Tumor-promoting effects of cannabinoid receptor type 1 in human melanoma cells

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Abstract

The role of endocannabinoid system in melanoma development and progression is actually not fully understood. The current study was aimed at clarifying whether cannabinoid-type 1 (CB1) receptor may function as tumor-promoting or -suppressing signal in human cutaneous melanoma. CB1 receptor expression was measured in human melanoma cell lines by real-time PCR. A genetic deletion of CB1 receptors in selected melanoma cells was carried out by using three different short hairpin RNAs (shRNAs). Performance of target gene silencing was verified by real-time PCR and western blot. The effects of CB1 receptor silencing on cell growth, clonogenicity, migration capability, cell cycle progression, and activation of mitogenic signals was tested. Lentiviral shRNAs vectors targeting different regions of the human CB1 gene led to a significant reduction in CB1 receptor mRNA and a near complete loss of CB1 receptor protein, compared to control vector (LV-c). The number of viable cells, the colony-forming ability and cell migration were significantly reduced in cells transduced with CB1 lentiviral shRNAs compared to LV-c. Cell cycle analyses showed arrest at G1/S phase. p-Akt and p-ERK expression were decreased in transduced versus control cells. Findings of the current study suggest that CB1 receptor might function as tumor-promoting signal in human cutaneous melanoma.

Keywords: Melanoma; cannabinoid receptor type 1; tumorigenic role.

Introduction

The endocannabinoid system is a ubiquitous complex signaling network with many biological activities both in the central and peripheral tissues (Hillard, 2015; Maccarrone et al., 2015; Minocci et al., 2011; Nieri et al., 2003a; 2003b). It is composed by endogenous ligands (anandamide and 2-arachidonoyglycerol), cannabinoid receptors type-1 (CB1) and type-2 (CB2), enzymes involved in endocannabinoid biosynthesis and metabolism, and more recently discovered members (Di Marzo, 2009).

Several studies have demonstrated that CB1 and CB2 receptor stimulation can block proliferation, motility, invasion, adhesion and induce apoptosis in various types of cancer, both in vitro and in vivo (Chakravarti et al., 2014; Pisanti et al., 2013). Nonetheless, the role of the endocannabinoid system in cancer pathophysiology appears to be controversial since several lines of evidence showed that it can be iper-activated in cancer (Velasco et al., 2015). With regard to this, much attention has been recently devoted to study the role of the endocannabinoid signaling in skin biology (Maccarrone et al., 2015). For example, it has been reported that genetic ablation of CB1 and CB2 receptors protected from ultraviolet light-induced skin carcinogenesis (Zheng et al., 2008). In apparent contrast with these findings are those obtained in in vivo models of melanoma showing that cannabinoids administration could inhibit tumor growth (Blázquez et al., 2006) and such an effect occurred in wild-type mice but not in those lacking CB1 and CB2 receptors (Glodde et al., 2015). Moreover, three studies carried out on the same melanoma cell line showed that CB1 selective agonists, mixed CB1/CB2 agonists and CB1 antagonists can decrease cell proliferation (Adinolfi et al., 2013; Blázquez et al., 2006; Carpi et al., 2015).

The current study was aimed at investigating whether genetic deletion of CB1 receptors by short harpin RNAs (shRNAs) may affect proliferation, clonogenicity and cell migration in well-characterized human melanoma cell lines. Understanding the precise role of CB1

receptors may provide further insights into molecular pathogenesis of human melanoma thus offering new potential therapeutic targets for innovative pharmacological strategies.

Materials and methods

Cell Culture

The human A375, MeWo and 501 Mel melanoma cells and the human embryonic kidney 293 cells (HEK-293T) were obtained from the American Type Culture Collection (ATCC). Melanoma cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Euroclone, Milan, Italy) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich, Milan, Italy). Normal human epidermal melanocytes (NHEM) (PromoCell GmbH, Germany) were cultured at 37 ˚C in a humidified atmosphere containing 5% CO2 in Melanocyte Growth Medium M2 (PromoCell GmbH, Germany). SSM2c cultures were obtained from the patient metastatic melanoma tissue after approved protocols by the Ethics Committee and were obtained as previously reported (Santini et al., 2014). HEK-293T cells were grown as specified (Santini et al., 2014). Cell morphology was examined under light microscopy.

