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# THE ANTIMITOGENIC EFFECT OF THE CANNABINOID RECEPTOR AGONIST WIN 55212-2 ON HUMAN MELANOMA CELLS IS MEDIATED BY THE MEMBRANE LIPID RAFT.

TESI DI DOTTORATO

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# General Index

# INTRODUCTION

Melanoma is the most fatal form of skin cancer. The incidence and mortality of this disease are increasing (Oliveria, 2007). The lifetime risk for the development of melanoma is now 1 in 41 for men and 1 in 61 for women, making this cancer a growing public health concern.

Surgery remains the prominent treatment of melanoma and it is curative in many cases. (Foster, 2008) Nevertheless, metastatic melanoma is a highly lethal malignancy with few good treatment options (Freudlsperger, 2008).

Such treatment failure is partly due to the broad chemoresistance of melanomas, related to an altered survival capacity and inactivation of apoptotic pathways and to genetic-related resistance of normal melanocytes. They are programmed to survive in order to respond to ionizing radiations, and, interestingly, are enhanced in this protective role by paracrine stimulation from bFGF-secreting fibroblasts, and keratinocytes, promoting melanocyte expression of Bcl-2 triggered by NGF and SCF. Together with Bcl-2, high levels in melanocytes of Bcl-xL and Mcl-1, other antiapoptotic members of the Bcl-2 family,

are features of an altered apoptotic mechanism (Soengas and Lowe, 2003), and, considering the connection between apoptosis and drug sensitivity, are the molecular base for the intrinsic drug resistance of melanoma cells (Freudlsperger, 2008).

The destabilization of programmed cell death in melanomas consist of three factors:

1) activation of antiapoptotic factors

Two members of IAP family (Inhibitor of Apoptosis Proteins), ML-IAP and survivin, and FLIP (FLICE/Casp8 inhibitory protein) are overexpressed in metastatic melanoma (Grossmann and Altieri, 2001). ML-IAP's effects on the apoptotic mitochondrial pathways are considered to be related to a direct inhibition of the factor Smac/Diablo, and the caspases 9 and 3 (Vucic, 2002).

2) inactivation of proapototic effectors

Although melanomas display a low frequency of p53 mutations, disruption of proapoptotic signalling downstream of p53 may alleviate pressure to mutate and, at the same time, decrease drug sensitivity for the inability of transduction of death signals. Apaf-1, binding *cyt c* and

ATP to form the "apoptosome" (**Fig. 1**) which in turn binds and activates Casp9, and its mRNA are downregulated in melanoma cell lines (Soengas and Lowe, 2003).

