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PII: S0014-2999(09)00515-9  
DOI: doi: [10.1016/j.ejphar.2009.06.004](https://doi.org/10.1016/j.ejphar.2009.06.004)  
Reference: EJP 65968

To appear in: *European Journal of Pharmacology*

Received date: 10 December 2008  
Revised date: 19 May 2009  
Accepted date: 3 June 2009



Please cite this article as: Luca, Tonia, Di Benedetto, Giulia, Scuderi, Mariagrazia Rita, Palumbo, Marco, Clementi, Silvia, Bernardini, Renato, Cantarella, Giuseppina, The CB<sub>1</sub>/CB<sub>2</sub> receptor agonist WIN-55,212-2 reduces viability of human Kaposi's sarcoma cells in vitro, *European Journal of Pharmacology* (2009), doi: [10.1016/j.ejphar.2009.06.004](https://doi.org/10.1016/j.ejphar.2009.06.004)

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**The CB<sub>1</sub>/CB<sub>2</sub> receptor agonist WIN-55,212-2 reduces viability of human Kaposi's sarcoma cells in vitro**

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**Abstract**

Kaposi's sarcoma is a highly vascularized mesenchymal neoplasm arising with multiple lesions of the skin. Endogenous cannabinoids have been shown to inhibit proliferation of a wide spectrum of tumor cells. We studied the effects of cannabinoids on human Kaposi's sarcoma cell proliferation in vitro. To do so, we first investigated the presence of the cannabinoid receptors CB<sub>1</sub> and CB<sub>2</sub> mRNAs in the human Kaposi's sarcoma cell line KS-IMM by RT-PCR and, subsequently, the effects of the mixed CB<sub>1</sub>/CB<sub>2</sub> agonist WIN-55,212-2 (WIN) on cell proliferation in vitro. WIN showed antimitogenic effects on Kaposi's sarcoma cells. Western blot analysis of Kaposi's sarcoma lysates suggested that WIN treatment induced activation of both caspase-3 and -6, as well as increased phosphorylation of the stress kinase p38 and JNK, along with transient phosphorylation of ERK<sub>1/2</sub>. To better characterize the involvement of each single CB receptor in cannabinoid-induced cell death, we incubated Kaposi's sarcoma cells with different selective cannabinoid receptor agonists, respectively ACEA (CB<sub>1</sub>) and JWH-133 (CB<sub>2</sub>). None of the agonists was able to induce KS-IMM cell apoptosis. Moreover, we co-incubated Kaposi's sarcoma cells with WIN-55,212-2 and either the CB<sub>1</sub> receptor antagonist AM251, the CB<sub>2</sub> receptor antagonist AM630, or a combination of both substances. The CB<sub>2</sub> receptor antagonist AM630 was able to significantly increase survival of Kaposi's sarcoma cells treated with WIN. In view of the antiproliferative effects of cannabinoids on KS-IMM cells, one could envision the cannabinoid system as a potential target for pharmacological treatment of Kaposi's sarcoma.

*Keywords:* Cannabinoid antagonists, Signal transduction, Kaposi's sarcoma, cell death

## 1. Introduction

Kaposi's sarcoma is a highly vascularized mesenchymal neoplasm arising with multiple independent cutaneous lesions. The development and growth of Kaposi's sarcoma is associated with the infection of human herpesvirus 8 (HHV-8), which is detectable in Kaposi's sarcoma cells (endothelial and spindle cells) (Antman et al., 2000). The most aggressive form of Kaposi's sarcoma is found in HIV-1-infected individuals (acquired immunodeficiency syndrome-associated Kaposi's sarcoma, AIDS- Kaposi's sarcoma). In the latter disease Kaposi's sarcoma cells are disseminated and localized in skin and visceral organs (Friedman-Kien, 1981; Haverkos et al., 1985).

Lesions from different forms of Kaposi's sarcoma share identical histopathological features such as neoangiogenesis, oedema, infiltration of lymphomononuclear cells, presence of activated proliferating endothelial cells forming abnormal blood vessels, extravasation of red blood cells, and growth of spindle-shaped cells (Kaposi's sarcoma spindle cells), that are considered to be the primitive tumour element of Kaposi's sarcoma (Martin et al., 1995).

Cannabinoids, pharmacologically active molecules contained in *Cannabis sativa*, exert pleiotropic effects which affect the central nervous system (CNS), as well as the cardiovascular, digestive, immune, reproductive and ocular systems (Felder et al., 1998; Pertwee, 2000). So far, two seven- transmembrane domains, G protein-coupled cannabinoid receptors, CB<sub>1</sub> and CB<sub>2</sub>, have been cloned. While CB<sub>1</sub> receptors are primarily found within the brain, CB<sub>2</sub> receptors are widely distributed to peripheral tissues, with special regard to cells of the immune system (Matsuda et al., 1990; Munro et al., 1993). The cannabinoid receptors function depends upon inhibition of adenylyl cyclase and of N- and Q-type calcium channels, whereas they activate rectifying potassium channels (Howlett et al., 1988; Caulfield et al., 1992; Felder et al., 1995). Following activation, cannabinoid receptors undergo phosphorylation (Garcia et al., 1998) and internalization, followed by recycling into the membrane (Hsieh et al., 1999).

