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RESEARCH ARTICLE

Evaluation of anti-invasion effect of cannabinoids on human hepatocarcinoma cells

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Department of Toxicology and Pharmacology, Faculty of Pharmacy, and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran

Abstract

Context: Cancer is a disease characterized by abnormal growth of cells. One of the most common types of liver cancers is called hepatocellular carcinoma (HCC) which is highly metastatic. As most of cannabinoids have shown anticancer effect against different cell lines in a number of reports, a biological investigation of two cannabinoids, CB65 (CB2 receptor agonist) and ACEA (CB1 receptor agonist) was carried out in this study.

Objective: In an attempt to find natural products as a new solution of cancer, this study was designed to investigate the potential antitumoral and anti-invasive activity of cannabinoids on HepG2 cells and the possible roles of matrix metalloproteinase-2 (MMP-2) and MMP-9 in its action.

Materials and methods: The researchers examined the effect of various concentrations of CB65 (CB2 receptor agonist) and ACEA (CB1 receptor agonist), on the cell proliferation, viability, and invasion as well as expression of MMP-2 and MMP-9 in HepG2 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay, matrigel invasion assay, and western blotting method.

Results: The results revealed that both cannabinoids reduce cell viability, cell invasion as well as MMP-2 and MMP-9 expression in higher dose of 20 nM. Furthermore, higher concentrations of examined cannabinoids were more effective.

Discussion: These data suggest ACEA and CB65 as an option for novel treatment of hepatocellular cancer.

Conclusion: Our findings may contribute to design of new therapeutic strategies for the management of HCC.

Keywords: Hepatocellular carcinoma (HCC), cannabinoids, HepG2 cell, invasion

Introduction

Cancer is identified as malignant growth of cells which tend to proliferate in an unpredictable way. Some cancers remain in one location; others tend to spread through the blood stream or lymphatic system throughout the body. Hepatocellular carcinoma (HCC) is known as an aggressive and deadly cancer. It is one of the most widespread solid tumors and the third leading cause of cancer-related death worldwide. There has not been a noticeable improvement of the overall survival of the patients with HCC in the last two decades. The current treatments can only be applied at the early stages of the

tumor development (Vara et al. 2011). Main treatments for patients with HCC are surgery, liver transplantation, and chemotherapy. Moreover, half of the patients suffer from tumor recurrence. Thus, additional studies are necessary to find out novel targets and mechanism-based agents with increased efficacy and low toxicity for prevention and treatment of HCC.

Mainly, cannabinoids are lipid mediators obtained from the hemp plant *Cannabis sativa* that produce their effects by activating primarily two G-protein-coupled receptors: cannabinoid receptor 1 (CB1), which is plentifully found in the brain, and cannabinoid receptor

Address for Correspondence: Prof. Seyed Nasser Ostad, Department of Toxicology and Pharmacology, Faculty of Pharmacy, and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran. Tel/Fax: +9866959105. E-mail: ostadnas@sina.tums.ac.ir

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2 (CB2), which is mostly expressed in non-neural tissues (Qamri et al. 2009). Overexpression of cannabinoid receptors can be related to improved prognosis in prostate, skin, and HCC (Blázquez et al. 2006; Sarfaraz et al. 2006; Xu et al. 2006). Moreover, it is reported that cannabinoids inhibit tumor angiogenesis and directly induce apoptosis or cell cycle arrest in neoplastic cells (Carracedo et al. 2006a; Casanova et al. 2003; Galve-Roperh et al. 2000; Sánchez et al. 2001). Recently, many studies have indicated that cannabinoids are potentially antitumoral drugs because of their capability to reduce tumors in different animal models including glioma, (Velasco et al. 2007) breast cancer (Qamri et al. 2009; Caffarel et al. 2006), and prostate cancer (Sarfaraz et al. 2005; Olea-Herrero et al. 2009). Recent studies have also shown that the cannabinoids can inhibit HCC growth, proliferation, and invasion of the cancer cells (Giuliano et al. 2009; Pellerito et al. 2010). It was also shown that the cannabinoid administration in animals causes the regression of lung adenocarcinomas (Munson et al. 1975), thyroid epitheliomas (Bifulco et al. 2001), lymphomas (McKallip et al. 2002), and skin carcinomas (Casanova et al. 2003).