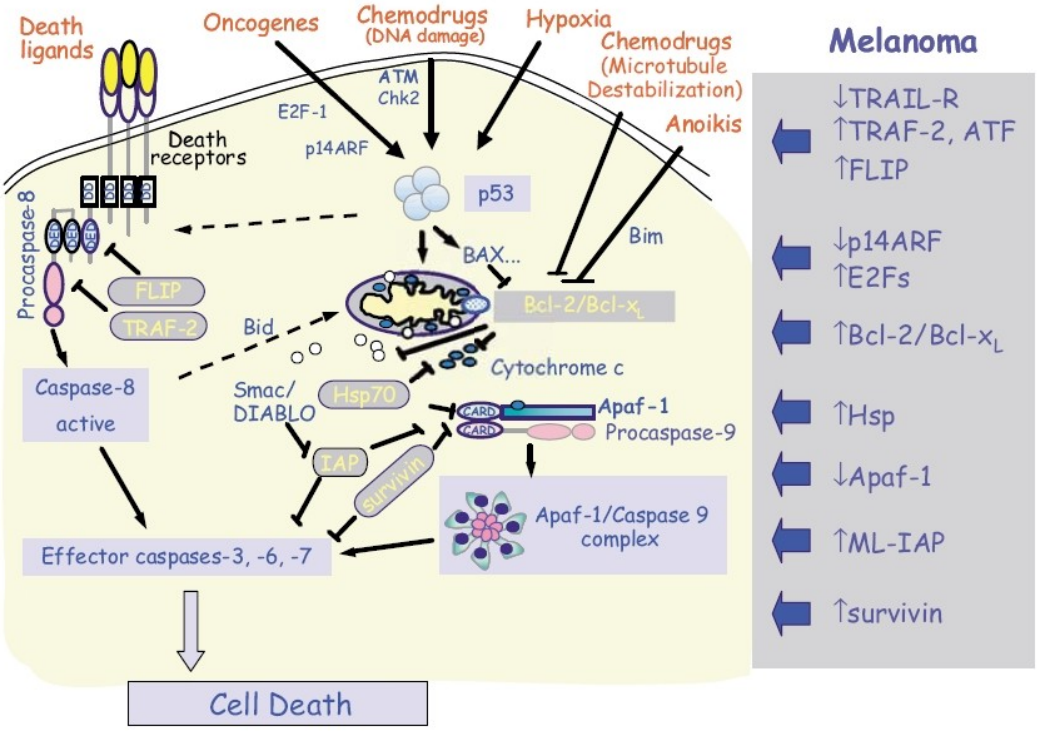



*Figure 1*

3) reinforcement of survival signals

In response to multiple mitogens (**Fig. 2**), such as integrins and growth factors, phosphoinositide 3-kinase ( PI3K) converts $PIP_2$ into $PIP_3$. $PIP_3$ activates AKT, which targets a plethora of survival signals, promoting the transcription of Bcl-xL and inactivating BAD and

Casp9. PTEN, a tumor suppressor that targets $PIP_3$ and prevents AKT activation, showed reduced expression in one-third of primary melanomas and about 50% of metastatic melanoma cell lines (Zhou, 2000).

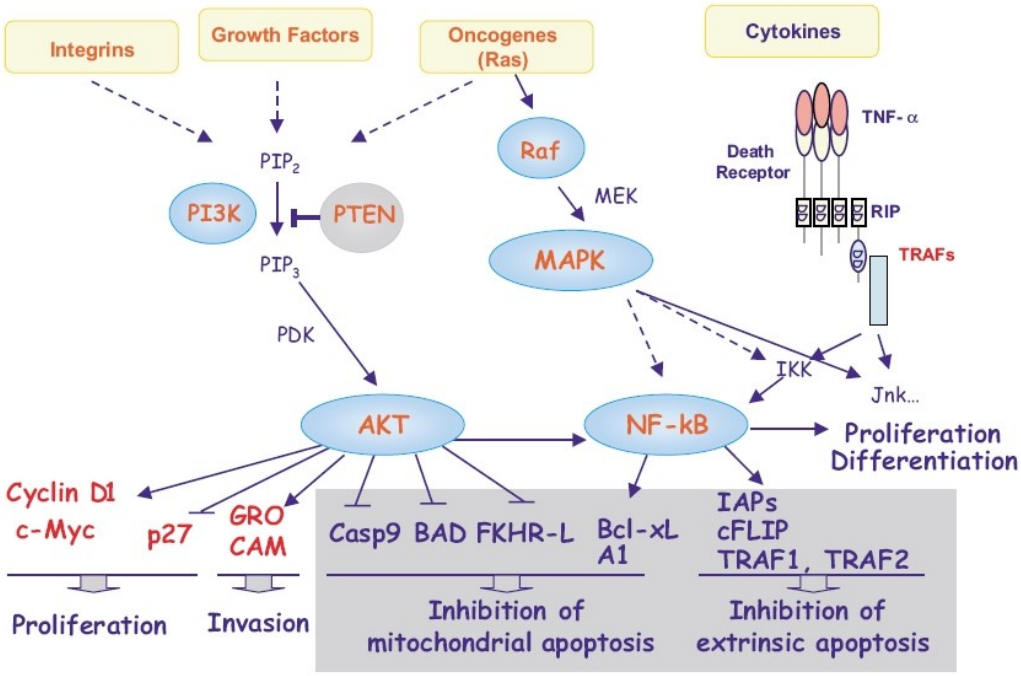



*Figure 2*

Preparations of cannabis have been used in medicine for many centuries, and currently, there is an intense renaissance in the study of the therapeutic effects of cannabinoids, the active components of Cannabis sativa, focused on the design of potent and selective synthetic cannabinoid agonists and antagonists (Pertwee, 2002).

Cannabinoids exert a wide array of effects on the central nervous system, as well as on peripheral sites, such as the immune, cardiovascular, digestive, reproductive, and ocular systems (Fowler 2010; Ligresti, 2009; Pandey, 2009). In addition, cannabinoids exert antimitogenic effects in various tumors in animal models (Llanos-Casanova, 2003) and directly induce apoptosis or cell cycle arrest in different transformed cells in vitro (Cianchi, 2008; Qamri, 2009).

