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SPECIAL REPORT

Cannabidiol inhibits human glioma cell migration through a cannabinoid receptor-independent mechanism

[1]Angelo Vaccani, [2]Paola Massi, [1]Arianna Colombo, [1]Tiziana Rubino & *,[1]Daniela Parolaro

[1]Department of Structural and Functional Biology, Pharmacology Section, Center of Neurosciences, University of Insubria, via A. da Giussano 10, Busto Arsizio (VA) 21052, Italy and [2]Department of Pharmacology, Chemotherapy and Medical Toxicology, University of Milan, via Vanvitelli 32, Milan 20129, Italy

We evaluated the ability of cannabidiol (CBD) to impair the migration of tumor cells stimulated by conditioned medium. CBD caused concentration-dependent inhibition of the migration of U87 glioma cells, quantified in a Boyden chamber. Since these cells express both cannabinoid CB1 and CB2 receptors in the membrane, we also evaluated their engagement in the antimigratory effect of CBD. The inhibition of cell was not antagonized either by the selective cannabinoid receptor antagonists SR141716 (CB1) and SR144528 (CB2) or by pretreatment with pertussis toxin, indicating no involvement of classical cannabinoid receptors and/or receptors coupled to Gi/o proteins. These results reinforce the evidence of antitumoral properties of CBD, demonstrating its ability to limit tumor invasion, although the mechanism of its pharmacological effects remains to be clarified.
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Abbreviations: ab-CBD, abnormal-cannabidiol; 2-AG, 2-arachidonoylglycerol; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; CBD, cannabidiol; CM, conditioned medium; CPZ, capsazepine; PMSF, phenylmethyl-sulfonylfluoride; PTX, Pertussis toxin; SR141716A, *N*-(piperidin-l-yl)-5-(4-chlorophenyl)-l-(2,4-dichlorophenyl)-4-methyl-H-pyrazole-3 carboxyamidehydrochloride; SR144528, *N*-[(l*S*)-endo-l,3,3-trimethylbicyclo[2,2,1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-l-(4-methylbenzyl)-pyrazole-3-carboxamide; THC, Δ^9-tetrahydrocannabinol; TRPV1, transient receptor potential channel vanilloid subfamily member 1

Introduction Cannabinoids, the active components of *Cannabis sativa,* and their derivatives, have a wide spectrum of pharmacological effects exerted through specific plasma membrane G-protein-coupled receptors. Two cannabinoid receptors, CB1 and CB2, have been cloned and characterized from mammalian tissues. Cannabinoids induce the inhibition of adenylate cyclases, influence ionic channels, and stimulate extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase, indicating that they may play a general role in the cell survival or death decision (Howlett *et al*., 2002).

Numerous recent reports state that cannabinoids inhibit the viability of various types of cancer cells *in vitro* and *in vivo* (Parolaro *et al*., 2002; Guzman, 2003). Cannabinoids induce apoptosis of malignant glioma cells, that is, from the most common primary cerebral tumor in adults. However, the psychotropic effects of these compounds limit their medicinal use.

In a previous work (Massi *et al*., 2004), we demonstrated that the nonpsychoactive cannabinoid compound, cannabidiol (CBD), can limit glioma cell growth *in vitro* or *in vivo*, by inducing programmed cell death. However, the characteristic diffuse infiltrative growth of gliomas makes surgical removal impossible and substantially complicates the clinical management of these patients.

In general, tumor cell invasion is a complex process involving adhesion to molecules of the extracellular matrix, degradation of matrix component, and subsequent active tumor cell locomotion (migration). Therefore, an understanding of the capacity of CBD to prevent glioma cell migration would be important and could probably improve its use as a potential antitumoral compound.