Normally, metastasis of cancer cells contains multiple processes and various cytophysiological changes, including altered adhesive capability between cells and the extracellular matrix and damaged intercellular interaction. Degradation of extracellular matrix by cancer cells through proteases such as serine proteinase, matrix metalloproteinases (MMPs), cathepsins, and plasminogen activator may lead to the separation of the intercellular matrix to promote cancer cell mobility, and may eventually lead to metastasis. Among the involved proteases, MMP-2, MMP-9, and u-plasminogen activator are most important for the degradation of base membranes, and are therefore deeply involved in cancer invasion and metastasis (Gialeli et al. 2011). The invasion of tumor and metastasis require increased expressions of MMPs (Liang et al. 2009). MMPs are a family of zinc-dependent endopeptidases which have the ability to degrade all necessary extracellular matrix components, including type IV collagen, laminin, entactin, proteoglycans, and glycosaminoglycans (Egeblad and Werb 2002; Nelson et al. 2000). MMP-2 and MMP-9 are particularly effective to degrade the basement membrane and have been previously shown to facilitate tumor invasion and metastasis. Recent reports from clinical pathological studies have demonstrated that upregulation of MMP-2 and MMP-9 mRNA levels correlate with later tumors/nodes/metastases (TNM) staging and metastasis in HCC (Chen et al. 2009) and their expression may be helpful in predicting poor prognosis in HCC patients after liver transplantation (Zhang et al. 2006). In addition, overexpression of PTEN (a tumor suppressor gene) in HepG2 cells results in cell growth suppression as well as MMP-2- and MMP-9-related suppression of cell invasion (Tian et al. 2010). These results suggested that MMP-2 and MMP-9 play an important role in HCC invasion and metastasis.

However, the mechanism by which MMP expression is controlled in HCC remains unclear. Based on these evidences, present study was designed to investigate the effect of two cannabinoids, ACEA, a selective cannabinoid CB1 receptor agonist and CB65, a selective cannabinoid CB2 receptor agonist on decreasing expression of MMP-2 and MMP-9 and invasion of HepG2 cells to find a new option for treating HCC.

Materials and methods

Materials

Selective agonists (ACEA and CB65) were purchased from Tocris Bioscience (Wiesbaden-Nordenstadt, Germany). Monoclonal antibody against MMP-2 and MMP-9 proteins were obtained from Sigma (Dorset, UK). PDDF membrane, Secondary Antibody (Anti-Rabbit/Mouse), and chemiluminescence detection were bought from Roche (Mannheim, Germany). Biomax films were purchased from Kodak (Rochester, NY, USA). Cell culture reagents, fetal bovine serum were purchased from Gibco (Paisley, UK). Culture flasks and plates were obtained from Nunc (Roskilde, Denmark). MTT was provided by Sigma (St Louis, MO).

Cell culture

Cells were obtained from Pasteur Institute (Tehran, Iran). They were cultured in RPMI-1640 containing 10% heat-activated fetal bovine serum, 1% penicillin (100 U/mL), and streptomycin (100 mg/mL) and were kept in humidified 5% CO₂ incubator at 37°C. Cells were subcultured every 3 days at a density of 4 × 10⁶ cells/flask 75 cm². Subculture number 20–25 was used for the experiment.

MTT assay

Cells were cultured on 24-well plates at a density of 5 × 10³ cells/well. Reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Mossmann 1983) test was carried out at the second day after cultures were incubated 2 days with different concentration of ACEA and CB65 (0.625, 1.25, 2.5, 5, 10, 20, 40 nM), and the viability was determined by MTT assay.