Two signalling pathways that have been reported to be either positively or negatively regulated by cannabinoids (depending on the cell type and cannabinoid studied) are the RAS-MAPK/ERK pathway and the PI3K-AKT pathway (Greenhough, 2007).

Cannabinoids act through two receptors: the CB1 receptor, mostly expressed in brain, and the CB2 receptor, expressed peripherally, for example in immune system cells. The mechanism by which

cannabinoids exerts their pro-apoptotic effects is not clear yet. Recent evidence suggests that their antiproliferative effect could be mediated through pathways alternative to those associated with CBRs. For example, some cannabinoid effects are mediated by the vanilloid receptor 1 (VR1) (Contassot, 2004; De Petrocellis, 2008), as well as by non-receptorial sites (Hinz, 2004; Yang, 2010).

In this line, an involvement of lipid rafts complexes, specialized domains within the cell membrane abundant in glycosphingolipids, saturated phospholipids and cholesterol, has been suggested. These cholesterol-rich regions can include or exclude proteins, and may serve as clues for recruitment and concentration of signaling molecules and thus have been implicated in signal transduction from cell surface receptors. Recently, a proteomics approach for various cancer cells revealed that two subsets of raft assemblage in cell membrane exist. One subset of raft is enriched with cholesterol-sphingomyeline-ganglioside (chol-raft) containing proteins mainly caveolins, CD44, and members of the RTKs family. “Chol-rafts” are responsible for cellular homeostasis, but when normal cellular signaling is dysregulated “cholrafts” promote cell transformation, tumor progression, angiogenesis and metastasis. Another subset of raft

is enriched with ceramide–sphingomyeline, “cer-raft” containing mainly, FAS, FASL, andCell. Mol. Life Sci the members of death-inducing signaling complex (DISC). “Cer-rafts” promote apoptosis (Bionda, 2008; Oh, 2007; Patra, 2007). Interestingly, depletion of cholesterol in lipid rafts attenuates antiblastic drug-induced apoptosis (Sarker, 2003; Van der Luit, 2007).

In light of these data, with the aim to identify novel molecular targets for melanoma therapy, this study was designed to characterize mechanisms through which the non-selective CB1/CB2 receptor agonist WIN 55,212-2 induces apoptosis in different human melanoma cell lines.

## MATERIALS AND METHODS

*Cell cultures and reagents*.

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

All human melanoma cell lines were a generous gift of the Laboratory of Immunology, National Cancer Institute "Regina Elena", Rome. SK-MEL28 is an HLA-allotyped, intermediate invasive adherent metastatic melanoma cell line; COLO38 is a malignant melanoma which expresses the MPG antigen; OCM-1 is a non-metastatic ocular choroidal melanoma cell line. Cells were grown in RPMI medium containing 2 mM glutamine, antibiotics and 10% fetal bovine serum (FCS). They were cultured at 37°C in humidified 5% CO2/95% atmosphere.

*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-1 oligo (dT)12-18 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 CB1 and human CB2 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 VR1 primers consisted of denaturation at 95° C for 45 sec, annealing at 55°C for 45 sec 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 CB1 using a primer pair composed of the sense primer 5'-CATCATCACACGTCTG-3' and the antisense primer 5'-ATGCTGTTATCCAGAGGCTGC-3'; the human CB2 using a primer pair composed of the sense primer 5'-TTTCCCACTGATCCCCAATG-3' and the antisense primer 5'-AGTTGATGAGGCACAGCATG-3'; the human VR1 using a primer pair composed of the sense primer 5'-X-3' and the antisense primer 5'-X-3'.

*Protein extraction*

In a set of experiments, Colo38, Ocm-1A and SKmel28 cells grown in 100 mm plastic Petri dishes were incubated for 48 h in basal condition. Membrane and cytosolic fractions of KS 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 freezed at –80°C for 5 min. After thawing, the lysed cells were centrifuged at max speed for 10 min at 4°C and the supernatans (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 fenilmetilsulfonifluride, 0.5 mg ml-1 leupeptin, 5 mg ml-1 aprotinin, 1 mg ml-1 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.