Cannabinoid agonists are involved in the control of cell proliferation by stimulating mitogen-activated protein kinases (MAPKs) (Bouaboula et al., 1995; Bouaboula et al., 1996; Rueda et al., 2000). One of the most intriguing and unexplored actions of cannabinoids is represented by their ability to inhibit the growth of a wide spectrum of transformed cells. Interestingly, cannabinoid administration to nude mice slows down the growth of various tumour xenografts (Guzmán et al., 2001). In laboratory species, cannabinoids administration is associated with regression of gliomas, by means of apoptosis of the tumour cells and without any substantial neurotoxic effect (Galve-Roperh et al., 2000). In addition, cannabinoids have also been shown to possess antiangiogenic properties (Blazquez et al., 2004).

Such background prompted us to consider the hypothesis that cannabinoids might have an antiproliferative effect on Kaposi's sarcoma cells. We explored the role of CB<sub>1</sub> and CB<sub>2</sub> receptors in the possible inhibition of tumour growth and investigated the molecular pathways potentially involved in the antiproliferative effect of cannabinoids on Kaposi's sarcoma, in a human cell line originating from non-AIDS patients which does not contain the HHV8 virus genome (Albini et al., 1996; Flamand et al., 1996), releases vascular endothelial growth factor (VEGF), and expresses receptors for the latter (Morini et al., 2000).

## 2. Materials and Methods

### 2.1 Cell cultures and reagents.

All materials and media were from Invitrogen Srl (San Giuliano Milanese, Italy) unless otherwise specified.

The human Kaposi's sarcoma KS-IMM lesionally-derived endothelial cell line, HHV8 negative, (Albini et al 1996; Flamand et al. 1996) was routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 50 µg/ml penicillin, and 100 µg/ml streptomycin. They were cultured at 37°C in humidified 5% CO<sub>2</sub>/95% atmosphere.

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords of healthy women undergone uncomplicated term pregnancies, as described elsewhere (Jaffe EA et al., 1973). HUVECs were grown in gelatin coated dishes in medium M199 supplemented with endothelial cell growth supplement (ECGS; 20 µg/ml) (Sigma-Aldrich, Milano, Italy), heparin (1625 U.I/ml) (Sigma-Aldrich, Milano, Italy) and 20 % FBS.

### 2.2 Reverse transcriptase-PCR

Total RNA was extracted from cells grown to 80% confluence using TRIzol, according to the manufacturer's instructions. For first strand cDNA synthesis, 1 µg of total RNA was reverse-transcribed using 25 µg/ml oligo (dT)<sub>12-18</sub> primer in a final volume of 20 µl, in the presence of 200 units of M-MLV reverse. The reaction was carried out at 37 °C for 1 h and heated at 95 °C for 10 min, and subsequently for 5 min at 4 °C. The reaction program for (a) human CB<sub>1</sub> and human CB<sub>2</sub> primers consisted of 35 runs of denaturation at 95 °C for 45 sec, annealing at 62 °C for 45 sec and elongation at 72 °C for 1 min.; (b) human GAPDH primers consisted of 25 runs of denaturation at 95 °C for 1min, annealing at 56 °C for 1 min, and elongation at 72 °C for 1 min. The cycle program was preceded by an initial denaturation at 95 °C for 3 min and followed by a final extension at 72 °C for 7 min. PCR products were analyzed by 1.0% agarose gel electrophoresis and visualized with ethidium bromide. The following RNA transcripts were detected via amplification of the corresponding cDNAs: the human CB<sub>1</sub> using a primer pair composed of the sense primer 5'-

CATCATCACACGTCTG-3' and the antisense primer 5'-ATGCTGTTATCCAGAGGCTGC-3'; the human CB<sub>2</sub> using a primer pair composed of the sense primer 5'-TTTCCCCTGATCCCCAATG-3' and the antisense primer 5'-AGTTGATGAGGCACAGCATG-3'; the human GAPDH, which was chosen as an endogenous expression RT-PCR standard, using a primer pair composed of the sense primer 5'-CCACCCATGGCAAATTCCATG-3' and the antisense primer 5'-TCTAGACGGCAGGTCAGGTCCACC-3'.

### 2.3 Viability assay

Kaposi's sarcoma cells were seeded at  $1,5 \times 10^3$  cells/well into 96-multiwell plates in DMEM with 10% FBS, penicillin and streptomycin.