Briefly, for MTT assay, at the end of treatment time, medium was discarded and MTT (5 mg/mL in phosphate-buffered saline) was added to the cells. Cells were incubated for 4 h at 37°C. After 4 h of incubation, formazan crystals were dissolved in dimethyl sulfoxide (DMSO; Sigma) and aliquots (150 µL) of the resulting solutions were transferred in 96-well plates and the absorbance was measured at 570 nm with a reference wavelength of 690 nm using the microplate reader (Biochrom Anthos 2020 Microplate Reader). Each experiment was carried out in triplicate. The amount of dye produced is proportional to the number of metabolically active live cells. Dose-response curves were plotted in computer after converting the mean data values to percentages of the control response. Then, concentrations of ACEA and CB65 (0.625, 2.5, 20 nM) were chosen for invasion assay and western blot analysis.

Matrigel invasion assay

Cell invasion assay was performed according to the Repesh method. HepG2 cells to be examined for invasion were detached from the plates, washed with phosphate-buffered saline, and resuspended in a serum-free RPMI-1640 medium (5×10^4 cells/200 μ L) with the presence or absence of ACEA and CB65 (0.625, 2.5, 20 nM) then seeded on to the upper chamber of matrigel-coated filter inserts (8- μ m pore size; Becton Dickinson, Franklin Lakes, NJ). Serum containing RPMI (500 μ L) was placed in the lower well of the chamber. After 48 h of incubation, filter inserts were removed from the wells. Cells from the upper surface of the chambers of the filter were wiped with a cotton swab. Filters were fixed for 10 min with methanol and stained with crystal violet for 1 h, and then the cells that invaded the lower surface of the filter were counted by counting 10 random fields under an inverted microscope (Repesh 1989).

Western blot analysis

HepG2 cells which were treated by ACEA (0.625, 2.5, 20 nM) and CB65 (0.625, 2.5, 20 nM) for 48 h harvested from culture flasks at passage 20–25, collected in phosphate-buffered saline, centrifuged at 400g for 5 min. The sample was resuspended in lysis buffer RIPA-L solution (Tris 1 M, $MgCl_2$ 1 M, KCl 1 M, Dithiothreitol (DTT) 0.1 M, phenylmethylsulfonyl fluoride (PMSF) 0.1 M, np40 100%, Sodium dodecyl sulfate (SDS) 10%, Na-deoxycolate, Leupeptine 500 μ g/mL) (Ghahremani et al. 2000).

The suspension was passed twice through 25G needle to shear the DNA and transferred to precooled microfuge tube. PMSF (10 μ g/mL) was added. The suspension was incubated on ice for 30 min, centrifuged at 6708g (10,000 rpm) for 10 min at 4°C. The supernatant was transferred to a clean precooled tube and stored at –80°C until analysis.

The protein extracted was determined by the method of Bradford using bovine serum albumin as a standard; 20–30 μ L (15–20 μ g) of proteins was loaded onto a 12% SDS polyacrylamide gel, using a 4% stacking gel (Sambrook et al. 1989). The proteins were then transferred on to polyvinylidene difluoride membrane in a semidry apparatus using transfer buffer (Tris 25 mM, Glycine 192 mM, Methanol 20% vol/vol at 1.5 mA/cm² for 75 min). Blots were blocked in Casein 1%, Tween-20 0.1% in TBS (Tris Buffer Saline, pH = 8) for 4 h and incubated with the mouse anti-MMP-2 and –MMP-9 primary antibodies (1:500 in Casein 1%) overnight at 4°C. After a series of washes (4 \times 10 min) with TBS, the blots were incubated with the secondary antibody (anti-mouse HRP-conjugated, 1:1000) in Casein 1% blocking solution for 1 h in room temperature. Blots were then washed, then MMP-2 and MMP-9 bands were detected using the Chemiluminescence's System (Roche). The bands were quantified by densitometric analysis using Scion software (Scion Corp, Fredrick, MD, USA) (Pellerito et al. 2010).

Statistical analyses

Statistical comparisons were carried out by unpaired *t*-test for the obtained data from HepG2 cells. The results are presented as % control \pm SD.

Results

The growth curve of HepG2 cells

Figure 1 shows that proliferation rate in HepG2 cells have decreased significantly in 216 h. Subsequently the doubling time has increased up to 168 h (Figure 1).