Lentiviral vector production and transduction

The lentiviral vectors used were pLKO.1-puro (LV-c) (Sigma-Aldrich, Milan, Italy), as a control, pLKO.1-puro-shCB1-1 (LV-shCB1-1) to target the coding region of CB1 receptor, and pLKO.1-puro-shCB1-2 (LV-shCB1-2) and pLKO.1-puro-shCB1-3 (LV-shCB1-3) to target the CB1 3'-untranslated region. The sequences were CCGGAGCTCATTAAGACGGTGTTTGCTCGAGCAAACACCGTCTTAATGAGCTTTTTTG for LV-shCB1-1, CCGGTGCTAATGTTTCCATAGTTTACTCGAGTAAACTATGGAAACATTAGCATTTTTG for LV-shCB1-2 and CCGGTAGTATCAGAGATGTCCATTTCTCGAGAAATGGACATCTCTGATACTATTTTTG for LV-

shCB1-3. All vectors were packaged in HEK-293T cells and produced as described previously (Stecca and Ruiz i Altaba, 2009). shCB1 stable cell lines were established by transducing A375 and 501Mel cells with purified virus. Puromycin, at 2 and 0.5 μg/ml, was used to select stable transduced pools of A375 and 501Mel cells, respectively.

Real-time PCR

Total RNA was isolated and retrotranscribed, as previously reported (Carpi et al., 2014). Quantitative PCR amplifications were carried out using TaqMan probe for CB1 receptors and 18S (Thermo Fisher Scientific, MA, USA) on MiniOpticon CFX 48 real-time PCR Detection System (Bio-Rad). Data were analyzed by δ-Ct method using β-actin and 18-S as housekeeping genes. Real-time PCR was performed using SsoFast Eva Green Supermix (Ref. 172–5201; Bio-Rad, California, USA). Samples were amplified using the following thermal profile: 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 15 s, followed by annealing for 30 s and 72 ° C for 30 s, with a final step at 65 °C for 5 s. β -actin primer sequences were 5'-AACTGGAACGGTGAAGGTGAC-3' (F) and 5'-GACTTCCTGTAACAACGCATCTC-3' (R) and the annealing temperatures (Ta) was 61 °C.

Western blot analysis

The expression of CB1 receptor was evaluated in melanoma cell lysates by western blot experiments as previously described (Carpi et al., 2014). Briefly, 30 μg of protein was diluted with Laemmli sample buffer 2x, boiled for 8 minutes at 96 °C and separated by 10% sodium dodecyl sulphate-polyacrylamide electrophoresis gels. Proteins were transferred to nitrocellulose membranes by electroblotting at 4 °C. To avoid non-specific immunodetection, membranes were incubated for 45 minutes in T-TBS (20 mM Tris, 500 mM NaCl, 0.1% Tween-20, pH 8) containing 5% non-fat milk. Blots were then incubated overnight at 4 °C with a rabbit anti-CB1 antibody (1:500 dilution, PA1-745; Thermo

Fischer), anti-ERK1 (p44)/ ERK2 (p42) (1:200 dilution, Ref. sc-514302, Total ERK, Santa Cruz Biotechnology), anti-phosphotyrosine 204-ERK1(p44)/ ERK2(p42) (1:500 dilution, Ref. sc-7383, Santa Cruz Biotechnology), anti-p-Akt1-2-3 (1:500 dilution, sc-7985-R Santa Cruz Biotechnology) and with a mouse anti-β-actin (1:5000 dilution; Merck-Millipore) diluted in T-TBS containing 1% non-fat milk. After extensive washes in T-TBS, immunoreactive bands were detected by incubation with horseradish peroxidase-conjugated secondary anti-bodies anti-rabbit (Merck-Millipore, # MAB201P) and anti-mouse (Sigma-Aldrich, A4416), respectively. Signals were revealed by chemiluminescent detection (ImageQuant LAS 4000, GE Healthcare) and densitometric analysis of bands was carried out with the ImageJ64 software (http://rsb.info.nih.gov/ij/download.html).

Cell growth assay

Growth curves were carried out by seeding 3×10^3-transduced cells per well on 12-well plates. Cell growth was assessed at 3, 5 and 7 days after seeding. Cells were stained by trypan blue dye (Sigma-Aldrich, Milan, Italy) and counted with Burker's camera.

Cell colony forming assay

Melanoma transduced cells were seeded at low density (500 cells/well) in triplicate and grown for 10 days in normal serum medium. Colonies of melanoma cells stably transduced with LV-c and LV-shCB1 were washed with PBS twice, fixed with methanol for 15 min, and stained with 0.05 % crystal violet for 15 min at room temperature. Colonies containing more than 50 individual cells are counted under light microscopy. Pictures were taken at a 4× magnification.