After 24 h, medium was replaced with DMEM supplemented with 1% FBS, penicillin and streptomycin and maintained for a further 24 h. They were incubated at 37 °C for 24 h, 48 h, 72 h and 96 h with the following concentrations of WIN-55,212-2 ( (R)-[+]-[2,3-Dihydro-5-methyl-3-((morpholinyl)methyl)pyrrolo(1,2,3-de)-1,4-benzoxazinyl]-[1-naphthalenyl]methanone)) (Sigma-Aldrich, Milano, Italy) in DMEM 1% FBS: 100 nM, 500 nM 1 μM, 1,5 μM, 2 μM, 2,5 μM, 5 μM. Control cells received 0,1% DMSO (Dimethyl Sulfoxide) in DMEM 1% FBS.

In other experiments Kaposi's sarcoma cells were treated for 96 h with a) the following concentrations of ACEA (N-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide) (Tocris Bioscience) and JWH-133 ((6aR,10aR)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro -6,6,9-trimethyl-6H-dibenzo[b,d]pyran) (Tocris Bioscience, Ellisville, MO, USA), 1 μM, 1,5 μM, 2 μM, 2,5 μM, 5 μM, in DMEM 1% FBS and b) 1 μM AM251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) (Tocris Bioscience, Ellisville, MO, USA) and 1 μM AM630 (6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone) (Tocris Bioscience) in DMEM 1% FBS, alone or in association with 2 μM WIN-55,212-2. Control cells received the equivalent dilution of DMSO in DMEM 1% FBS. Cell proliferation was evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. Briefly, after drug exposure, MTT (10 μL) solution (5 mg/ml) was added to

each well. The reaction was allowed to proceed for 3 to 4 h at 37°C. The culture medium was removed and formazan crystals were dissolved by adding DMSO (200 µL). The absorbance of each well was read at 570 nm and directly correlated with the number of remaining viable cells.

#### *2.4 Staining of apoptotic cells with bis-benzimide*

Cells were plated on coverslips at a density of 20,000 cells/well, washed with PBS and fixed in 4 % paraformaldehyde in PBS for 20 min, washed once in PBS, and incubated with bis-benzimide (Hoechst 33258) 2:1000 in PBS at 37°C for 15 min. Cells were washed three times with bidistilled water and the coverslips were mounted and examined under UV illumination using a Leica microscope. Cells with condensed chromatin or fragmented nuclei were considered to be apoptotic.

#### *2.5 Protein extraction*

In a set of experiments, Kaposi's sarcoma cells grown in 100 mm plastic Petri dishes were serum-starved for 24 h and then incubated in the presence of WIN-55,212-2 (2 µM) for 24 h, 48 h, 72 h and 96 h.

Membrane and cytosolic fractions of Kaposi's sarcoma cells were prepared as described. Cells were washed and scraped with 1 ml ice-cold PBS and centrifuged at 2500 rpm for 10 min at 4°C. The cell pellet was resuspended in ice-cold hypotonic PBS (0,1 X) and frozen at -80°C for 5 min. After thawing, the lysed cells were centrifuged at max speed for 10 min at 4°C and the supernatants (cytosolic proteins) were stored at -80 °C until use. The pellets were resuspended in lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml leupeptin, 5 µg/ml aprotinin, 1 µg/ml pepstatin), incubated 30 min at 4°C and centrifuged at max speed for 10 min at 4°C. The supernatants (membrane proteins) were stored at -80 °C until use.

In other experiments cells grown in 60 mm plastic Petri dishes, starved for 24 h, were incubated in the presence of WIN-55,212-2 (2 µM) for 15 min, 1 h, 3 h, 12 h and 24 h or for 5 min, 15 min, 30 min, 1 h, 6 h and 24 h. Cells were then lysed in NP-40 lysis buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 50µM NaF, 2 mM EDTA, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1% NP-40, 2mM phenylmethylsulfonyl fluoride).

### 2.6 Western blot analysis.

Proteins obtained after different treatments, according to the experiment, were separated in acrylamide gels and electrotransferred to nitrocellulose blots (Amersham Italia S.r.l., Milan, Italy). Membranes were incubated with 5% fat-free milk, 0.1% Tween-20 in PBS and incubated with the following antibodies: rabbit polyclonal CB<sub>1</sub> and CB<sub>2</sub> receptor (Cayman Chemicals, Ann Arbor, MI, USA), rabbit polyclonal anti Phospho-p42/44 Map kinase (Cell Signaling Technology, Inc., Danvers, MA, USA), mouse monoclonal anti-p-JNK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-p-P38 (Promega Italia S.r.l. Milan, Italy), rabbit polyclonal  $\beta$ -tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The latter was used as a loading control. Peroxidase-labeled secondary antibodies and an enhanced chemiluminescence kit (Amersham Italia S.r.l., Milan, Italy) were used for immunodetection. Western blot analysis was performed on samples from three separated experiments.

