Original Article

Anti-proliferative and Anti-angiogenic Effects of CB_2R Agonist (JWH-133) in Non-small Lung Cancer Cells (A549) and Human Umbilical Vein Endothelial Cells: an *in Vitro* Investigation

(angiogenesis / cell proliferation / matrix metalloproteinase)

B. VIDINSKÝ[1], P. GÁL[2,5,6], M. PILÁTOVÁ[1], Z. VIDOVÁ[1,3], P. SOLÁR[4], L. VARINSKÁ[1], L. IVANOVÁ[1], J. MOJŽIŠ[1]

[1]Department of Pharmacology, Pavol Jozef Šafárik University, Košice, Slovak Republic
[2]Department of Pathological Anatomy, University of Veterinary Medicine and Pharmacy, Košice, Slovak Republic
[3]1st Department of Psychiatry, Louis Pasteur University Hospital and Pavol Jozef Šafárik University, Košice, Slovak Republic
[4]Institute of Biology and Ecology, Pavol Jozef Šafárik University, Košice, Slovak Republic
[5]East-Slovak Institute of Cardiovascular Diseases, Department for Biomedical Research, Košice, Slovak Republic
[6]Institute of Histology and Embryology, 1st Faculty of Medicine, Charles University in Prague, CzechRepublic

Abstract. Non-small cell lung cancer has one of the highest mortality rates among cancer-suffering patients. It is well known that the unwanted psychotropic effects of cannabinoids (CBs) are mediated via the CB_1 receptor (R), and selective targeting of the CB_2R would thus avoid side effects in cancer treatment. Therefore, the aim of our study was to evaluate the effect of selective CB_2R agonist, JWH-133, on A549 cells (non-small lung cancer) and human umbilical vein endothelial cells (HUVECs). Cytotoxicity assay and DNA fragmentation assay were employed to evaluate the influence of JWH-133 (3-(1,1-dimethylbutyl)-1-deoxy-Δ8-tetrahydrocannabinol) on investigated cancer cells. In addition, migration assay and gelatinase zymography were performed in HUVECs to asses JWH-133 anti-angiogenic activity. Our study showed that JWH-133 exerted cytotoxic effect only at the highest concentration used (10^{-4} mol/l), while inhibition of colony formation was also detected at the non-toxic concentrations (10^{-5}–10^{-8} mol/l). JWH-133 was also found to be able to induce weak DNA fragmentation in A549 cells. Furthermore, JWH-133 at non-toxic concentrations inhibited some steps in the process of angiogenesis. It significantly inhibited endothelial cell migration after 17 h of incubation at concentrations of 10^{-4}–10^{-6} mol/l. In addition, JWH-133 inhibited MMP-2 secretion as assessed by gelatinase zymography. The present study demonstrates the *in vitro* anti-proliferative and anti-angiogenic potential of CB_2R agonist, JWH-133, in non-small lung cancer cells and HUVECs. Our results generate a rationale for further *in vivo* efficacy studies with this compound in preclinical cancer models.

Introduction

Non-small cell lung cancer is one of the leading causes of cancer deaths worldwide (Preet et al., 2011). In spite of this fact, only limited therapeutic options are available in the current clinical practice. Although the first anticancer effect of cannabinoids (CBs) was shown in the 1970s (Grotenhermen, 2005), their use in modern medicine has remained controversial until today. The revolution in CB research started when the endocannabinoid system (ECS) was discovered followed by cloning of G-protein-coupled CB receptors (CBR), CB_1R in 1990 (Matsuda et al., 1990) and CB_2R three years later (Munro et al., 1993). CB_1R is expressed mainly in the central nervous system (CNS) and mediates all psycho-

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Corresponding author: Peter Gál, East-Slovak Institute of Cardiovascular Diseases, Department for Biomedical Research, Ondavská 8, 040 11 Košice, Slovak Republic. e-mail: galovci@yahoo.com or pgal@vusch.sk

Abbreviations: CBR – CB receptor, CBs – cannabinoids, CNS – central nervous system, ECGF – endothelial cell growth factor, ECS – endocannabinoid system, HUVEC – human umbilical vein endothelial cell, IFN-α – interferon α , MMP – metalloproteinase, THC – tetrahydrocannabinol.

active effects of cannabinoids, whereas CB_2R is localized in the periphery and has no psychoactive effects (Rodriguez de Fonseca et al., 2005).