The cannabinoids have complex effects on cell migration, stimulating or inhibiting depending on the cell type studied. Little is known about their action on tumor cell motility. Anandamide inhibits the migration of human colon carcinoma cells (SW480) through a CB1-dependent mechanism (Joseph *et al*., 2004). In contrast, 2-AG increases the migration of HL-60 cells (Kishimoto *et al*., 2003), and murine myeloid leukemia cells (Jordà *et al*., 2002). Since CBD's effects on malignant glioma cell migration have not been investigated yet, we investigated how this compound influences the motility of human glioma cells, and also considered the pharmacological viewpoint, assessing the role of cannabinoid receptors CB1 and CB2.

Methods and materials CBD was a generous gift from GW Pharm (Salisbury, U.K.). It was initially dissolved in ethanol to a concentration of 100 mM and stored at −20°C. It was further diluted with tissue culture medium to the desired concentration for *in vitro* studies, keeping the ethanol concentration below 0.001%. SR141716A and SR144528 were kindly supplied by Dr F. Barth (Sanofi-Synthélabo Recherche, Montpellier, France). Pertussis toxin (PTX) was purchased from Sigma-Aldrich (St Louis, MO, U.S.A.). Tissue culture media and all supplements were obtained from Sigma-Aldrich.

*Author for correspondence; E-mail: daniela.parolaro@uninsubria.it
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Cell culture We used U87 human glioma cells, maintained at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. Cells were cultured in 75-cm^2 flasks in DMEM supplemented with 4 mM L-glutamine, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, and 10% heat-inactivated foetal bovine serum.

Cell migration assay U87 cell migration was examined in a chemotaxis experiment using a 48-well modified Boyden chamber whose upper and lower compartments were separated by a polycarbonate filter (Biomap, Agrate Brianza, Italy), pore diameter 8 μm, coated with 15 μg ml^{-1} of fibronectin. Conditioned medium (CM) served as the chemoattractant, made by incubating a subconfluent culture of U87 cells with complete medium for 3 days. The coated filter was placed over the bottom chamber containing the CM. Serum-free medium was used as negative control. Cells were treated with CBD or vehicle for 30 min, and then seeded in the upper chamber at a concentration of 3×10^4 cells well^{-1}. After 6 h incubation at 37°C, the nonmigrated cells were scraped off the upper surface of the filter. The migrated cells on the lower side of the filter were stained with Diff-Quick stain (VWR, Scientific Products, Bridgeport, NJ, U.S.A.) and five to eight unit fields per filter were counted at $\times$400 magnification using a Zeiss microscope.

For the experiments using the selective antagonists SR141716 and SR144528, cells were pretreated with the antagonists for 30 min, treated with CBD for another 30 min, and then seeded in the upper chamber. In the PTX studies, cells were preincubated with PTX at a concentration of 100 ng ml^{-1} in a 25-cm^2 flask for 4 h, and then harvested and exposed to CBD as reported above.

Western immunoblotting We prepared cell extracts from subconfluent cells grown in 75-cm^2 flasks. Cells were washed twice in phosphate-buffered saline, and pelleted by centrifugation. Pellets were resuspended in lysis buffer (10 mM Tris–HCl, pH 7.4; 1 mM phenylmethyl-sulfonylfluoride, PMSF; 2 μM aprotinin and 10 μM leupeptin), disrupted by sonication, and centrifuged at 10,000 $\times g$ for 1 h at 4°C. The protein concentration was determined by BCA assay (Pierce, IL, U.S.A.) and loaded at a concentration of 40 μg lane^{-1} in 10% SDS–PAGE. The gel was transferred to a nitrocellulose membrane, blocked with 7.5% milk in Tris-buffered saline/Tween-20 overnight. The filters were then probed with the receptor polyclonal antibodies (Cayman, Ann Arbor, MI, U.S.A.) at a dilution of 1 : 800 for CB1 and 1 : 400 for CB2. Immunoreactive proteins were detected by incubation with horseradish peroxidase-conjugated anti-rabbit IG (Dako, Denmark, 1 : 3000) using the enhanced chemiluminescence system (ECL, Amersham, U.K.).