*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 anti CB1 (Cayman chemical Ann Arbor, MI, USA), rabbit polyclonal anti CB2 (Cayman chemical Ann Arbor, MI, USA), rabbit polyclonal anti Phospho-p42/44 Map kinase (Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit polyclonal β-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.

*Viability assay*

Colo38 and Ocm-1A were seeded at 2 x $10^3$ cells/well into 96-multiwell plates in RPMI with 10% FBS, penicillin and streptomycin. After 24 h, medium was replaced with RPMI supplemented with 1% FBS, penicillin and streptomycin and maintained for a further 24 h. They were incubated at 37 °C for 24 hr, 48 hr, 72 hr and 96 hr with the following concentrations of WIN-55,212-2 (Sigma-Aldrich,

Milano, Italy) in RPMI 1% FBS: 500 nM, 2 μM, 5 μM. Control cells received 0,1% DMSO in RPMI 1% FBS.

In a second experiment Colo38 and Ocm-1A were treated for 48h, 72h and 96h with the following concentrations of a) WIN-55,212-2 500 nM, 2 μM, 5 μM in RPMI 1% FBS b) ACEA (Tocris Bioscience, Ellisville, MO, USA) and JWH-133 (Tocris Bioscience, Ellisville, MO, USA) alone or in combination 500 nM, 2 μM, 5 μM in RPMI 1% FBS and b) AM251 (Tocris Bioscience, Ellisville, MO, USA) and AM630 (Tocris Bioscience) alone or in combination 500 nM, 2 μM, 5 μM in RPMI 1% FBS, alone or in association with 2 μM WIN-55,212-2. Control cells received the equivalent dilution of DMSO in RPMI 1% FBS.

In a third experiment Colo38 and Ocm1a were treated as it follows: a)capsaicin for 24 h at the concentration of 50 μM, 100μM, 150μM and 200μM; b) capsaicin at the concentration of 100 μM for 24, 48, 72 and 96 h; c) SB366791 (Tocris Bioscience, Ellisville, MO, USA) at the concentration of 20 nM alone or in association with 500nM WIN-55,212-2 for 96h and with 5μM WIN-55,212-2 for 48h. Control cells received the equivalent dilution of DMSO in RPMI 1% FBS.

In other experiments Colo38 and Ocm-1A were treated with 5μM

WIN-55,212-2 for 24h alone or in association with 1 mM methyl-β cyclodextrin (MCD, Sigma-Aldrich, Milano, Italy) with the following modalities: 30 minutes prior to, concurrently with, or 10 – 30 – 60 minutes after Win.

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 hours 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.

*Acridine orange/ethidium bromide staining*

Morphological assessment of apoptotic cells was performed using the acridine orange (AO)/ethidium bromide (EB) double-staining method. Colo38 and Ocm-1A cells were seeded in 24-well microtiter plates (10 000 cells per well). After 24-h incubation, cells were treated with WIN-55,212-2 2 μM, staurosporin (0.5 μM), H2O2 (1μM) and incubated at 37 °C in a 5% CO2 atmosphere for 72 h. Freshly isolated

cells were harvested in an Eppendorf centrifuge tube, spun for 5 min at $10^6$ g and suspended in phosphate-buffered saline containing fluorescence dye AO/EB (both AO and EB were at the concentration of 100 mg/L in phosphate-buffered saline). Then, cells were prepared and dropped onto slides. The morphology of the cells was observed under fluorescence light microscope (DMIRB, Leica, Germany) and photographed. Viable cells were stained green with intact nuclei. Chromatin was stained stained bright orange chromatin in non-viable cells. Apoptosis was demonstrated by the appearance of cell shrinkage with condensation and fragmentation of the nuclei. Necrotic cells appeared orange with a normal nuclear structure.

*Caspase-8, Caspase-6, Caspase-3 and Caspase-7 assay*

The activity of the caspase was measured in Ocm-1A cells. Cells were exposed to WIN-55,212-2 5 μM and to WIN-55,212-2 5 μM plus methyl-β cyclodextrin 1 mM for 6 hr, 16 hr and 24 hr. caspase 3 - 8 and 9 activity was determined in whole cell lysates 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,IETD for caspase-8 and LEHD for caspase-9) 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 IETDase or LEHDase activity present in the sample. Fold-increase in caspase activity can be determined by comparing these results with the level of the uninduced control.