### 2.7 Caspase-3 and Caspase-6 assay.

The activity of the proteases caspase-3 and caspase-6 was measured in Kaposi's sarcoma cells. Cells were exposed to WIN-55,212-2 2  $\mu$ M for 15', 30', 1 h, 6 h and 24 h,  $1-5 \times 10^6$  cells were counted and pelleted and caspase-3/6 activity in whole cell lysates was determined by using the Colorimetric Caspase Assay Kit (Alexis Biochemicals, San Diego CA, USA), following the manufacturer's instructions.

This assay provides a colorimetric substrate (DEVD for caspase-3 and VEID for caspase-6) labelled with the chromophore p-nitroaniline (p-NA), which is released from the substrate on cleavage by caspase. Free p-nitroaniline produces a yellow colour that is measured by a photometer at 405 nm. The amount of yellow colour produced is proportional to the amount of DEVDase or VEIDase activity present in the sample. Fold-increase in caspase activity can be determined by comparing these results with the level of the uninduced control.

### 3. Results

#### 3.1 Cannabinoid receptors are expressed in Kaposi's sarcoma cells and influence their viability.

We first studied CB<sub>1</sub> and CB<sub>2</sub> receptors expression in Kaposi's sarcoma cells. RT-PCR analysis showed that both mRNAs are present in KS-IMM cells. Experimental samples from human umbilical vein endothelial cells (HUVEC) were used as an internal positive control (Fig. 1, panel A). CB<sub>1</sub> and CB<sub>2</sub> proteins were also expressed by Kaposi's sarcoma cells (Fig. 1, panel B).

To evaluate the role of CB receptors in Kaposi's sarcoma cell viability, the latter were incubated with graded concentrations of the mixed CB<sub>1</sub>/CB<sub>2</sub> receptor agonist WIN-55,212-2. WIN-55,212-2 induced cell death either in a concentration- and in a time-dependent manner. The most effective concentration of WIN-55,212-2 was 2  $\mu$ M. The antiproliferative effects reached its maximum at 72 h and lasted up to 96 h (Fig. 2 panel A). In order to compare the sensitivity of Kaposi's sarcoma cells to WIN-55,212-2, HUVEC were incubate with equal concentrations of the cannabinoid agonist in the same experiment. Data indicate that WIN 55,212-2 was more effective in inhibiting proliferation of HUVEC cells than that of Kaposi's sarcoma cells. The most effective concentration of WIN-55,212-2 was 25 nM. The antiproliferative effect of 25 nM WIN-55,212-2 on HUVEC reached its maximum at 96 h (Fig. 2, panel B).

The increase in Kaposi's sarcoma cell death rate induced by WIN-55,212-2 was associated with substantial morphological changes occurring after 72 h incubation. The number of cells decreased at 72 h and reached a minimum at 96 h of incubation with WIN-55,212-2 (Fig. 3, panel A).

In addition, DNA fluorochrome bis-benzimide (Hoechst 33258) staining of the same cells revealed the presence of fragmented nuclei, suggesting that WIN-55,212-2 was able to induce apoptosis in Kaposi's sarcoma cells (Fig. 3, panel B).

### **3.2 WIN-55,212-2 modulates the activity of caspases and mitogen-activated protein kinases in Kaposi's sarcoma cells.**

In order to elucidate molecular mechanisms underlying WIN-55,212-2-induced Kaposi's sarcoma cell apoptosis, activation of caspase-3 and caspase-6 was checked was investigated in specific experiments.

KS-IMM cells were exposed to 2  $\mu$ M WIN-55,212-2 at different times up to 24 h. The cleaved form of caspase-3 was significantly increased after 15 min incubation of Kaposi's sarcoma cells with WIN-55,212-2 and eventually increased thereafter. The activity of the enzyme returned to basal levels after 1 h. (Fig. 4, panel A). Similarly, an increase of the cleaved form of caspase-6 occurred after 15 min of incubation of Kaposi's sarcoma cells with WIN-55,212-2 and lasted up to 30 min. The activity of the enzyme returned to basal levels after 1 h. (Fig. 4, panel B).

To better elucidate the signalling events occurring after activation of cannabinoid receptors, we also analysed the expression of apoptosis-associated mitogen-activated protein kinases (MAPKs) in KS-IMM cells treated with WIN-55,212-2 (2  $\mu$ M). Western blot analysis was performed on protein lysates to detect the phosphorylated forms of extracellular signal-regulated kinase 1/2 (ERK<sub>1/2</sub>), p-38 kinase, and JNK. Increased levels of phosphorylated stress kinases (p-P38 and p-JNK-1) were detected after 3 and up to 12 h of treatment (Fig. 5, respective blots). An early, transiently increased expression of p-ERK<sub>1/2</sub> was observed after 15 min of treatment, followed by its progressive decline until 12 h. Phosphorylation of ERK<sub>1/2</sub> was increased again after 24 h of treatment with WIN-55,212-2 (Fig. 5, respective blot).