Effect of ACEA and CB65 on cell viability

Viability of cells were evaluated in ACEA and CB65 concentrations (0.625, 1.25, 2.5, 5, 10, 20, 40 nM) using MTT test (Figure 2). As it is seen in Figure 2A, in the treated cells ACEA could significantly decrease the cell viability in the concentration of 0.625 nM using MTT assay when compared with untreated cells. However, it has not affected the cell viability at the concentration range of 5–40 nM using MTT analysis. According to Figure 2B, the treatment with 0.625 nM CB65 could keep the cell viability in compared with untreated cells and in the higher doses increased the cell viability.

The invasive action of ACEA and CB65 on HepG2

Cannabinoids have been shown inhibitory effect on invasion of some human cancer cells. To examine whether ACEA and CB65 also have the same effect in hepatoma cells, HepG2 cells were treated with ACEA and CB65 in concentration of (0.625, 2.5, 20 nM) and then were analyzed by matrigel invasion assay (Figure 3). As it is shown in Figure 3A, the treatment with ACEA could considerably decrease the HepG2 cells invasive action in the concentration of 20 and 2.5 nM in the treated cells compared with control group. Nevertheless, it has not affected the cell invasion at the concentration

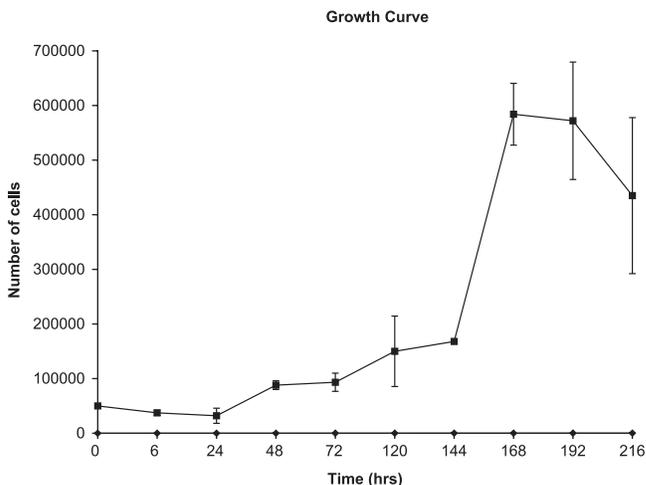


Figure 1. The growth curve of HepG2 cells using trypan blue method. Cells were cultured in 24-well plates in triplicate and growth was evaluated for 8 days. The data presented as mean \pm SD ($n = 6$).

of 0.625 nM using invasion analysis. Figure 2B indicated that, the treatment with CB65 has reduction effect on invasive action remarkably in all doses compared with untreated cells.