Caspase-7 was determined by using the Caspase-Glo 3/7 luminescent assay (Promega Corporation, Madison, WI, USA), following the manufacturer's instructions. The assay provides a luminogenic caspase-3/7 substrate which contains the tetrapeptide sequence DEVD. Adding a single Caspase-Glo 3/7 reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate and generation of a luminescent signal, product by luciferase. Luminescence is proportional to the amount of caspase activity present.

## RESULTS

### WIN55212-2 induces apoptotic cell death in human melanoma cells which express both CB1 and CB2 cannabinoid receptors.

In order to verify possible responsiveness of various melanoma cell types to cannabinoids, the presence of canonical cannabinoid receptor CB1 and CB2 in cell lysates by means of either northern blot and western blot was first assessed. All the three lines studied, COLO38, SKMEL28 and OCM1A contained mRNA corresponding to CB1 and CB2 gene (**Fig. 3, panel A**). The corresponding proteins were also expressed by the three cell lines. The CB1 protein was mostly expressed by the cytosolic fraction of cell lysates, whereas it was expressed to a lesser extent at the level of cell membrane in all cell lines. While the CB2 protein expression profile was similar to that of CB1 in COLO38 cells, it was inverted in SKMEL28 and OCM1A cells, where the higher level of expression was found onto the plasma membrane, whereas it was scarce at the level of cytosol**.** (**Fig. 4, panel B**).

To understand the functional significance of both CB1 and CB2 receptors in melanoma cells, the metastatic human melanoma cell line COLO38 and the non metastatic OCM1A cell line were incubated

with graded concentrations of the mixed cannabinoid receptor agonist WIN 55212-2 at different times. WIN55212-2 induced cell death in a concentration- and time-dependent fashion in both cell lines. Both in COLO28 and OCM1A, WIN exerted its maximal antiproliferative effect at the concentration of 5 μM after 24 of incubation. The effect of WIN lasted until 96 h incubation. A more pronounced effect of WIN was observed on OCM1A cells (non metastatic cells), in which WIN was effective already at the concentration of 500 nM after 96 h incubation (**Fig. 4, panels A and B**).

Then, in order to classify the cell death type to which cells had undergone after the treatment with WIN55212-2, both the acridine orange and the ethidium bromide test were applied to cultures treated with WIN55212-2 and various proapoptotic compounds.

Both tests, performed on the Ocm1A cell line revealed the presence of apoptotic nuclei in cultures treated with WIN55212-2 (2 μM) for 72 hours. The number of apoptotic cells in the WIN55212-2 experiments was comparable to that resulting after treatment with the proapoptotic agent staurosporine (**Fig. 5**). Similar results were obtained with Colo38 cells (data not shown).

**None of both CB1 and CB2 cannabinoid receptors, nor vanilloid receptors mediate apoptotic death caused by WIN55212-2 in human melanoma cell lines.**

With the aim to identify which one of the two canonical cannabinoid receptor CB1 or CB2 could mediate the apoptotic death of melanomatous cells induced by the mixed agonist WIN55212-2, both the selective CB1 agonist ACEA or the selective CB2 agonist JWH133 were added for 96 h to the two melanoma cell line cultures.

Neither ACEA, nor JWH133 were able to cause apoptotic cell death in both human melanoma cell lines at all the concentrations used over 96 h incubation. Similarly, when the two drugs were used in combination, no effect on cell proliferation occurred (**Fig. 6, panel A**).

To confirm that CB1 and CB2 receptors are not involved in melanoma cells apoptotic death caused by WIN55212-2, the specific CB1 receptor antagonist AM251, as well as the specific CB2 receptor antagonist AM630 were co-incubated at proper concentrations with WIN55212-2 in melanoma cell cultures with different time schedule.

Results indicate that none of the two cannabinoid receptor antagonists was able to prevent the proapoptotic effects of WIN55212-2 upon both melanoma cell lines. Protection from WIN55212-2-

induced cell death did not occur even in those cultures where a combination of AM251 and AM630 was coincubated with WIN55212-2. Both AM251 and AM630 were uneffective when used alone (**Fig. 6, Panel B**).