Since cloning of the CBRs the influence of their modulators on tumour cell proliferation has started to be investigated more intensively. Numerous studies have been published describing both anti- and pro-cancer effects of CBs (Vidinský et al., 2006). At present, the medications based on CBs are registered as useful adjuvants to conventional anti-tumour chemotherapy (Sarfaraz et al., 2008). However, still more studies point to the antiproliferative effects of CBs on tumour cells (Bifulco et al., 2006).

It is well known that the unwanted psychotropic effects of CBs are mediated via the CB_1R. From this point of view, selective targeting of CB_2R would avoid side effects in cancer treatment. This study was, therefore, aimed at assessing the effect of highly selective CB_2R agonist, JWH-133 (3-(1,1-dimethylbutyl)-1-deoxy-Δ8--tetrahydrocannabinol), on non-small lung cancer cells and on human umbilical vein endothelial cells, in which the expression of this receptor has previously been confirmed (Schley et al., 2009; Preet et al., 2011).

Material and Methods

JWH-133

JWH-133 (99 % purity determined by GLC) was a kind gift of Prof. J.W. Huffman (Clemson University, Clemson, SC).

A549 non-small lung cancer cells

A549 cells were kindly provided by Dr. M. Hajdúch (Palacký University, Olomouc, Czech Republic). Cells were routinely maintained in RPMI 1640 medium with Glutamax-I supplemented with 10% foetal calf serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml) (all from Invitrogen, Grand Island, NY) in the atmosphere of 5% CO_2 in humidified air at 37 °C. Cell viability, estimated by trypan blue (Sigma-Aldrich, St. Louis, MO) exclusion, was greater than 95 % prior to each experiment.

Isolation and in vitro cultivation of HUVECs

HUVECs were isolated, cultured, and characterized as previously described (van Hinsbergh et al., 1987; Defilippi et al., 1991). Cells were cultured on gelatin-coated dishes in cM199 (M199 medium supplemented with 10 % human serum, 10 % heat-inactivated newborn calf serum (NBCS) (all from Invitrogen), 150 μg/ml crude endothelial cell growth factor (ECGF), 5 U/ml heparin, 100 IU/ml penicillin, and 100 μg/ml streptomycin, all from Cambrex (Verviers, Belgium) at 37 °C under 5 % CO_2/95 % air atmosphere.

Colony formation assay

For colony formation assay, A459 cells were seeded in six-well plates at a density of 1,000 cells per well and allowed to adhere for 10 h before treatment. Culture medium containing variable concentrations of JWH-133 was added to the cells and incubated for 14 days. The cells were then fixed in buffered formalin (pH 7.2) and stained with 0.01 % crystal violet. The crystal violet stain was then extracted with 10% acetic acid for 60 min and read at 540 nm. Cell survival at each drug concentration was expressed as a percentage of survival of controls (no drug added).

Cytotoxicity assay (MTT)

The cytotoxic effects of the tested compounds were studied by using colorimetric microculture assay with the 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) end-point. In this assay, the amount of MTT reduced to formazan is proportional to the number of viable cells (Mosmann, 1983; Nowakowska et al., 2010). Briefly, the cells (1×10^4 of cells per well) were plated in 96-well polystyrene microplates (Sarstedt, Nümbrecht, Germany) in the culture medium containing the tested chemicals at final concentrations 10^{-4}–10^{-9} mol/l. After 72 h of incubation, 10 μl of MTT (5 $mg.ml^{-1}$) (Sigma-Aldrich) were added into each well. After additional 4 h, during which insoluble formazan was produced, 100 μl of 10% sodium dodecylsulphate were added into each well and another 12 h were allowed for the formazan to be dissolved. The absorbance was measured at 540 nm using the automated MRX microplate reader (Dynatech Laboratories, West Sussex, UK). The absorbance of the control wells was taken as 100 % and the results were expressed as a percentage of the control.