Statistical analysis Inhibition of cell migration was expressed as a percentage of inhibition with the vehicle (maximal stimulation). One-way ANOVA followed by Dunnett's test was used to compare groups. In the studies with antagonists, one-way ANOVA followed by Tukey's test was used.

Results *Inhibition of human glioma cell migration by CBD* As shown in Figure 1, the addition of CBD to the culture medium of human glioma cells for 6 h resulted in concentration-dependent inhibition of the migration induced by CM, with an estimated IC_{50} of 5.05 $\pm$ 1.1 μM in a concentration range of 0.01–9 μM, that had no effect on cell viability, as we have already reported (Massi *et al*., 2004). Similar results were observed when cells were incubated for 4 h (data not shown).

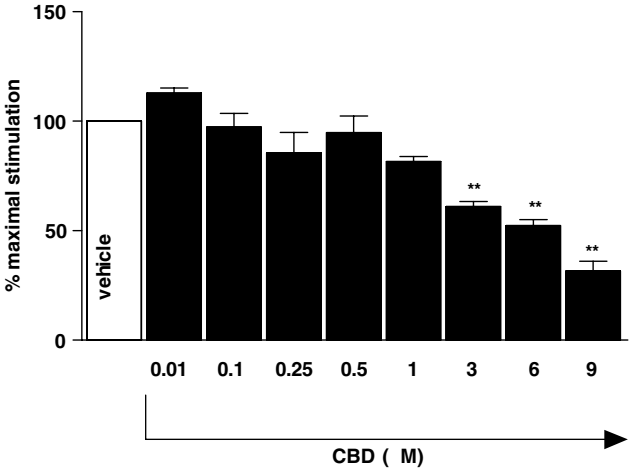

Figure 1 Concentration-dependent inhibition of U87 migration by CBD. CM was added in the lower compartment of the Boyden chamber and cells were added in the upper part in the presence of CBD, and then U87 cell migration was quantified. Cells were exposed to increasing concentrations of CBD from 0.01 to 9 μM (black column) and migration was evaluated after 6 h of incubation. Results are expressed as percentages of control migration (U87 cell migration in presence of vehicle, open column). Values are mean $\pm$ s.e.m. of at least three independent experiments. *$P<0.05$, **$P<0.01$ vs vehicle Dunnett's *t*-test.

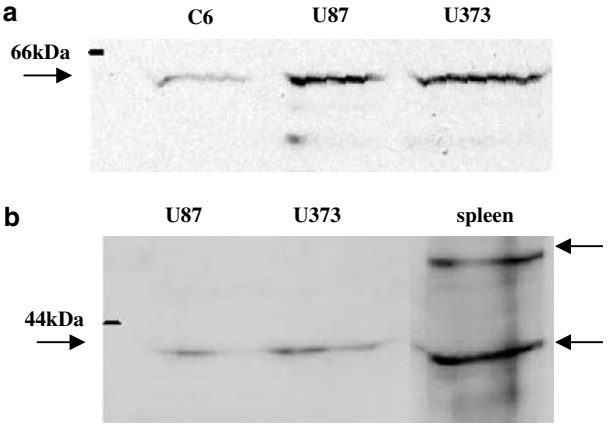

Figure 2 CB1 and CB2 expression in U87 human glioma cells. (a) Western immunoblotting of protein homogenates from U87 and U373 cells for CB1. Proteins (40 μg lane^{-1}) from lysates of C6 (as positive control), U87 and U373 cells reacted with anti-CB1 antibody gave an immunoreactive band at 60 kDa corresponding to the native form of the CB1 receptor (black arrow). (b) Western immunoblotting of protein homogenates from U87 and U373 cells for CB2. Proteins (40 μg lane^{-1}) from lysates of splenocytes (as positive control), U87 and U373 cells reacted with anti-CB2 antibody gave an immunoreactive band at 40 kDa corresponding to the native form of the CB2 receptor.