Cell cycle assay

Cells (2×10^5) were seeded on 6-well plates, incubated in medium with 1% FBS for 24 h and then with 10 % FBS for 24 h. To determine cell cycle distribution, cells were centrifuged for 5 min at 5,000 rpm. Cells were resuspended in 500 µl of propidium iodide solution (trisodium citrate 0.1 % w/v, NP40 0.1 % w/v, PI 50 µg/ml) (Merck Millipore, Calbiochem, #537059), incubated for 30 min at 4 °C in darkness and subjected to flow cytometry analysis. Flow cytometry was performed by using a FACSCanto II (BD Bioscience). The acquired FACS data were analyzed by ModFit LT software (Verity Software House, Inc.) to determine the percentage of the population in the G0/G1, S and G2/M phase.

Cell migration assay

An IBIDI culture insert (IBIDI GmbH) consisting of two reservoirs separated by a 500 µm thick wall was used. An equal number of control and CB1-silenced melanoma cells (70 µl; 3×105 cells/ml) were added into the two reservoirs of the same insert and incubated at 37 °C/5 % CO2. After 24 h, the insert was gently removed creating a gap of 500 µm. Dishes were washed twice with PBS to remove the detached cells, and incubated using the complete growth medium. Wound closure (cells migrating into the scratched empty space) was monitored by light microscopy. Pictures were taken at a 4× magnification after 12 h. The percentage of scratch area was calculated using WimScratch program (Wimasis S.L., Córdoba, Spain).

Statistic analysis

Data were presented as mean ± standard error of the mean (SEM) from at least three independent experiments. Statistical analysis was performed by one-way or two-way ANOVA followed by Dunnetts's or Bonferroni's multiple comparison test. P values less then 0.05 were considered as statistically significant.

Results

Expression of CB1 receptor in human melanoma cells

The mRNA expression of CB1 receptors was measured in a panel of different melanoma cell lines by qRT-PCR analyses and protein levels were analyzed by Western blot. All tested cells were found to express CB1 receptor at both transcriptional and translational levels. A375 and 501Mel cells were selected for the current study since they expressed significant levels of CB1 receptor ($p< 0.05$) compared to NHEM (Fig. 1A,B). Western blot experiments showed that the levels of CB1 receptor protein expression in A375 and 501 Mel cells were comparable.

Silencing of CB1 receptor inhibits cell growth

To evaluate the role of CB1 receptor in melanoma, we knocked-down CB1 in A375 and 501Mel melanoma cells using three independent lentiviral shRNAs vectors (LV-shCB1-1, LV-shCB1-2 and LV-shCB1-3) targeting different regions of CB1. All three shRNAs led to a significant ($p< 0.05$) reduction in CB1 mRNA levels and a near complete loss of CB1 protein compared to control LV-c in both cell lines (Fig. 2). Silencing of CB1 receptor significantly decreased the number of viable cells in both melanoma cell lines, as compared to control cells (LV-c) (Fig. 3A and B).

To investigate whether CB1 depletion affects cell cycle progression, we quantified cells in different cell cycle phases by flow cytometry analysis. Cell cycle experiments showed that CB1 receptor knockdown increased the number of cells in G0/G1 phase ($p<0.01$) and decreased those in S phase in both melanoma cell lines compared to controls (Fig. 3C and D) suggesting cell cycle arrest at the G1/S phase.

Colony formation assays was performed in both A375 and 501Mel cells transduced with CB1 lentiviral shRNAs or LV-c control vectors and cell colonies were counted after 10 days

culture period. The colony-forming ability was significantly ($p<0.05$) reduced in CB1-depleted cell lines compared to controls (Fig. 4).

Silencing of CB1 receptor inhibits cell migration

Wound-healing assay on A375 and 501Mel cells transduced with different CB1 lentiviral shRNAs or LV-c were performed and data recorded at 12 h. Cell migration was significantly reduced in the three shRNA-transduced A375 and 501Mel cells as compared to LV-c (Fig. 5) suggesting a role of CB1 receptors also in the control of cell motility.