DNA fragmentation assay

Treated (for 72 h) and untreated cells (1×10^6) were washed twice with 1× PBS, calcium and magnesium free. Lysis of cells was performed in a lysis buffer containing 10 mmol/l TRIS, 10 mmol/l EDTA, 0.5% Triton X-100. Proteinase K (1 mg/ml) was added and the cells were incubated at 37 °C for 1 h. Then they were heated at 70 °C for 10 min, and after adding RNAase (200 μg/ml) the cells were again incubated at 37 °C for 1 h. Samples were transferred to 2% agarose gel and run at 40 V for 3 h. DNA fragments were visualized by a UV illuminator.

Two dimensional migration (wound healing) assay

The motility of HUVECs was assayed using wound-healing assay (Martínez-Poveda et al., 2005). Briefly, HUVECs were cultured in a 24-well plate in the cM199 medium to reach confluence. A 2 mm pipette tip was used to wound the monolayer of cells. Afterwards, the medium was replaced with fresh ECGF and human serum-free medium containing the studied compound at different concentrations in the presence of 25 ng/ml of recombinant VEGF-A (Biosource, Camarillo, CA). The wounded area was photographed at the start (t = 0 h) and at time point t = 17 h. Quantification of cell migration was performed as previously described (Cheung and Li, 2002). The percentage of remaining scratched area was

calculated after being marked and quantified by the histogram function of the Adobe Photoshop 5.5 software (Adobe Systems Incorporated, CA). The experiments were performed in duplicate wells and repeated three times.

Gelatinase zymography

Gelatinolytic activities of secreted matrix metalloproteinases (MMPs) were analysed by zymography on gelatin-containing polyacrylamide gels as previously described (Newcomb et al., 2005). Using this technique both active and latent species can be visualized.

Media were obtained by incubating cells in 10 cm^2 dishes for 48 h with 1 ml of serum-free cultivation medium to which appropriate concentration of JWH-133 was added. Phorbol 12-myristate 13-acetate was used as a positive control. The conditioned media were centrifuged for 20 min at 1000 *g* in a microfuge to remove cells and cellular debris, and samples were frozen at -80 °C until use. Samples were placed into the buffer containing 3% (w/v) SDS and 10% (w/v) glycerol and then applied to 10% (w/v) polyacrylamide gels co-polymerized with 2 mg/ml gelatin. After electrophoresis the gels were renatured in 2.5 % Triton X-100 (2 × 15 min), then incubated overnight at 37 °C in development buffer (50 mM Tris-HCl, pH 7.6; 10 mM CaCl2; 50 mM NaCl; 0.05 % Brij35 [MP Biomedicals]). Gels were stained with 0.5 % Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid for 40 min at room temperature and then destained for 1 h in 50% methanol and 10% acetic acid. Data from three independent experiments were pooled for statistical analysis.

Statistical analysis

For all experiments, mean values and standard deviations (from three experiments) were calculated. To evaluate the statistical significance observed between groups, one-way ANOVA followed by Tukey-Kramer post-hoc test were employed. Significance was considered to be present if $P < 0.05$.

Results

Colony formation assay

Compared to the control, JWH-133 significantly inhibited the capacity of producing colonies in both cell lines in all tested concentrations (10^{-4}–10^{-8} mol/l; $P < 0.001$, $P < 0.01$). These results thus suggest that cannabinoid treatment is highly effective in suppressing the colony-forming ability of human cancer cells (Fig. 1a).