Effects of cannabinoid receptor antagonists on CBD-induced inhibition of cell migration Most of the effects of cannabinoids on the central nervous system described so far are

believed to be exerted through the cannabinoid receptor. To determine whether CBD-induced inhibition of cell migration was dependent on the stimulation of these receptors, first we checked the presence of the receptors in our cell lines.

Figure 2a shows the results of immunoblot experiments for CB1 and CB2 receptors. A single band of approximately 59 kDa molecular weight (Song & Howlett, 1995) was obtained for U87 cells, comparable with the band observed in murine glioma cells C6 and human glioma cell line U373 used as positive control, where CB1 have already been demonstrated (Sanchez *et al*., 1998). Figure 2b reports the expression of the cannabinoid receptor CB2 in human glioma cells, with a molecular weight of approximately 40 kDa, which is comparable with CB2 receptors in the spleen that express two forms differing in molecular weight possibly because of different glycosylated forms of the receptors (Carlisle *et al*., 2002).

We then proceeded using specific antagonists selective for CB1 and CB2 receptors, respectively, SR141716A and SR144528. As reported in Figure 3, CBD virtually halved cell migration. This inhibition was not prevented by either of the antagonist, both used at two concentrations that did not alter cell viability and migration (data not shown), indicating that CBD's effect was not mediated by the classical cannabinoid receptors. In addition, we tested either the combination of the two cannabinoid receptor antagonists and the vanilloid antagonist, capsazepine, for the CBD-induced inhibition on cell migration. As reported in Figure 3b, the combination of the two cannabinoid antagonists (both at a concentration of 1 μM) failed to limit the inhibition induced by CBD. Also the TRPV1 antagonist, capsazepine used at a concentration of 0.625 μM (that did not alter cell motility *per se*, data not shown), failed to antagonize the CBD effects (Figure 3b).

Inhibitory effect of CBD on cell migration after PTX treatment To further exclude any interaction with classical cannabinoid receptors and/or Gi/o-protein-coupled receptors, in subsequent experiments, we pretreated cells with 100 ng ml^{-1} of PTX for 4 h. This partially impairs cell migration by about 15% compared with unpretreated cells (data not shown). As reported in Figure 4, PTX did not prevent CBD inhibiting tumor cell migration.

Discussion Malignant gliomas are highly infiltrating, proliferative tumors. Glioma cells follow a characteristic pattern of growth, invading the adjacent normal brain structures and surrounding large blood vessels. Inhibiting migration is