CB1 silencing decreases ERK1/2 and AKT phosphorylation

Recent data suggest an involvement of CB1 receptors in PI3K/AKT and MEK/ERK signaling pathways in retinal neurons (Kokona and Thermos, 2015). RAS/RAF/MEK/ERK and AKT signaling pathways are often aberrantly activated in melanoma, therefore we assessed the effect of CB1 silencing on phosphorylation levels of ERK1/2 and Akt by Western blot. ERK1/2 phosphorylation (Tyr204) was reduced in A375 and 501Mel cells transduced with CB1 shRNAs compared to LV-c (Fig. 6A). Phosphorylation of Akt (Ser473) was also strongly inhibited in silenced versus control cells (Fig. 6B). Total amount of ERK and was not affected by CB1 silencing (Fig. 6A and B). These data altogether suggest that CB1 receptor positively modulate MEK/ERK1/2 and AKT signaling pathways in melanoma cells.

Discussion

The present study provides evidence that CB1 receptor function may contribute to the neoplastic phenotype of human melanoma by promoting cell growth, clonogenicity, cell cycle progression, migration and activation of ERK and Akt signaling pathways. These lines of evidence were obtained using a selective stable genetic deletion of CB1 receptor with three different shRNAs in two different melanoma cell lines. Such an approach may offer

some advantages over functional studies using selective CB1 receptor agonists and antagonists because these compounds may also act through off-target mechanisms, which can make data interpretation difficult and ambiguous (Fogli et al., 2006; Pertwee, 2010; Scuderi et al., 2011). In line with this concept, a double-knockout mice model was used to elucidate the role of CB1/CB2 receptors in UV-induced inflammation and skin cancer development (Zheng et al., 2008).

In the current study, CB1 expression was assessed in several human melanoma cells by real-time PCR and by Western blot. We found that A375 and 501Mel cells had the highest levels of CB1 expression and these findings were in line with those showing the presence of the endocannabinoid system in other melanoma cells and melanocytes (Blàzquez et al., 2006; Glodde et al., 2015; Kenessey et al., 2012; Pucci et al., 2012; Scuderi et al., 2011). To our knowledge, no report has been published on CB1 expression in melanoma patients. Nonetheless, some information can be retrieved from the r2 platform by selecting the "Tumor Skin Cutaneous Melanoma" dataset (http://r2.amc.nl http://r2platform.com), where CB1 receptors were found to be expressed both at the early stages and in the advanced disease, suggesting an important role of the endocannabinoid system in the pathophysiology of human melanoma.

Our findings demonstrated that silencing of CB1 receptor induced cell cycle arrest at the G1-S transition and such an effect was not accompanied by apoptosis or necrosis. In agreement with this, the anti-proliferative effect of the CB1 receptor antagonist, SR141716, in human breast cancer cells was not accompanied by apoptosis or necrosis and was characterized by a G1/S-phase cell cycle arrest (Sarnataro et al., 2006). Furthermore, it has been reported that CB1 receptor inactivation by SR141716 induced G1 phase stasis and reduced the growth of glioma xenograft (Pisanti et al., 2015). Finally, we previously demonstrated that treatment of A375 cells with the selective CB1 receptor antagonist AM251 inhibited cell proliferation inducing G2/M cell cycle arrest (Carpi et al., 2015).

Overall, these lines of evidence reinforce the importance of CB1 receptor activation in the control of cell cycle progression.

The epidermal growth factor receptor signaling has a pivotal role in the pathogenesis of human melanoma (Bardeesy et al., 2005). It acts through two distinct pathways, the MAPK pathway and the PI3K pathway that, in turn, critically regulate G1 cell cycle progression (Torii et al., 2006). Our findings demonstrated that cell cycle arrest at the G1/S phase was paralleled by inhibition of ERK and AKT phosphorylation in CB1-silenced versus control cells and evidence supporting the role of CB1/CB2 receptors in mediating activation of ERK signaling cascades in response to UVB treatment has been provided (Zheng et al., 2008). Noteworthy, activation of the PI3K/Akt/GSK-3 signaling pathway has been related to the in vivo neuroprotective properties of cannabinoids and phosphorylation of Akt was specifically mediated by CB1 receptors (Ozaita et al., 2007). Alterations in the PI3K pathway occur frequently in human cutaneous melanomas (X. P. Zhou et al., 2000) and silencing of CB1 receptor almost abolished Akt phosphorylation in our experimental setting, suggesting the presence of a possible mechanistic link between CB1 receptor function, cell cycle progression and ERK/AKT activation in human melanoma.