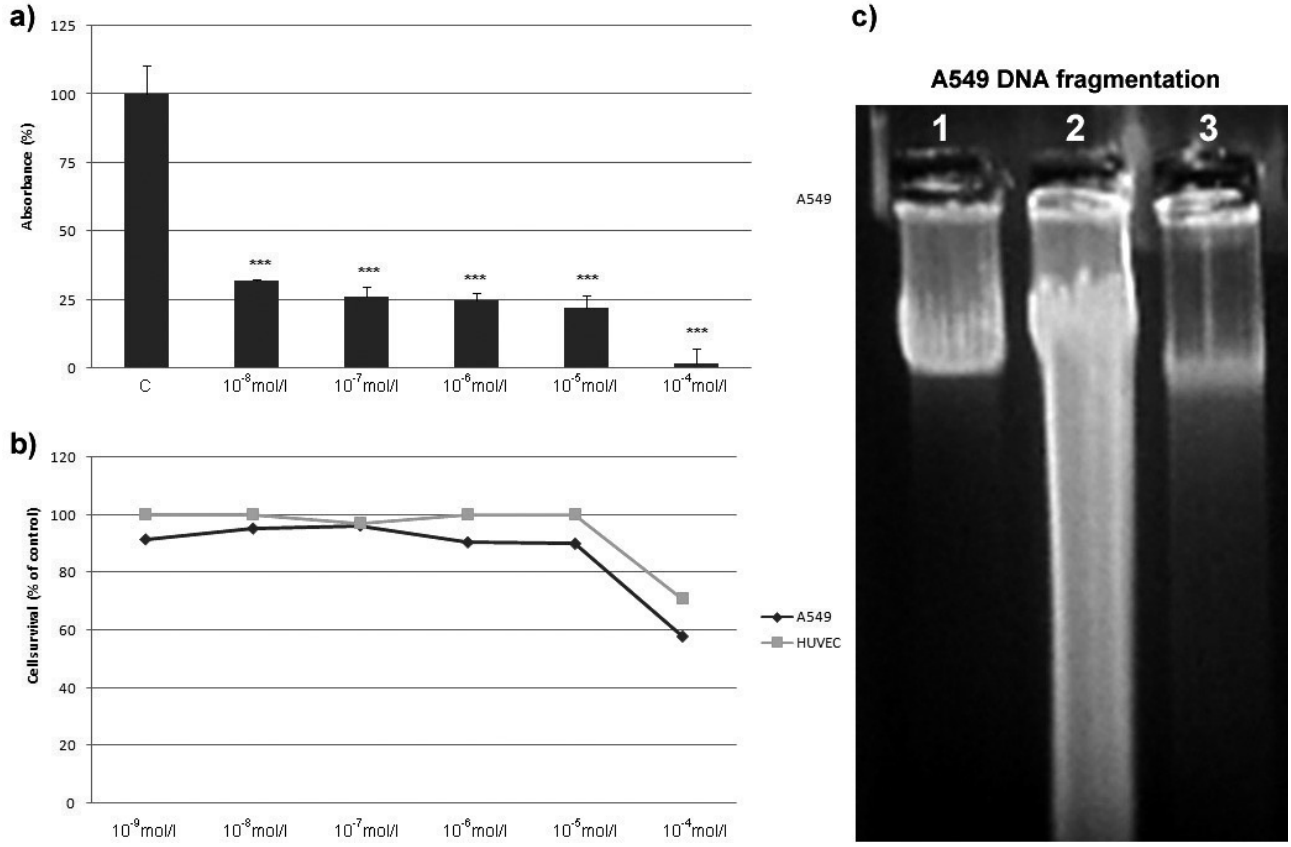

Fig. 1. **a**) Colony formation assay. Cell survival at each drug concentration was expressed as a percentage of survival of controls (no drug added). JWH-133 significantly decreased colony formation in A549 cells; **b**) Effect of JWH-133 on viability of cancer (A549) and endothelial (HUVEC) cells after 72 h incubation at JWH concentrations 10^{-4}–10^{-9} mol/l detected by MTT test; **c**) DNA fragmentation of A549 cells after 72 h incubation with JWH 133 at the concentration 10^{-4} mol/l. Lanes indicate different treatments: Lane 1 – negative control; Lane 2 – positive control (etoposide 50 µl/ml); Lane 3 – DNA fragmentation after 72 h of incubation with JWH 133.