### **3.3 Pharmacological characterization of the effects of selective cannabinoid-receptor agonists and antagonists on Kaposi's Sarcoma cell viability.**

To better characterize the involvement of each single CB receptor in cannabinoid-induced cell death, we incubated KS-IMM cells with different selective cannabinoid receptor agonists. We used either the selective CB<sub>1</sub> receptor agonist ACEA, or the selective CB<sub>2</sub> receptor agonist JWH-

133. Neither ACEA, nor JWH-133 exerted an effect upon KS cells during a 96 h incubation (Fig. 6, panels A and B respectively).

In other experiments, cells were treated with different concentrations of both agonists for 96 h. No antiproliferative effect was observed following the combined use of the two drugs (Fig. 7).

Moreover, we co-incubated Kaposi's sarcoma cells with WIN-55,212-2 and either the CB<sub>1</sub> receptor antagonist AM251, the CB<sub>2</sub> receptor antagonist AM630, or a combination of both substances.

Among all combinations used, only the CB<sub>2</sub> receptor antagonist AM630 was able to significantly increase survival of Kaposi's sarcoma cells treated with WIN-55,212-2, although it did not rescue cells from death totally. The CB<sub>1</sub> receptor antagonist AM251 was unable to produce such an effect (Fig. 8).

#### 4. Discussion

Here we show that the Kaposi's sarcoma cell line KS-IMM, which expresses both CB<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors, respond to agonists with decreased proliferation. In fact, the mixed CB<sub>1</sub>/CB<sub>2</sub> agonist WIN-55,212-2 can induce Kaposi's sarcoma cell death after 72 h of treatment. Morphological changes of Kaposi's sarcoma cells and bis-benzimide nuclear staining indicates that such cannabinoid-induced death was of the apoptotic type.

In fact, cannabinoids have a role in cell proliferation, growth arrest or apoptosis in a variety of cells, including lymphocytes and neurons (Kogan, 2005). The pharmacological effects of cannabinoids depend on different experimental conditions, such as drug concentration, timing of drug delivery, and cell type. Particularly, cannabinoids have been shown to induce apoptotic death in HUVEC (Blazquez et al., 2003) in C6 rat glioma cells (Jacobsson et al., 2001; Sanchez et al., 1998), PC-12 rat pheochromocytoma cells (Sarker et al., 2000), breast cancer cells (De Petrocellis et al., 1998), CHP100 neuroblastoma cells (Maccarrone et al., 2000), and hippocampal neurons (Chan et al., 1998) in vitro. Moreover, regression of C6 glioma cells has also been reported after administration of either endogenous or synthetic cannabinoids in vivo (Sánchez et al., 2001).

Our data show that WIN-55,212-2-induced apoptosis was preceded by activation of caspases -3 and -6 in KS cells, an event associated with the apoptotic process (Massi et al., 2006).

Even though molecular mechanisms that are responsible of the antiproliferative effect of cannabinoids have not been entirely elucidated, recently progress has been made into the understanding of the intracellular signalling pathways underlying the anti-tumor effects of cannabinoid receptor agonists. For example, it is now established that of  $\Delta^9$ -tetrahydrocannabinol (THC) and endocannabinoids stimulate the activity of proteins that are downstream activation of p21ras, that is, the MAPKs (Sarker et al., 2000). Indeed, an array of data report the involvement of both SAPKs (stress-activated protein kinases) and ERK<sub>1/2</sub> in several in vitro tumour models, which undergo apoptosis following different stimuli. In general, the same stimulus activates SAPKs and ERK<sub>1/2</sub> with opposite cellular effects. It is generally accepted that the activation of ERK cascade

leads to cell proliferation (McCubrey et al., 2007; Chambard et al., 2007). However, the relationship between activation of the ERK cascade and cell survival depends upon the duration of the stimulus. Indeed, it has been reported that the apoptotic action of THC relies on long term ERK activation (Galve-Roperh et al., 2000).

On this basis, we have shown that WIN-55,212-2 induces the phosphorylation of ERK<sub>1/2</sub> at the beginning of its action. Then, the noxious stimulus may trigger activation of the stress kinases JNK and p38, which, in turn, activate downstream mechanisms leading Kaposi's sarcoma cells to apoptotic death (Xia et al., 1995).

Such early activation of ERK<sub>1/2</sub> has been already reported in breast cancer cells (Liu, 2007) and is followed by a second delayed phosphorylation phase, which confirms the biphasic activation of ERK<sub>1/2</sub> observed in other in vitro models. Moreover, rapid inactivation of ERK<sub>1/2</sub> implies the existence of a tightly controlled regulatory mechanism (Guerra, 2004).