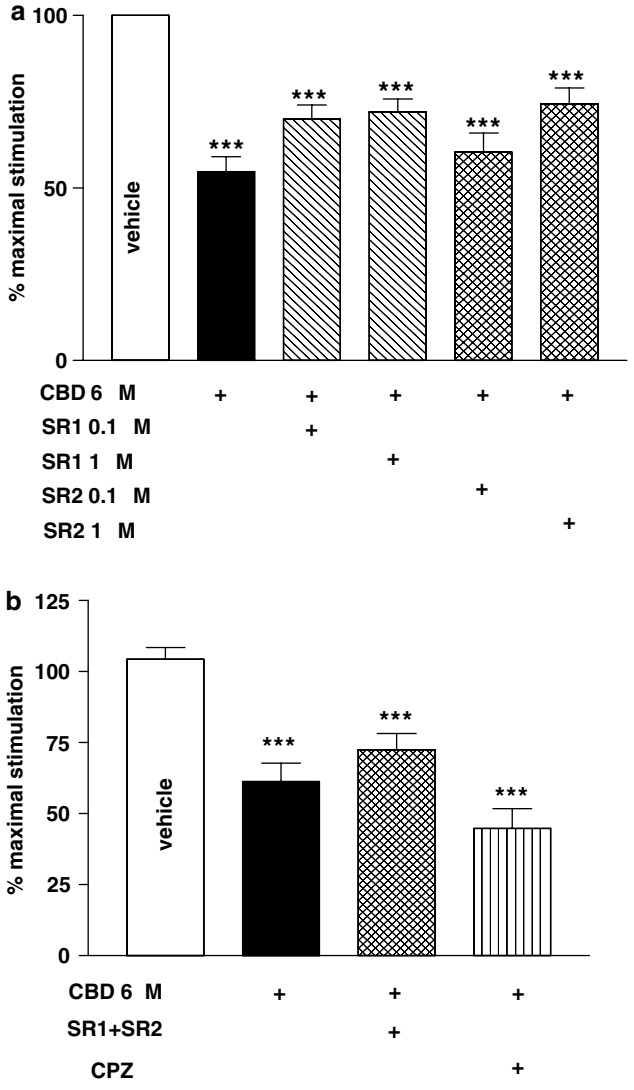

Figure 3 (a) CBD inhibition of cell migration is not prevented by pretreatment with selective antagonists for CB1 and CB2, respectively, SR141716 (diagonal bars) and SR144528 (cross-hatched bars). Cells were preincubated for 30 min with the antagonists, then 30 min with CBD (black bar, CBD alone) and loaded in the upper compartment of the Boyden chamber. (b) CBD inhibition of cell migration is not prevented by the combination of the two cannabinoid receptor antagonists (SR1 + SR2) and capsazepine (CPZ). Results are expressed as percentages of control migration (U87 cell migration in presence of vehicle, open column). Values are mean ± s.e.m. of at least three independent experiments. *$P<0.05$, **$P<0.01$ vs vehicle Tukey's *t*-test.

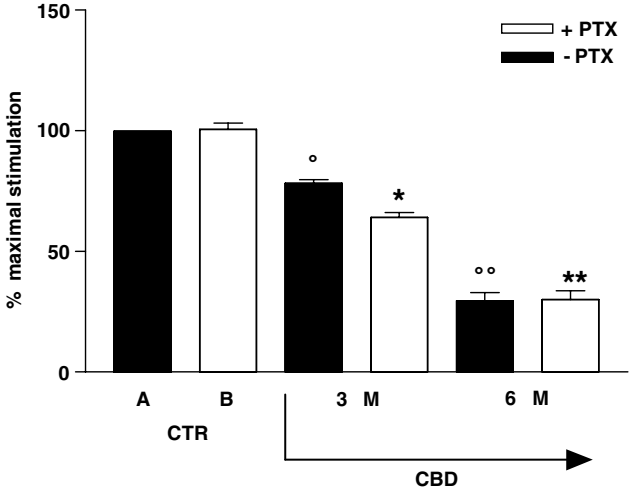

Figure 4 CBD-induced cell migration is not prevented by pretreatment with PTX. Cells were pretreated with 100 ng ml^{-1} PTX for 4 h, then treated with CBD or vehicle B for 30 min (open columns) and loaded in the upper compartment of the Boyden chamber. Black columns represent cells without PTX treatment, with CBD or vehicle A alone. Results are expressed as percentages of control migration (U87 cell migration in the presence of vehicle). Values are mean ± s.e.m. of at least three independent experiments. *$P<0.05$, **$P<0.01$ vs vehicle B; °$P<0.05$, °°$P<0.01$ vs vehicle A Tukey's *t*-test.

therefore a vital step towards improving the prognosis of patients with malignant gliomas. In a previous study, we demonstrated that CBD, a nonpsychotropic derivative of marijuana, impaired the viability of human glioma cell lines U87 and U373 *in vitro* and *in vivo*, suggesting its potential medical use.

The present study shows, for the first time, that CBD can inhibit the migration of U87 human glioma cells *in vitro*. The cannabinoids' effects on cell migration have already been reported, although the main studies were on immune cells or endothelial cells, resulting sometimes in conflicting data depending on the cell type. Stimulation of the CB2 receptor by the endocannabinoid 2-AG was reported to increase the migration of HL60 and mouse microglia (Kishimoto *et al*., 2003; Walter *et al*., 2003). In contrast, THC or the synthetic agonist CP-55,940 inhibited macrophage migration, involving both receptors, CB1 and CB2 (Sacerdote *et al*., 2000; Roth *et al*., 2002). Anandamide has also been described as being able to inhibit the migration of SW480 colon carcinoma cells (Joseph *et al*., 2004).