MTT assay

Survival of tumour cells and HUVECs exposed to various concentrations of JWH-133 is shown in Fig. 1b. JWH-133 markedly decreased cell survival at the concentration 10^{-4} mol/l in A549 cells and HUVECs.

DNA fragmentation assay

The detection of internucleosomal DNA cleavage (DNA laddering) is considered to be an indicator of apoptosis. In our experiments, 72 h of incubation of A549 cells with JWH-133 at a concentration of 10^{-4} mol/l led to only weak DNA fragmentation (Fig. 1c).

Wound healing assay

After wounding with a pipette tip, solvent controls reformed a confluent monolayer within 24 h of incubation. JWH-133 was added at concentrations of 10^{-4}–10^{-8} mol/l (concentrations of 10^{-5} and 10^{-8} mol/l were non-toxic). In the presence of this cannabinoid a potent dose-dependent inhibition of endothelial cell migration was observed at concentrations 10^{-4}–10^{-5} mol/l ($P < 0.001$) and 10^{-6} mol/l ($P < 0.01$) (Fig. 2a, b).

Gelatinase zymography

The effect of JWH-133 on MMP-2 expression/activity in HUVECs is shown in Fig. 2c. JWH-133 inhibited enzyme secretion at 10^{-4} mol/l. The bands of the standard for MMP-2 reflect both the inactive and the active form of the studied enzyme. We observed only the pro-enzyme of MMP-2 in our samples, not the active form of this enzyme. Therefore, we can claim that JWH-133 treatment decreased the secretion of MMP-2 without inhibiting MMP activity.

Discussion

Despite recent advances in the early detection and therapy, lung cancer-related mortality still remains very high (Preet et al., 2011). Surgical removal of the malignancy presents in most of cases the ultimate way for complete remission of the disease (Belák et al., 2010).