A number of hypotheses have been formulated to explain receptor-mediated mechanisms by which cannabinoids induce death in transformed cells. Using different biochemical and pharmacological approaches, the role of the CB<sub>1</sub> receptor has been shown in various cell types, such as C6, PC-12 and primary hippocampal neurons (Portella et al., 2003; Downer et al., 2003). Moreover, the involvement of the CB<sub>2</sub> receptor has also been demonstrated in cannabinoid-induced apoptosis in HUVEC (Blazquez et al., 2003) and C6 rat glioma cells (Sanchez et al., 1998). In Kaposi's sarcoma cells, both CB<sub>1</sub> and CB<sub>2</sub> receptors did not mediate WIN-55,212 effects, although the latter was able to induce apoptosis. In fact, when selective CB<sub>1</sub> and CB<sub>2</sub> agonists were added to cultures alone or in combination, they were not able to induce cell death even at high concentrations. Moreover, incubation of Kaposi's sarcoma cells with the selective CB<sub>2</sub> antagonist AM630 could only partially prevent WIN-55,212-2-induced cell death, whereas a similar effect was not exerted by the specific CB<sub>1</sub> antagonist AM251. From the data obtained, it appears that the apoptotic death induced by WIN 52,212 is not, or at least not completely, mediated through cannabinoid receptors. Other mechanisms are probably involved in this WIN-55,212-dependent

effect, such as, for instance, the pathway related to the membrane lipidic raft (Hinz et al., 2004; DeMorrow et al., 2007), an hypothesis which however needs to be confirmed by additional work. Such alternate mechanism could eventually explain the higher concentrations of WIN needed to inhibit Kaposi's sarcoma cell proliferation compared to HUVEC cells where the antiproliferative effects are mediated by CB receptors (Blazquez et al., 2003). Finally, we have demonstrated the mixed cannabinoid agonist WIN-55,212 induces apoptosis in KS-IMM cells, an effect associated with caspase and stress kinase activation, as well as with activation of proliferative kinases, such as ERK<sub>1/2</sub>.

In view of the dual antiproliferative and antiangiogenic effects of cannabinoids, as well as of the endothelial origin of the Kaposi's sarcoma cells, one could envision the cannabinoid system as a potential target for pharmacological treatment of this proliferative disorders.

## 5. Acknowledgements

This work was partly funded by PRA University of Catania grant to Professor Renato Bernardini

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**Legend to figures**

**Fig. 1. Expression of cannabinoid receptor mRNAs and proteins (panels A and B, respectively) in cultured Kaposi's sarcoma cells (KS-IMM):** 1 and 5 lanes: CB<sub>1</sub> and CB<sub>2</sub> mRNAs in control cells (HUVE cells); 2 and 6 lanes: CB<sub>1</sub> and CB<sub>2</sub> mRNAs in KS-IMM cells.; 3 and 7 lanes: negative control (H<sub>2</sub>O); MK: marker.

**Fig. 2. Concentration-dependent effect of WIN-55,212-2 on HUVEC and Kaposi's sarcoma cell proliferation:** Cells were cultured with the agonist for 24 h, 48 h, 72 h, 96 h at graded concentrations. Vertical bars are means + S.E. of at least three separate experiments, in triplicate. \*P<0.05, significantly different from vehicle incubations (CTRL) using Student's "t" test after one-way ANOVA.

**Fig. 3. WIN-55,212-2-induced apoptosis in Kaposi's sarcoma cells:** Representative light microscopy (panel A) and Hoechst staining (panel B) of vehicle treated Kaposi's sarcoma cells and cells incubated with WIN-55,212-2 (2 μM).

**Fig. 4. Time-dependent increase in caspase activity in Kaposi's sarcoma cells treated with cannabinoids :** Kaposi's sarcoma Cells were cultured with vehicle (CTRL) or WIN-55,212-2 (2 μM). Caspase activation was evaluated after 15', 30', 1 h, 6 h and 24 h. \*P<0.05 vs. control values (One way ANOVA followed by a Student's "t" test).

**Fig. 5. Time course phosphorylation and dephosphorylation of MAPKs in Kaposi's sarcoma cells:** cells were incubated with vehicle (CTRL) or 2 μM WIN-55,212-2; expression of p-P38 kinase, p-JNK and p-ERK<sub>1/2</sub> were evaluated at different times. Loading control was carried out with an anti-β-tubulin antibody.