Here, we found that CBD caused concentration-related inhibition of glioma cell migration in a range of concentrations starting from 0.01 up to 9 μM, which did not affect cell viability, as we have already reported (Massi *et al*., 2004). In a first attempt to clarify the mechanism of action of CBD, we investigated whether the inhibition of tumor cell migration was due to the classical cannabinoid receptors. Neither the CB1 antagonist SR141716 nor the CB2 antagonist SR144528 prevented the cell migration inhibition in response to CBD. Also, the combination of the two cannabinoid antagonists or the TRPV1 receptor antagonist capsazepine failed to antagonize the inhibitory effects of CBD, strengthen our previous data (Massi *et al*., 2004) on the antiproliferative effects of CBD that resulted independent of cannabinoid and vanilloid receptors.

The role of cannabinoid receptors in CBD's pharmacological effects is anyway controversial. Binding studies have shown it could bind to cannabinoid receptors (Bisogno *et al*., 2001), but in our pharmacological study, the selective antagonists did not block CBD's effects *in vitro* (Massi *et al*., 2004). Since the existence of a third type of cannabinoid receptor has been suggested (Pertwee & Ross, 2002) to which CBD could potentially bind, with a view to excluding any interaction with Gi/o-coupled receptors, we evaluated the effect of CBD on cell migration also in the presence of PTX which, however, was unable to prevent CBD-induced inhibition, meaning that CBD does not act through cannabinoid and/or other receptors coupled to Gi/o proteins. These data are in line with our previous results about the proapoptotic effect of CBD on human glioma cells, which was not related to its interaction with cannabinoid receptors.

Since CBD has been reported to stimulate mouse microglia BV-2 cell migration (Walter *et al*., 2003) through potential binding with a still not fully characterized receptor named 'endothelial receptor sensitive to Ab-CBD' (Jarai *et al*., 1999), we could speculate that CBD's effect in glioma might be due to an interaction with a similar type of receptor showing inverse agonist properties.

In conclusion, the present study demonstrates, for the first time, that CBD can inhibit the migration of tumoral cells. Although the mechanism of this action is not clear at the moment, we can exclude any engagement of classical cannabinoid receptors and/or Gi/o-coupled receptors. Our data further support the use of cannabinoids as antimetastatic drugs as previously demonstrated for met-fluoro-anandamide on rat thyroid cancer cell (Portella *et al*., 2003). This antimigratory property, together with the known antiproliferative and apoptotic features of CBD (Massi *et al*., 2004), strengthen the evidence for its use as a potential antitumoral agent.

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References

BISOGNO, T., HANUS, L., DE PETROCELLIS, L., TCHILIBON, S., PONDE, D.E., BRANDI, I., MORIELLO, A.S., DAVIS, J.B., MECHOULAM, R. & DI MARZO, V. (2001). Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. *Br. J. Pharmacol.,* **134,** 845–852.

CARLISLE, S.J., MARCIANO-CABRAL, F., STAAB, A., LUDWICK, C. & CABRAL, G.A. (2002). Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation. *Int. Immunopharmacol.,* **2,** 69–82.

GUZMAN, M. (2003). Cannabinoids: potential anticancer agents. *Nat. Rev. Cancer,* **3,** 745–755.

HOWLETT, A.C., BARTH, F., BONNER, T.I., CABRAL, G., CASELLAS, P., DEVANE, W.A., FELDER, C.C., HERKENHAM, M., MACKIE, K., MARTIN, B.R., MECHOULAM, R. & PERTWEE, R.G. (2002). International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol. Rev.,* **54,** 161–202.