The molecular mechanisms for CB1-mediated activation of ERK1/2 and AKT pathways have been clearly described in neural progenitor cells. Specifically, ERK activation results from coupling of CB1 to heterotrimeric Gi/o proteins and reduction in cAMP levels that prevents the inhibitory effect of PKA over Raf-1, whereas PI3K activation is a Gβγ-dependent mechanism {GalveRoperh:2013fe}. It is conceivable that such a signaling network is conserved in melanoma cells because both nervous cells and normal melanocytes derive from the neural crest (Shakhova, 2014). In addition, neural crest stem cells and melanoma cells have common molecular mechanisms that regulate self-renewal (Shakhova, 2014). Cell migration represents an important step for cancer spread and metastasis and our results showed that CB1 receptor function could be important in the control of cell motility.

To our knowledge, this is the first evidence showing a role of CB1 receptor in promoting melanoma cell migration and recent studies highlighted the importance of the endocannabinoid system in neuronal migration (Y. Zhou et al., 2014). These biological properties may account, at least partially, for the ability of melanoma cells to reach and penetrate the central nervous system and the consequent high percentage of patients who develop brain metastasis (i.e., 20% at diagnosis of metastatic melanoma and nearly 50% in the course of the metastatic disease) (Spagnolo et al., 2016).

In apparent contrast with this notion, several studies demonstrated the ability of cannabinoid agonists to reduce tumor growth, in vitro and in vivo. For example, methanandamide (selective CB1 receptor agonist) demonstrated to inhibit both cell proliferation and migration in breast cancer models (De Petrocellis et al., 1998; Grimaldi et al., 2006; Guindon and Hohmann, 2011; Laezza et al., 2010), whereas a decrease in the number of viable cells and cell cycle arrest at the G1-S transition with no evidence of apoptosis was reported after treatment of A375 cells with mixed CB1/CB2 receptor agonists (Blázquez et al., 2006).

To explain the complex role of CB1 receptor in human melanoma cell progression, one could assume the presence of a cannabinoid-dependent autocrine loop. In in vitro models, this loop could be characterized by the endocannabinoid release in the cell supernatant, activation of CB receptors and cell cycle progression. According to these results and assumptions, one could hypothesize that CB receptor overstimulation and down-regulation by receptor agonists or inactivation by receptor antagonists or gene silencing strategies may alter endocannabinoid system dynamics in human melanoma cells thus causing cell cycle arrest. In line with this hypothesis, a marked peak of anandamide and other endocannabinoid-like molecules at the G1 phase of the cell cycle was observed in a rat model of liver regeneration and, in the same experimental model, CB1 receptor blockade inhibited cell cycle progression (Pisanti et al., 2015). The role of CB1 receptor in the control

of liver regeneration was also demonstrated by the use of CB1 knockout mice and with wild-type mice treated with SR141716 (Jeong et al., 2008; Mukhopadhyay et al., 2010).

In conclusion, findings of the current study provide evidence on the tumor-promoting effect of CB1 receptor in human melanoma cells. Improving the knowledge about the endocannabinoid system in the molecular pathophysiology of human melanoma may be important to develop novel targeted strategies against this devastating disease.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This project was supported by the Associazione contro il melanoma (ACM_ONLUS). Sara Carpi was supported by a grant fellowship from the Italian Society of Pharmacology and Merck & Co.

REFERENCES

Adinolfi, B., Romanini, A., Vanni, A., Martinotti, E., Chicca, A., Fogli, S., Nieri, P., 2013. Anticancer activity of anandamide in human cutaneous melanoma cells. Eur. J. Pharmacol. 718, 154–159. doi:10.1016/j.ejphar.2013.08.039

Bardeesy, N., Kim, M., Xu, J., Kim, R.-S., Shen, Q., Bosenberg, M.W., Wong, W.H., Chin, L., 2005. Role of epidermal growth factor receptor signaling in RAS-driven melanoma. Mol. Cell. Biol. 25, 4176–4188. doi:10.1128/MCB.25.10.4176-4188.2005

Blázquez, C., Carracedo, A., Barrado, L., Real, P.J., Fernández-Luna, J.L., Velasco, G., Malumbres, M., Guzmán, M., 2006. Cannabinoid receptors as novel targets for the treatment of melanoma. FASEB J. 20, 2633–2635. doi:10.1096/fj.06-6638fje

Carpi, S., Fogli, S., Giannetti, A., Adinolfi, B., Tombelli, S., Da Pozzo, E., Vanni, A., Martinotti, E., Martini, C., Breschi, M.C., Pellegrino, M., Nieri, P., Baldini, F., 2014. Theranostic properties of a survivin-directed molecular beacon in human melanoma cells. PLoS ONE 9, e114588. doi:10.1371/journal.pone.0114588