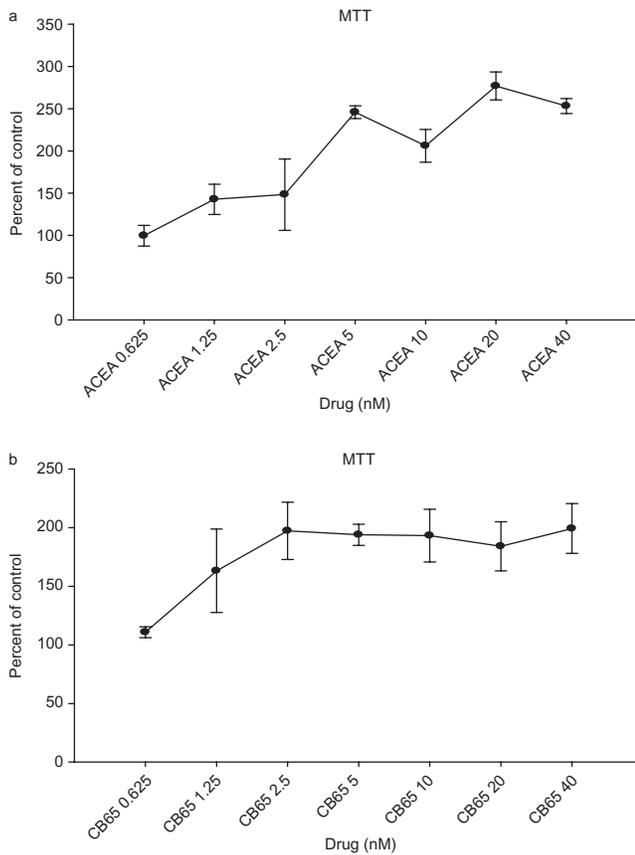


Figure 2. The effect of ACEA and CB65 on the viability of HepG2 cells. Cells were plated in 24-well plates in the presence of various concentrations of (A) ACEA (0.625, 1.25, 2.5, 5, 10, 20, 40 nM) and (B) CB65 (0.625, 1.25, 2.5, 5, 10, 20, 40 nM) for 2 days. Viability measured using MTT assay and data presented as mean \pm SD of 6 wells ($n = 6$). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide.

Effects of ACEA and CB65 on MMP-2 and MMP-9 expression

The effect of ACEA and CB65 on MMP-2 and MMP-9 expression in HepG2 cells was measured by western blot analysis (Figure 4). In Figure 4A, the 72 kD bands correspond to MMP-2 were detected and western blot analysis revealed that for the same amount of proteins load, ACEA in the concentration of 20 nM and CB65 in concentration of 2.5 and 20 nM lowered MMP-2 expression in HepG2 cells in a significant manner compared with the absence of antagonists of the CB1 or CB2 cannabinoid receptors.

In addition, according to Figure 4B, the 92 kD bands correspond to MMP-9 were detected and for the same amount of proteins load, ACEA in the concentration of 20 nM and CB65 in the concentrations of 20 and 2.5 nM decreased MMP-9 expression in HepG2 cells significantly compared with the absence of antagonists of the CB1 or CB2 cannabinoid receptors.

Discussion

HCC is a common malignant neoplasm and the third leading cause of cancer-related death worldwide. The second half of the twentieth century has been involved by growing number of this malignancy with half of this patients suffer from tumor recurrence. Therefore, more studies are necessary to determine novel targets and mechanism-based agents in order to increase efficacy of HCC treatment. Recent studies proposed cannabinoids as potential antitumor agents on a broad spectrum of human tumor cell lines (including melanoma, glioma, and breast cancer) in culture and in xenograft studies (Blázquez et al. 2006; Xu et al. 2006; Sánchez et al. 2001; Sarfaraz et al. 2008; Chen et al. 2005; Bifulco et al. 2006; Caffarel et al. 2006; Guzmán 2003).

Cannabinoids are proved to have unique-based action on their targeted cancer cells with an ability to spare normal cells. Normally, the differential expression of CB1 and CB2 receptors is the main reason for the

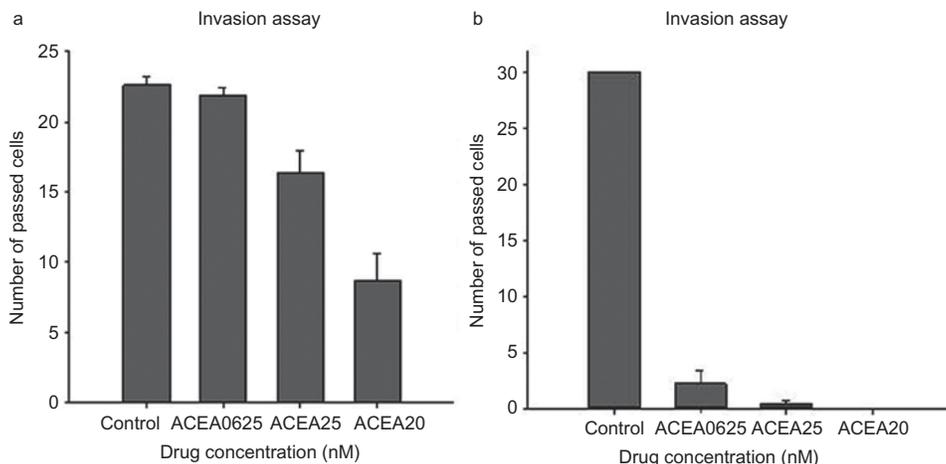


Figure 3. Effect of ACEA and CB65 on invasion in HepG2 cells. Cells were treated with (A) ACEA and (B) CB65 in concentration of (0.625, 2.5, 20 nM) for 2 days and analyzed by matrigel invasion assay and data results presented as mean \pm SD ($n = 3$).