JARAI, Z., WAGNER, J.A., VARGA, K., LAKE, K.D., COMPTON, D.R., MARTIN, B.R., ZIMMER, A.M., BONNER, T.I., BUCKLEY, N.E., MEZEY, E., RAZDAN, R.K., ZIMMER, A. & KUNOS, G. (1999). Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. *Proc. Natl. Acad. Sci. U.S.A.,* **96,** 14136–14141.

JORDÀ, M.A., VERBAKEL, S.E., VALK, P.J., VANKAN-BERKHOUDT, Y.V., MACCARRONE, M., FINAZZI-AGRO, A., LOWENBERG, B. & DELWEL, R. (2002). Hematopoietic cells expressing the peripheral cannabinoid receptor migrate in response to the endocannabinoid 2-arachidonoylglycerol. *Blood,* **99,** 2786–2793.

JOSEPH, J., NIGGEMANN, B., ZAENKER, K.S. & ENTSCHLADEN, F. (2004). Anandamide is an endogenous inhibitor for the migration of tumor cells and T lymphocytes. *Cancer Immunol. Immunother.,* **1853,** 723–728.

KISHIMOTO, S., GOKOH, M., OKA, S., MURAMATSU, M., KAJIWARA, T., WAKU, K. & SUGIURA, T. (2003). 2-Arachidonoylglycerol induces the migration of HL-60 cells differentiated into macrophage-like cells and human peripheral blood monocytes through the cannabinoid CB2 receptor-dependent mechanism. *J. Biol. Chem.,* **278,** 24469–24475.

MASSI, P., VACCANI, A., CERUTI, S., COLOMBO, A., ABBRACCHIO, M.P. & PAROLARO, D. (2004). Antitumor effects of cannabidiol, a nonpsychoactive cannabinoid, on human glioma cell lines. *J. Pharmacol. Exp. Ther.,* **308,** 838–845.

PAROLARO, D., MASSI, P., RUBINO, T. & MONTI, E. (2002). Endocannabinoids in the immune system and cancer. *Prostaglandins Leukot. Essent. Fatty Acids,* **66,** 319–332.

PERTWEE, R.G. & ROSS, R.A. (2002). Cannabinoid receptors and their ligands. *Prostaglandins Leukot. Essent. Fatty Acids,* **66,** 101–121.

PORTELLA, G., LAEZZA, C., LACCETTI, P., DE PETROCELLIS, L., DI MARZO, V. & BIFULCO, M. (2003). Inhibitory effects of cannabinoid CB1 receptor stimulation on tumor growth and metastatic spreading: actions on signals involved in angiogenesis and metastasis. *FASEB J.,* **17,** 1771–1773 (Epub 2003 Jul 03).

ROTH, M.D., BALDWIN, G.C. & TASHKIN, D.P. (2002). Effects of delta-9-tetrahydrocannabinol on human immune function and host defense. *Chem. Phys. Lipids,* **121,** 229–239.

SACERDOTE, P., MASSI, P., PANERAI, A.E. & PAROLARO, D. (2000). *In vivo* and *in vitro* treatment with the synthetic cannabinoid CP55, 940 decreases the *in vitro* migration of macrophages in the rat: involvement of both CB1 and CB2 receptors. *J. Neuroimmunol.,* **109,** 155–163.

SANCHEZ, C., GALVE-ROPERH, I., CANOVA, C., BRACHET, P. & GUZMAN, M. (1998). Delta9-tetrahydrocannabinol induces apoptosis in C6 glioma cells. *FEBS Lett.,* **436,** 6–10.

SONG, C. & HOWLETT, A.C. (1995). Rat brain cannabinoid receptors are N-linked glycosylated proteins. *Life Sci.,* **56,** 1983–1989.

WALTER, L., FRANKLIN, A., WITTING, A., WADE, C., XIE, Y., KUNOS, G., MACKIE, K. & STELLA, N. (2003). Nonpsychotropic cannabinoid receptors regulate microglial cell migration. *J. Neurosci.,* **23,** 1398–1405.

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