Carpi, S., Fogli, S., Romanini, A., Pellegrino, M., Adinolfi, B., Podestà, A., Costa, B., Da Pozzo, E., Martini, C., Breschi, M.C., Nieri, P., 2015. AM251 induces apoptosis and G2/M cell cycle arrest in A375 human melanoma cells. Anticancer Drugs Publish Ahead of Print, 1. doi:10.1097/CAD.0000000000000246

Chakravarti, B., Ravi, J., Ganju, R.K., 2014. Cannabinoids as therapeutic agents in cancer: current status and future implications. Oncotarget 5, 5852–5872. doi:10.18632/oncotarget.2233

De Petrocellis, L., Melck, D., Palmisano, A., Bisogno, T., Laezza, C., Bifulco, M., Di Marzo, V., 1998. The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation. Proc. Natl. Acad. Sci. U.S.A. 95, 8375–8380.

Di Marzo, V., 2009. The endocannabinoid system: its general strategy of action, tools for its pharmacological manipulation and potential therapeutic exploitation. Pharmacol. Res. 60, 77–84. doi:10.1016/j.phrs.2009.02.010

Fogli, S., Nieri, P., Chicca, A., Adinolfi, B., Mariotti, V., Iacopetti, P., Breschi, M.C., Pellegrini, S., 2006. Cannabinoid derivatives induce cell death in pancreatic MIA PaCa-2 cells via a receptor-independent mechanism. FEBS Lett. 580, 1733–1739. doi:10.1016/j.febslet.2006.02.024

Glodde, N., Jakobs, M., Bald, T., Tüting, T., Gaffal, E., 2015. Differential role of cannabinoids in the pathogenesis of skin cancer. Life Sci. 138, 35–40. doi:10.1016/j.lfs.2015.04.003

Grimaldi, C., Pisanti, S., Laezza, C., Malfitano, A.M., Santoro, A., Vitale, M., Caruso, M.G., Notarnicola, M., Iacuzzo, I., Portella, G., Di Marzo, V., Bifulco, M., 2006. Anandamide inhibits adhesion and migration of breast cancer cells. Exp. Cell Res. 312, 363–373. doi:10.1016/j.yexcr.2005.10.024

Guindon, J., Hohmann, A.G., 2011. The endocannabinoid system and cancer: therapeutic implication. Br. J. Pharmacol. 163, 1447–1463. doi:10.1111/j.1476-5381.2011.01327.x

Hillard, C.J., 2015. Endocannabinoids and the Endocrine System in Health and Disease. Handb Exp Pharmacol 231, 317–339. doi:10.1007/978-3-319-20825-1_11

Jeong, W.-I., Osei-Hyiaman, D., Park, O., Liu, J., Bátkai, S., Mukhopadhyay, P., Horiguchi, N., Harvey-White, J., Marsicano, G., Lutz, B., Gao, B., Kunos, G., 2008. Paracrine activation of hepatic CB1 receptors by stellate cell-derived endocannabinoids mediates alcoholic fatty liver. Cell Metab. 7, 227–235. doi:10.1016/j.cmet.2007.12.007

Kokona, D., Thermos, K., 2015. Synthetic and endogenous cannabinoids protect retinal neurons from AMPA excitotoxicity in vivo, via activation of CB1 receptors: Involvement of PI3K/Akt and MEK/ERK signaling pathways. Exp. Eye Res. 136, 45–58. doi:10.1016/j.exer.2015.05.007

Laezza, C., Malfitano, A.M., Proto, M.C., Esposito, I., Gazzerro, P., Formisano, P., Pisanti, S.,

Santoro, A., Caruso, M.G., Bifulco, M., 2010. Inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity and of Ras farnesylation mediate antitumor effects of anandamide in human breast cancer cells. Endocr. Relat. Cancer 17, 495–503. doi:10.1677/ERC-10-0009

Maccarrone, M., Bab, I., Bíró, T., Cabral, G.A., Dey, S.K., Di Marzo, V., Konje, J.C., Kunos, G., Mechoulam, R., Pacher, P., Sharkey, K.A., Zimmer, A., 2015. Endocannabinoid signaling at the periphery: 50 years after THC. Trends Pharmacol. Sci. 36, 277–296. doi:10.1016/j.tips.2015.02.008

