% staining for CD31) and apoptosis (positive TUNEL staining) with treatment with cannabinoids. (*, $P < 0.05$; **, $P < 0.001$; compared to untreated animals), C. Receptor-mediated inhibition of metastatic lesions and lung weight.

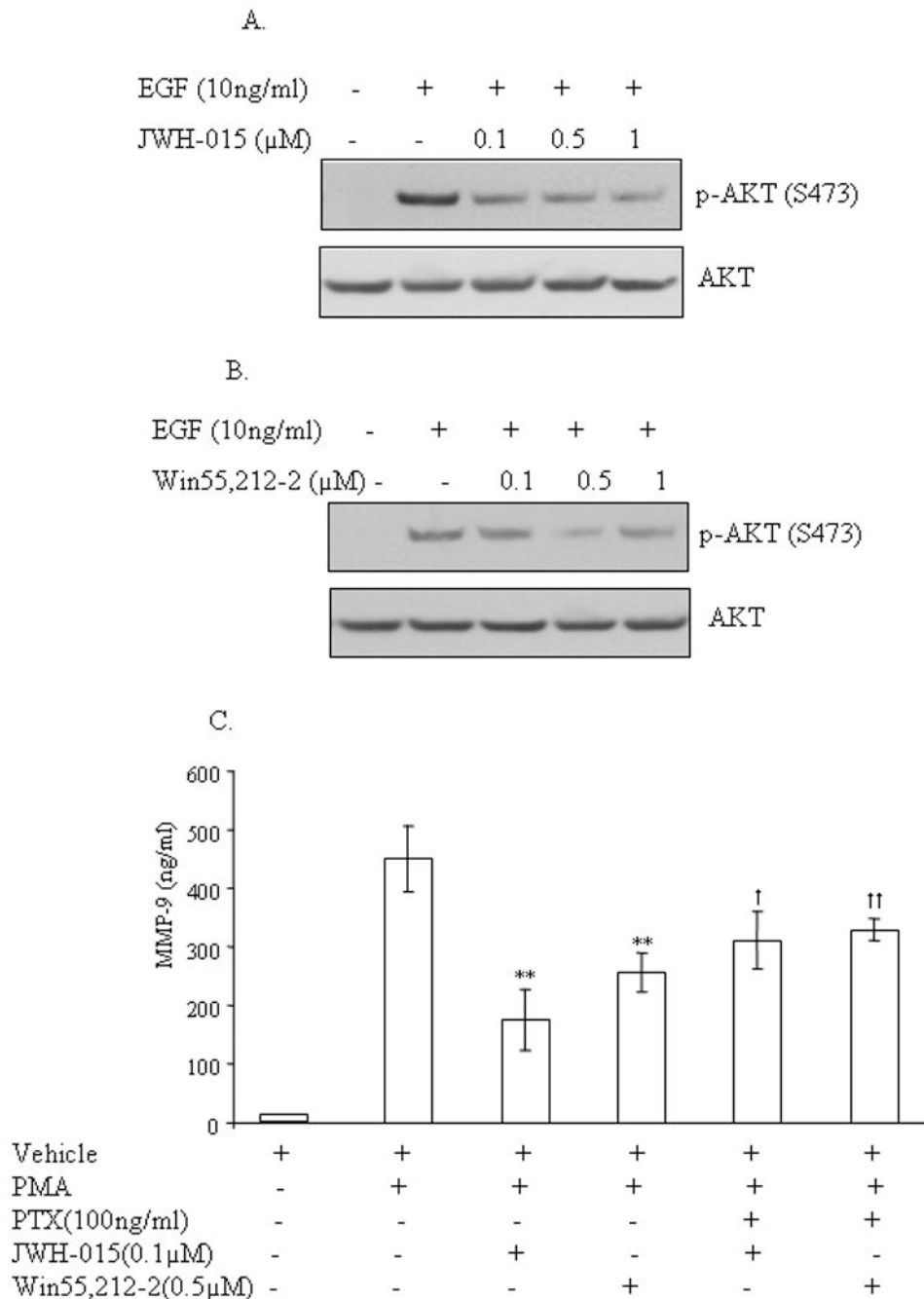


Figure 6. CB1 and CB2 receptor specific ligands inhibit the EGF-induced phosphorylation of AKT and PMA-induced secretion of MMP-9 in NSCLC cells

A549 cells were incubated overnight with different concentrations of JWH-015 and Win55,212-2 (0.1, 0.5 and 1 μ M) in serum-free medium supplemented with 0.1% FBS and stimulated for 10 min with 10 ng/ml EGF. The phosphorylation of AKT was analyzed in the cells by Western blot analysis using phospho-specific antibodies against AKT. Total protein levels in each lane are shown in the lower lane. WB: Western blot. Stimulations were repeated three times and representative blots are shown.

Analysis for secreted levels of MMP-9 by A549 cells through ELISA shows inhibitory effect of cannabinoids in comparison to the corresponding vehicle-treated cells. A549 cells

were treated with different concentrations of JWH-015 and Win55,212-2 for 24 h in serum-free growth medium. Supernatants were collected and levels of MMP-9 were measured by ELISA. (*, $P < 0.05$; **, $P < 0.001$; compared to PMA-treated or untreated cells, †, $P < 0.05$; ††, $P < 0.001$; to cannabinoid treated cells).