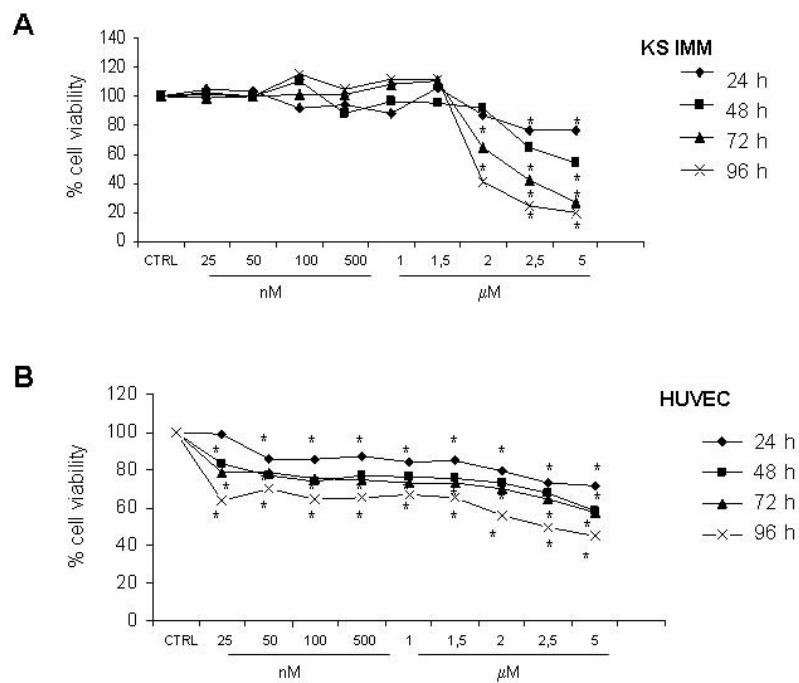
**Fig. 6. Effect of increasing concentration of cannabinoid agonists on the growth of Kaposi's sarcoma cells:** cells were cultured for 96 h with a CB<sub>1</sub> agonist (panel A) or a CB<sub>2</sub> agonist (panel B).

Vertical bars are means + S.E. of at least three separate experiments, in triplicate. \*P<0.05 vs control values (One way ANOVA followed by Student's "t" test).

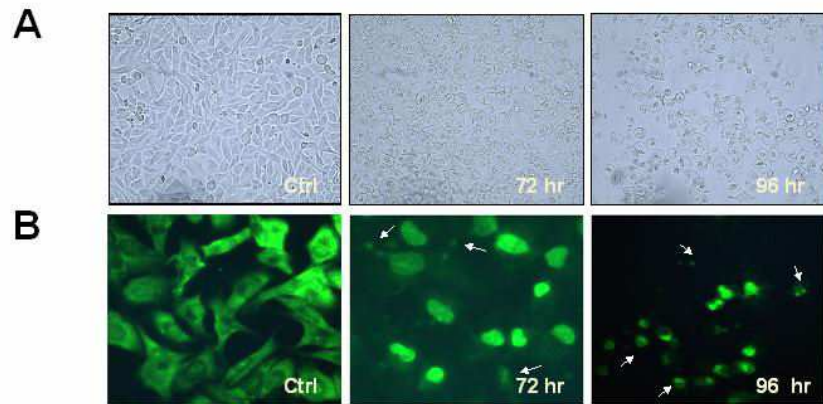
**Fig. 7. Effect of the combination of cannabinoid agonists on the growth of Kaposi's sarcoma cells:** cells were incubated for 96 h in the presence of 5  $\mu$ M ACEA (CB<sub>1</sub> agonist) and 5  $\mu$ M JWH-133 (CB<sub>2</sub> agonist), alone or in combination. Incubations were also performed with 2  $\mu$ M WIN-55,212-2. Vertical bars are means + S.E. of at least three separate experiments, in triplicate. \*P<0.05 vs, control values (One way ANOVA followed by Student's "t" test).

**Fig. 8. Effect of selective cannabinoid antagonists on the growth of Kaposi's sarcoma cells:** cells were incubated for 96 h in the presence of 1  $\mu$ M AM251 (CB<sub>1</sub> antagonist) and 1  $\mu$ M AM630 (CB<sub>2</sub> antagonist), alone or in combination. AM251 and AM630 incubations were also performed in the presence of 2  $\mu$ M WIN-55,212-2. Vertical bars are means + S.E. of at least three separate experiments, in triplicate. \*P<0.05, vs control values (One way ANOVA followed Student's "t" test); #P<0.05 vs. WIN-55,212-2 values (One way ANOVA followed by Student's "t" test).