Minocci, D., Massei, J., Martino, A., Milianti, M., Piz, L., Di Bello, D., Sbrana, A., Martinotti, E., Rossi, A.M., Nieri, P., 2011. Genetic association between bipolar disorder and 524A>C (Leu133Ile) polymorphism of CNR2 gene, encoding for CB2 cannabinoid receptor. J Affect Disord 134, 427–430. doi:10.1016/j.jad.2011.05.023

Mukhopadhyay, B., Liu, J., Osei-Hyiaman, D., Godlewski, G., Mukhopadhyay, P., Wang, L., Jeong, W.-I., Gao, B., Duester, G., Mackie, K., Kojima, S., Kunos, G., 2010. Transcriptional regulation of cannabinoid receptor-1 expression in the liver by retinoic acid acting via retinoic acid receptor-gamma. J. Biol. Chem. 285, 19002–19011. doi:10.1074/jbc.M109.068460

Nieri, P., Greco, R., Adinolfi, B., Breschi, M.C., Martinotti, E., Nannetti, C., Podestà, A., 2003a. CB1- and CB2-cannabinoid receptor-independent lipolysis induced by WIN 55,212-2 in male rat adipocytes. Naunyn Schmiedebergs Arch. Pharmacol. 368, 352–359. doi:10.1007/s00210-003-0831-3

Nieri, P., Martinotti, E., Testai, L., Adinolfi, B., Calderone, V., Breschi, M.C., 2003b. R+-methanandamide inhibits tracheal response to endogenously released acetylcholine via capsazepine-sensitive receptors. Eur. J. Pharmacol. 459, 75–81.

Ozaita, A., Puighermanal, E., Maldonado, R., 2007. Regulation of PI3K/Akt/GSK-3 pathway by cannabinoids in the brain. J. Neurochem. 102, 1105–1114. doi:10.1111/j.1471-4159.2007.04642.x

Pertwee, R.G., 2010. Receptors and channels targeted by synthetic cannabinoid receptor agonists and antagonists. CMC 17, 1360–1381.

Pisanti, S., Picardi, P., D'Alessandro, A., Laezza, C., Bifulco, M., 2013. The endocannabinoid signaling system in cancer. Trends Pharmacol. Sci. 34, 273–282. doi:10.1016/j.tips.2013.03.003

Pisanti, S., Picardi, P., Pallottini, V., Martini, C., Petrosino, S., Proto, M.C., Vitale, M., Laezza, C., Gazzerro, P., Di Marzo, V., Bifulco, M., 2015. Anandamide drives cell cycle progression through CB1 receptors in a rat model of synchronized liver regeneration. J. Cell. Physiol. 230, 2905–2914. doi:10.1002/jcp.24959

Santini, R., Pietrobono, S., Pandolfi, S., Montagnani, V., D'Amico, M., Penachioni, J.Y., Vinci, M.C., Borgognoni, L., Stecca, B., 2014. SOX2 regulates self-renewal and tumorigenicity of human melanoma-initiating cells. Oncogene 33, 4697–4708. doi:10.1038/onc.2014.71

Sarnataro, D., Pisanti, S., Santoro, A., Gazzerro, P., Malfitano, A.M., Laezza, C., Bifulco, M., 2006. The cannabinoid CB1 receptor antagonist rimonabant (SR141716) inhibits human breast cancer cell proliferation through a lipid raft-mediated mechanism. Mol. Pharmacol. 70, 1298–1306. doi:10.1124/mol.106.025601

Scuderi, M.R., Cantarella, G., Scollo, M., Lempereur, L., Palumbo, M., Saccani-Jotti, G., Bernardini, R., 2011. The antimitogenic effect of the cannabinoid receptor agonist WIN55212-2 on human melanoma cells is mediated by the membrane lipid raft. Cancer Lett. 310, 240–249. doi:10.1016/j.canlet.2011.07.008

Shakhova, O., 2014. Neural crest stem cells in melanoma development. Curr Opin Oncol 26, 215–221. doi:10.1097/CCO.0000000000000046