However, CB1 and CB2 receptors are not the only capable of binding WIN55212-2 and mediate its effects. In fact, cannabinoid molecules can also bind to vanilloid receptors, such as VR1 (Contassot, 2004; De Petrocellis, 2008). Thus, possible interactions of WIN55212-2 with the VR1 receptor were studied in melanoma cell cultures. To do so, first of all the presence of the VR1 receptor mRNA was assessed into the different cell line. Both COLO 38 and OCM1A contained the VR1 receptor mRNA (**Fig. 7, panel A**).

In addition, to assess the role of VR1 receptor in the cell lines studied, the receptor agonist capsaicin was added at graded concentration to the cultures, resulting in concentration-dependent melanoma cell death in both lines used. Capsaicin was effective after 24 h incubation at a concentration of 50 M, and reached a maximum effect at 200 μM. The effect of capsaicin was time-dependent, with maximum cell death at 96 h (**Fig. 7, panel B**).

Subsequently both melanoma cell lines were incubated with

WIN55212-2, in the presence or the absence of proper concentrations of the specific VR1 receptor antagonist SB366791.

Similarly to CB receptor antagonists, SB366791 was unable to abrogate the apoptotic effect of WIN55212-2 upon melanoma cell lines (**Fig. 7, panel C**).

**The membrane lipid raft mediates the proapoptotic effects of WIN55212-2 on melanoma cell lines via the intrinsic caspase pathway.**

It has been reported that the CB1 receptor may function in a complementary way with the membrane lipid raft (Bari, 2008; Rimmerman, 2008; Sarnataro, 2005). Thus, to better understand whether the latter could be involved in the WIN proapoptotic effect, melanoma cells were incubated with the cannabinoid agonist in the presence or in the absence of the lipid raft disruptor methyl-beta-cyclodestrin (MCD).

MCD was able to partially rescue melanoma cell lines from apoptotic death caused by WIN. Such abrogative effect of MCD appeared after 24h incubation and reached a peak at 48h. (**Fig.8**)

In another set of experiments, incubation with WIN alone was

associated with activation of the caspase 9 pathway, which reached its peak after 16 h. In different experiments in which WIN was combined with MCD, significant activation of caspase 8 occurred with a maximum after 16h incubation. Both caspases 8 and 9 activity returned to baseline after 24h incubation, either in cultures incubated with WIN alone or with a combination of WIN and MCD (**Fig. 9, panel A**).

The complex of effector caspases 3 and 7 was also activated in both cell lines treated with WIN, either alone or in combination with MCD. Peak of the effect occurred after 24h. The effect of WIN at 24 h was significantly greater than that of the combined treatment (**Fig. 9 panel B**).

ERK phosphorylation has been described in cells undergoing the apoptotic process and related to caspase cascade activation in melanoma cells (Kim, 2008; Lee, 2008; Osada, 2001). Thus, in addition, expression of phosporylated ERK was evaluated in both melanoma cell lines in different conditions of treatment, i.e. with WIN alone or combined with MCD, at different time points.

Results indicate that ERK phosphorylation was increased in cells (both lines) incubated with either WIN or the combination of

WIN and MCD. Peaks of the effect of both treatments occurred at 24 h. The effect of WIN was significantly greater than that of the combined treatment (**Fig. 10**).

## DISCUSSION AND CONCLUSIONS

In this paper we show that cannabinoids exert antimitogenic effect upon human melanoma cells via a mechanism involving membrane lipids. Although cannabinoid receptors mediate inhibition of proliferation and angiogenesis in non melanoma skin tumors, scanty data existed relating cannabinoids to the utmost aggressive among cutaneous neoplasms (Llanos-Casanova, 2003).

In our hands, different melanoma cells contained mRNA for cannabinoid receptors CB1 and CB2 and express the corresponding proteins. Interestingly, in basal conditions, the cellular distribution of the receptorial proteins is quite variated, as CB1 is present predominantly in the cytosol, confirming the intravescicular localization reported in the literature (Bari, 2008; Sarnataro, 2005), whereas the CB2 receptor expression prevails within the cell membrane in two of the cell lines studied, OCM1A and SKMEL28, and in the cytosol of COLO38 cells.