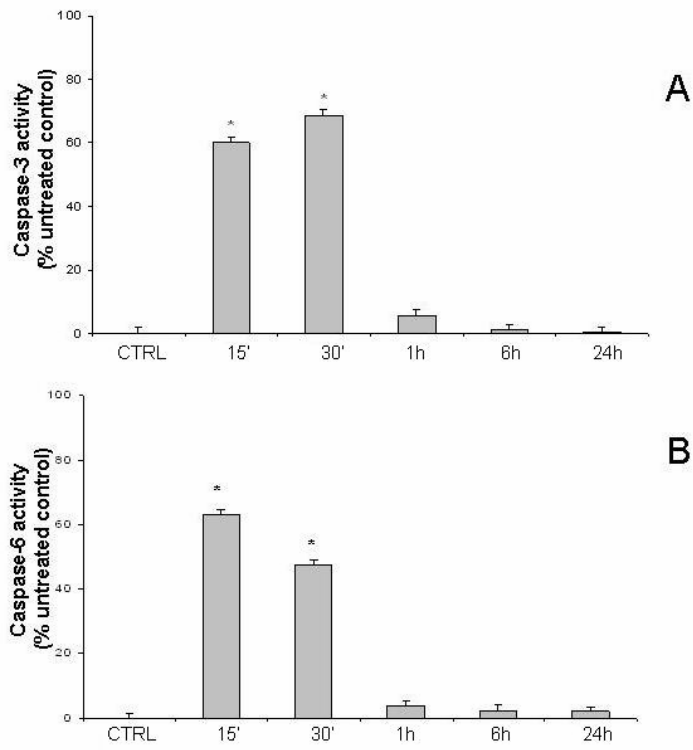




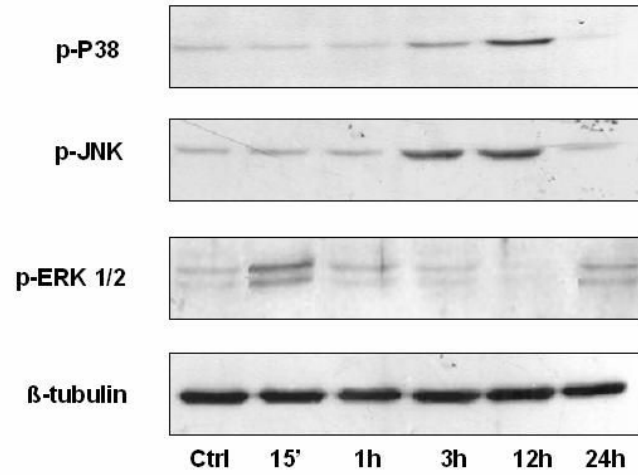
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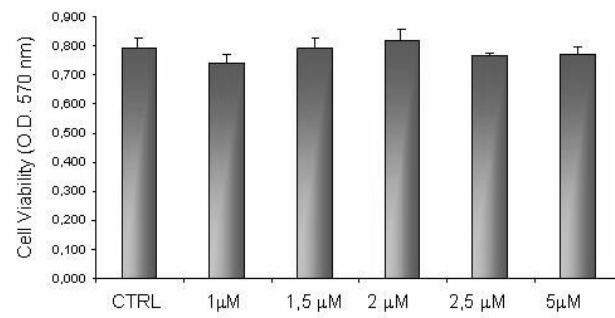
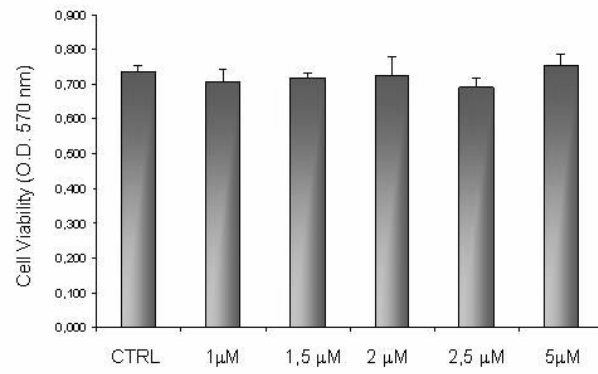
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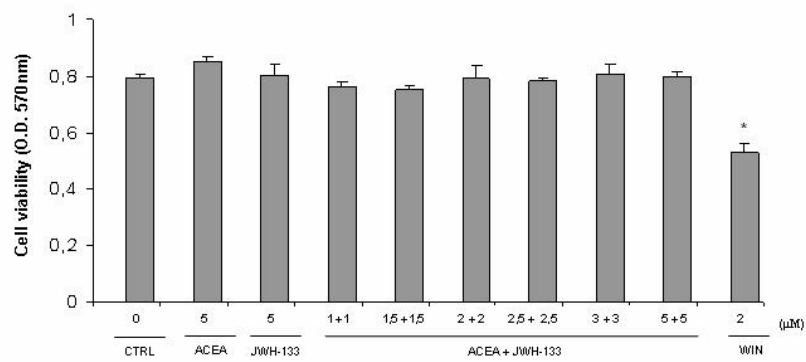
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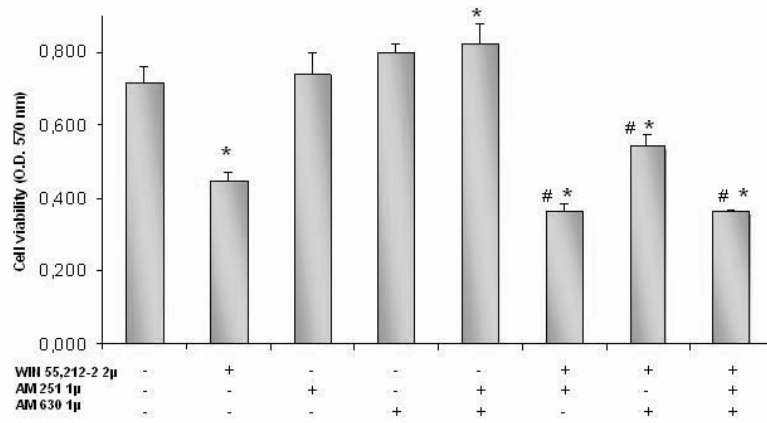
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