Spagnolo, F., Picasso, V., Lambertini, M., Ottaviano, V., Dozin, B., Queirolo, P., 2016. Survival of patients with metastatic melanoma and brain metastases in the era of MAP-kinase

inhibitors and immunologic checkpoint blockade antibodies: A systematic review. Cancer Treat. Rev. 45, 38–45. doi:10.1016/j.ctrv.2016.03.003
Stecca, B., Ruiz i Altaba, A., 2009. A GLI1-p53 inhibitory loop controls neural stem cell and tumour cell numbers. EMBO J. 28, 663–676. doi:10.1038/emboj.2009.16
Torii, S., Yamamoto, T., Tsuchiya, Y., Nishida, E., 2006. ERK MAP kinase in G cell cycle progression and cancer. Cancer Sci. 97, 697–702. doi:10.1111/j.1349-7006.2006.00244.x
Velasco, G., Sánchez, C., Guzmán, M., 2015. Endocannabinoids and Cancer. Handb Exp Pharmacol 231, 449–472. doi:10.1007/978-3-319-20825-1_16
Zheng, D., Bode, A.M., Zhao, Q., Cho, Y.-Y., Zhu, F., Ma, W.-Y., Dong, Z., 2008. The cannabinoid receptors are required for ultraviolet-induced inflammation and skin cancer development. Cancer Res. 68, 3992–3998. doi:10.1158/0008-5472.CAN-07-6594
Zhou, X.P., Gimm, O., Hampel, H., Niemann, T., Walker, M.J., Eng, C., 2000. Epigenetic PTEN silencing in malignant melanomas without PTEN mutation. Am. J. Pathol. 157, 1123–1128. doi:10.1016/S0002-9440(10)64627-5
Zhou, Y., Falenta, K., Lalli, G., 2014. Endocannabinoid signalling in neuronal migration. Int. J. Biochem. Cell Biol. 47, 104–108. doi:10.1016/j.biocel.2013.12.007

FIGURE LEGENDS

Figure 1. Expression of CB1 receptors in human melanoma cells. (a) Endogenous CB1 mRNA expression in healthy melanocytes (NHEM) and in melanoma cells (A375, 501Mel, ssM2c and MeWo) normalized with two housekeeping genes (18s and β-actin). (b) Western blot analysis of CB1 protein in healthy melanocytes (NHEM) and in melanoma cells (A375, 501Mel, ssM2c and MeWo). β-actin was used as a loading control. Data were presented as mean ± SEM of three independent experiments. *p<0.05 ***p<0.001, as compared to NHEM (one-way ANOVA followed by Dunnetts's multiple comparison test).

Figure 2. shCB1 inhibits CB1 expression. (a) mRNA and (b) protein expression levels for CB1 receptor in melanoma cells stably transduced with LV-shCB1-1, LV-shCB1-2 and LV-shCB1-3 or LV-c (control) cells. β-actin was used as a loading control in western blot analyses. Data were presented as mean ± SEM of three independent experiments. **p<0.01 ***p<0.001, as compared to control (one-way ANOVA followed by Dunnetts's multiple comparison test).

Figure 3. Silencing of CB1 receptor reduces melanoma cell growth. Growth curves for (a) A375 and (b) 501Mel cells transduced with lentiviral CB1 shRNAs or LV-c (control). Cell cycle phase distribution for (c) A375 and (d) 501Mel cells transduced with lentiviral CB1 shRNAs or LV-c. Data were presented as mean ± SEM of three independent experiments. *p< 0.05, **p<0.01, ***p<0.001 (two-way ANOVA followed by Bonferroni's multiple comparison test).

Figure 4. Silencing of CB1 receptor and clonogenicity. (a) A representative image of cells stained with crystal violet and (b) the number of colonies transduced with lentiviral CB1 shRNAs or LV-c (control). Data were presented as mean ± SEM of three independent experiments. *p< 0.05, **p<0.01, as compared to control (one-way ANOVA followed by

Dunnetts's multiple comparison test).

Figure 5. Silencing of CB1 receptor and cell migration. Scratch assays for (a) A375 and (b) 501Mel cells transduced with lentiviral CB1 shRNAs or LV-c (control). Data were presented as mean ± SEM of three independent experiments. *p<0.05, as compared to control (one-way ANOVA followed by Dunnetts's multiple comparison test).

Figure 6. Silencing of CB1 receptor reduces ERK1/2 and AKT phosphorylation levels. Western blot experiments evaluating phosphorylation of ERK and Akt in melanoma A375 (a) and 501Mel (b) cells transduced with lentiviral CB1 shRNAs or LV-c (control). Data are representative of three independent experiments. Data were presented as mean ± SD of three independent experiments. *p< 0.05, **p<0.01, ***p<0.001 (two-way ANOVA followed by Bonferroni's multiple comparison test).