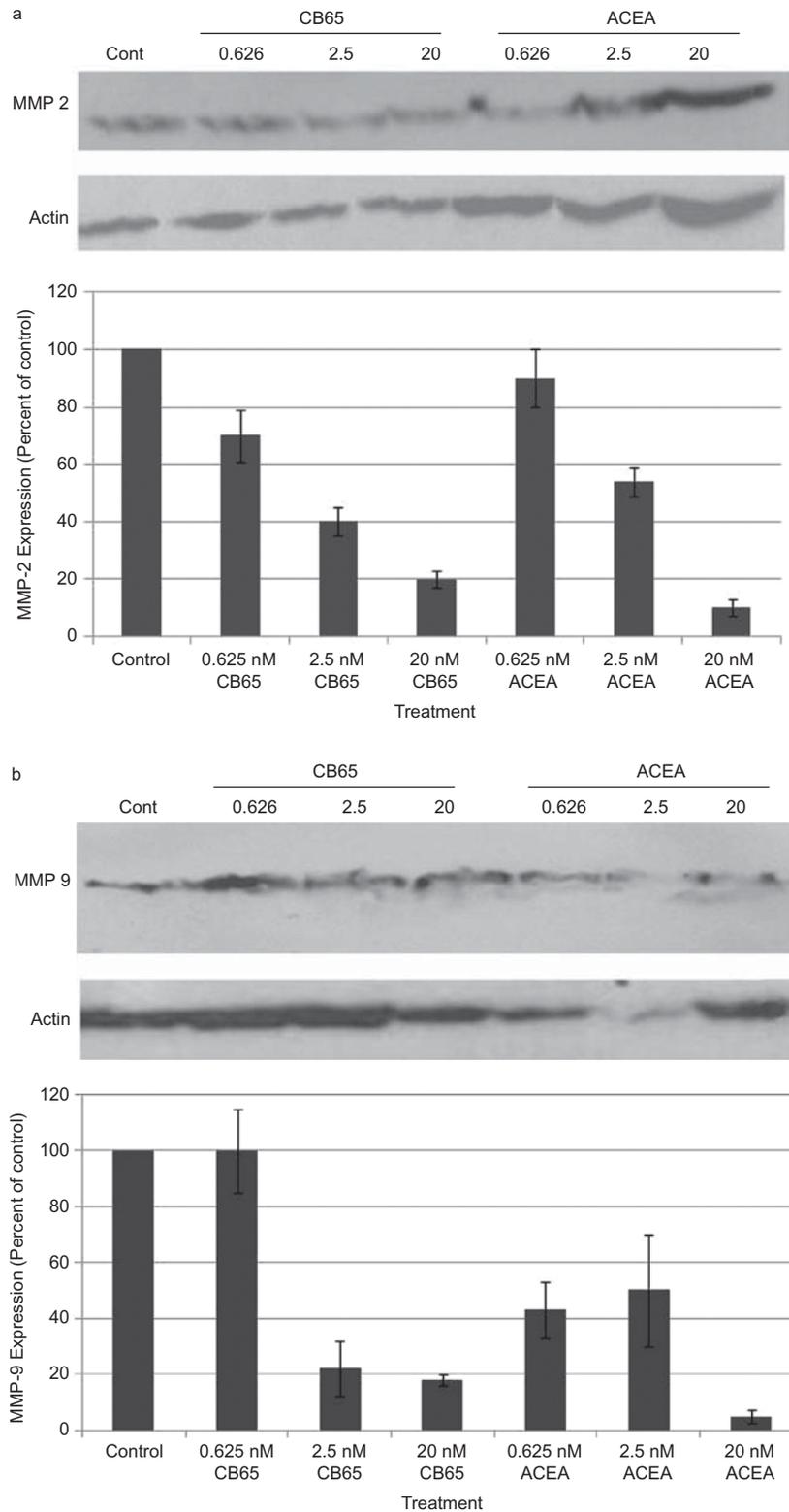


Figure 4. Effect of ACEA and CB65 on MMP-2 and MMP-9 expression in HepG2 cells. Cells were grown in the presence of (0.625, 2.5, 20 nM) ACEA and CB65 for 2 days. Protein was extracted from both treated and control cells and measured by western blotting. Immunoblotting was performed with (A) MMP-2 and (B) MMP-9 antibodies, using ACEA (lane A), CB65 (lane B). Each lane contains 30 μ L protein from cell lysate. The bands were quantified by densitometric analysis and expression was calculated as ratio to actin. Results presented as mean \pm SD for three independent experiments ($n = 3$). MMP, matrix metalloproteinase.

change in the effects of cannabinoids in different cell lines and tumor model. In this study, we observed that viability of HepG2 cells treated with ACEA and CB65 significantly decreased in comparison to the untreated cells by using MTT assay which is in favor of the results of Vara et al. study which showed that JWH-015 (a cannabinoid receptor 2 (CB2), cannabinoid receptor-selective agonist) reduced the viability of HepG2 cells (Vara et al. 2011).

We also showed that ACEA and CB65 have inhibitory effect on invasion of HCC cells. CB65 could decrease the HepG2 cells invasive action in all doses and ACEA has reduction effect on invasive action in the concentration of 2.5 and 20 nM by matrigel invasion assay.

In recent studies, molecular mechanisms for these effects were analyzed, and it was found that cannabinoids inhibited tumor cell growth and induced apoptosis by modulating different cell signaling pathways in gliomas and lymphomas, prostate, breast, lung, skin, and pancreatic cancer cells (Carracedo et al. 2006b; Gustafsson et al. 2006; Sarfaraz et al. 2006; Ligresti et al. 2006; Carracedo et al. 2006a). Antiadhesion and anti-migration effects of cannabinoids as well as their modulatory effects on metastasis has been also demonstrated (Guzmán 2003). Since