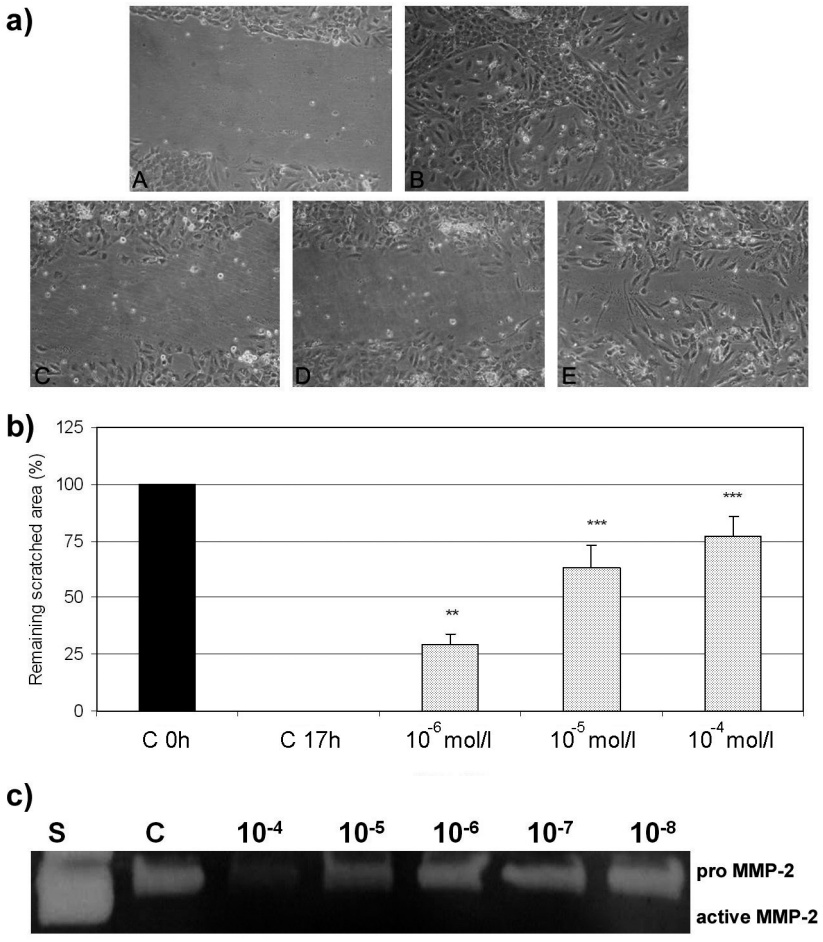

Fig. 2. **a**) Representative photographs showing the control (A – control 0 h, B – control 17 h) and the effect of JWH-133 (C – 10^{-4}, D – 10^{-5}, E – 10^{-6} mol/l) on HUVEC migration after 17 h of incubation; **b**) Percentage of remaining scratched area. Experiments were performed in triplicate; (*** $P < 0.001$; ** $P < 0.01$ vs. C 17 h); **c**) Figure shows that JWH-133 down-regulates secretion of MMP-2 at the concentration 10^{-4} mol/l. Cells were incubated in the absence (Lane 2) or presence (Lanes 3–7) of different concentrations of JWH-133, and the level of MMP-2 protein secreted into the medium was measured by gelatin zymography. This is a representative gel electrophoresis picture of one of three independent experiments with similar results; (S – standard MMP-2; C – control; JWH-133 concentrations in mol/l (10^{-4}–10^{-8})).

Therefore, there is an emerging request for alternative therapies with increased efficacy. For the first time, Munson et al. (1975) reported lung adenocarcinoma growth retardation following oral administration of CBs. Later, it was found that CBs suppressed lung adenocarcinoma growth via inhibition of DNA synthesis (White et al., 1976; Friedman et al., 1977). Another study conducted with A549 cells showed that micromolar concentrations of Δ9-tetrahydrocannabinol are able to increase expression of interferon α (IFN-α) (Berdyshev et al., 1997), an important modulator of anti-tumour immunity (Krejčová et al., 2009). In parallel, we found a significant decrease in A-549 cell viability as well as limited DNA fragmentation after treatment with JWH-133.

On the other hand, in experiments with the cancer cell line, more interesting effects of the tested agonist were observed by colony-forming inhibition than by direct cytotoxic effects observed during the MTT assay. JWH-133 significantly reduced the ability of cancer cells to create colonies also at nano-molar concentrations. Since it is well known that solid tumours do not grow as monolayers but as colonies, our results generate a rationale for *in vivo* efficacy studies with JWH-133 in preclinical animal cancer models.

At the present time, inhibition of angiogenesis in solid tumours is one of the current topics in the area of cancer research and/or treatment (Ferrara and Kerbel, 2005). Previously, it has been shown that a non-selective CBR agonist inhibits angiogenesis (Blazquez et al., 2003, 2004). Accordingly, in our current work we tested the effect of JWH-133 on endothelial cells and found inhibition of cell migration. Moreover, administration of Δ9-tetrahydrocannabinol (THC), a non-selective CBR agonist, down-regulated MMP-2 expression in gliomas generated in mice (Blazquez et al., 2008). In our study we bring new information since we have shown that JWH-133, a selective CB_2R agonist, reduces MMP-2 secretion as well.

In conclusion, our study showed that JWH-133, a selective CB_2R agonist, displays anti-tumour activity at micro-molar concentrations in A549 cells and down-regulates MMP-2 activity, thus providing novel information on targeting the second cannabinoid receptor. Since JWH-133 is a non-psychoactive agent, further *in vivo* research should be performed to facilitate its clinical use.

Acknowledgement

There is no conflict of interest.

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