In basal conditions, CB1 receptor was present mainly in the cytoplasm of all the cell lines studied, whereas CB2 expression was higher in the cytoplasm of COLO cells and in the membrane of OCM and SKMEL cells. In fact, partitioning of CB1 receptors into

specialized membrane domains was postulated from a C-terminal palmytoilation domain required for proper interactions with lipid raft-associated G proteins. It has been reported that the internalization of CB1 receptors in HEK293-CB1 transfected cells occurred via both clathrin-coated pits and caveolae, although the latter was absent in N18TG2 neuroblastoma cells natively expressing 1 recepotrs or CB1 receptor-transfected AtT20 cells (Hsieh, 1999). Bari, 2008 showed that in rat C6 glioma cells, CB1 receptor binding and signalling were doubled following lipid raft disruption by cholesterol depletion with methyl-b-cyclodestrins, while cholesterol enrichment of C6 glioma cells led to reduced CB1 receptor binding efficiency. Consistent with these findings, Sarnataro, 2005 showed that in MDA-MB-231 breast cancer cells, CB1 receptors associated with lipid raft fractions and were redistributed mostly to non-lipid raft fractions following treatment.

The CB mixed agonist WIN induced time and concentration dependent cell death in all the lines studied, and the death was of the apoptotic type, as assessed by the ethidium bromide method.

It is known that either CB1 and CB2 cannabinoid receptors mediate cell death in cancerous cells (Cianchi, 2008; Llanos-Casanova, 2003),

as well as in normal cells (Alvaro-Bartolomé, 2010; Lim, 2010; Viscomi, 2010). WIN is an agonist of both receptors and is thus expected to induce cell death. However, when we used the specific CB receptors agonists ACEA or JWH-133, we were unable to induce cell death in melanomatous cells in culture, suggesting that neither CB1 receptors, nor CB2 appear involved in this process. In fact, CB1 receptors induce cell death in different model, similarly to that happening under activation of CB2, although the two receptor systems are, using different signal transduction mechamism (Demuth, 2006). Similarly, when we used the specific CB receptors antagonist in the attempt to identify which one of the two receptors was mediating WIN-dependent melanoma cell death, we failed observing inhibition of the cell death rate.

The results obtained were convincing about the plausible hypothesis that CB receptors could not be involved in melanomatous cell death induced by WIN.

Now, it is known that CB receptors are not the only signal transductors of cannabinoid effects on the cells (Hinz, 2004; Yang, 2010), as other receptors, such as vanilloid receptors (Contassot, 2004; De Petrocellis, 2008), which are able to mediate effects such as, for

example, modulation of pain transmission and of tumor cell survival (Contassot, 2004; De Petrocellis, 2008). The natural ligand of vanilloid receptors, capsaicin, induces in addition to its effects upon neural cells (Mishra, 2010; Ro, 2009), also other effects upon different cells, including, for example expression of androgen receptor via PI3K and MAPK pathways in prostate LNCaP cells (Malagarie-Cazenave, 2009), as well as apoptosis in prostate PC3 cells (Sánchez, 2009). Interestingly, all melanomatous cell lines examined contained mRNA for vanilloid receptor VR1, a presence associated with functional effects of the protein, considered that incubation of cells with capsaicin resulted in increased cell death rate.

Effects of capsaicin mediated by the VR1 receptors are antagonized by specific VR1 receptor antagonists, such as SB366791 (Gunthorpe, 2004).

Now, it has also been reported that eicosanoids induce apoptosis of human glioma cells through vanilloid receptor-1 (Contassot, 2004) and that the same receptors mediate disruption of anandamide-induced behaviour (Panlilio, 2009).

Eventually, SB366791 was unable to prevent cell death induced by WIN, suggesting that not only the apoptotic effects of WIN are not

mediated by CB receptor, but VR1 receptor antagonism may not be claimed to explain such a phenomenon.

Some membrane extra-receptor structures are able to bind cannabinoid-related molecules and eventually mediate some of the effects of the latter upon different cell system. A number of reports indicate that cannabinoid-dependent cell death is induced with a mechanisms dependent upon the membrane lipid rafts (Yang, 2010). Many effect of cannabinoids are under control of the latter. For example, CB1 receptor-dependent signalling relies upon activation of the membrane lipid raft machinery in neurons (Bari, 2008). Most of the lipid raft mediated events are affected by the membrane disruptors of the cyclodextrine family (Patra, 2007).

In this line, the addition of methylcyclodextrin to different melanomatous cell cultures resulted in a time-dependent decrease of WIN-induced cell death rate, serving as a proof that disruption of membranes enhances cancer cell proliferation (Patra, 2007).