invasive properties of tumors and metastasis require increased expressions of MMPs (Liang et al. 2009) and based on observed anti-invasive effect of cannabinoids in different cell lines like pancreatic tumor cells (Casanova et al. 2003), skin tumor (Carracedo et al. 2006a), and glioma (Sánchez et al. 2001) ACEA and CB65 may effective on MMPs expression in HepG2 cells. We found that ACEA in the concentration of 20 nM and CB65 in the concentrations of 2.5 and 20 nM could significantly decrease MMP-2 expression in HepG2 cells. In addition, we have shown that ACEA in the concentration of 20 nM and CB65 in the concentrations of 2.5 and 20 nM decreased MMP-9 expression in HepG2 cells.

These results are in positive correlation with recent reports from clinical pathological studies have demonstrated that upregulation of MMP-2 and MMP-9 mRNA levels correlate with later TNM staging and metastasis in HCC (Chen et al. 2009) and other anti-metastasis experiments, such as nuclear factor- κ B inhibition (Wu et al. 2009), treatment with pravastatin (Taras et al. 2007), and Spred-1 overexpression (Yoshida et al. 2006), have been suggested that the invasive and metastatic abilities of HCC are suppressed by decreased expression of MMP-2 and MMP-9. These results suggested that MMP-2 and MMP-9 play an important role in HCC invasion and metastasis.

Our results showed that the two cannabinoids, CB65 and ACEA have inhibitory effect on cell growth and invasive properties of HepG2 cells by involvement of both MMP-2 and MMP-9. This study suggest that cannabinoids with similar mechanisms of ACEA and CB65 may be considered an extra option in treatment of hepatocellular cancer if they fulfill all safety regulations.

Declaration of interest

This study was supported by grant no. 13203 from Deputy of Research, Tehran University of Medical Sciences. The authors declare no conflicts of interest.

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