In a similar fashion, MCD prevents anticancer phospholipids agent from killing lymphoma cells (Van der Luit, 2007) and inhibits cell viability in cholesterol depleted prostatic cells (Oh, 2007).

In addition, whereas treatment of cells with WIN activates the

caspase-9-dependent intrinsic pathway, the addition of MCD induced in similar conditions, activation of the caspase-8-dependent extrinsic pathway. On the other hand, both executive caspases 7 and 3 are equally activated by WIN or after addition of MCD. Recent reports have demonstrated that CB1R functions in the context of lipid raft and that MCD treatment increases the accessibility of CBR1 to its specific antibodies and doubles the binding efficiency of CB1R and thus the CB1R-dependent actions of endocannabinoids. Thus, based upon our results, it appears plausible to hypothesize that, as CB1 is not functioning in an intact lipid ratf, WIN could promote apoptosis via activation of the intrinsic pathway. On the other hand, disruption of the lipid raft with MCD would result in activation of CB1 and the extrinsic pathway of apoptosis, as shown by recruitment of residual cells to apoptotis following treatment with WIN.

WIN also induced phosphorylation of ERK. Although it is widely known that the extracellular signal-regulated kinase (ERK) pathway stimulates cell growth thus protecting cells from death, evidence suggests a proapoptotic action of ERK phosphorylation (Osada, 2001). For example, TrkA overexpression induced substantial cell death even in the absence of NGF, by stimulating ERK phosphorylation and

caspase-7 activation leading to PARP cleavage and, in addition, induces mitochondria-mediated apoptosis (Jung, 2008).

In synthesis, we showed that the CB mixed agonist WIN induces a CB1-, CB2- and VR-1 independent human melanomatous cell death. The membrane lipid rafts machinery appears involved in such antimitogenic effects of WIN, associated with cleavage of caspases 9 and 7 and ERK phosphorylation. In the presence of a disrupted lipid raft, the CB1 receptor would mediate the proapoptotic effect of WIN. Unravelling the lipid raft mediated endocannabinoid-induced apoptosis of human melanomatous cells may be suggestive of novel molecular targets for candidate drugs in treatment of either primitive or metastatic melanomas.

## LEGEND TO FIGURES

**Figure 1.** Apoptotic pathways in eucaryotic cells. Up and down-regulated factors in melanoma are showed (Soengas and Lowe, 2003).

**Figure 2.** Survival mechanisms in melanoma and their impact on inhibition of both intrinsic and extrinsic apoptotic pathway (Soengas and Lowe, 2003).

**Figure 3**. *Panel A:* Reverse transcriptase PCR for CB1 and CB2 receptors mRNAs in different human melanoma cells. *Panel B:* Western blot analysis for CB1 and CB2 receptors proteins in different human melanoma cells. *Panel C:* Densitometric analysis of the western blots shown in panel B. Bars are means ± SE; *$p<0.05$ (one-way ANOVA followed by a Fisher's test).

**Figure 4.** *Panel A:* Time-related mitogenic effect of graded concentrations of the mixed CB1/CB2 receptor agonist WIN 55212-2 upon the human melanoma cell line COLO38. Bars are means ± SE; *$p<0.05$ (two-way ANOVA test) . *Panel B:* Time-related mitogenic effect of graded concentrations of the mixed CB1/CB2 receptor agonist WIN 55212-2 upon the human melanoma cell line OCM1A in

vitro. Bars are means ± SE; *p<0.05 (two-way ANOVA test).

**Figure 5.** Representative tests with acridine orange (upper panels) or ethidium bromide (lower panels) staining in the human melanoma cell line OCM1A treated with the mixed CB1/CB2 receptor agonist WIN 55212-2. CTRL: untreated cells; H2O2: cells treated for 72 hours with hydrogen peroxide 300 μM; staurosporine: cells treated for 72 hours with the inducer of apoptosis staurosporin at the concentration of 1 μM; WIN: cells treated for 72 hours with the mixed CB1/CB2 receptor agonist WIN 55212.

**Figure 6**. *Panel A*: Effects of either selective CB1 (ACEA) or CB2 (JWH133) receptor agonists at graded concentrations upon cell viability in COLO38 and OCM-1 human cells incubated 96 h in vitro. *p<0.05 compared to untreated cells; vertical bars are SE of means (one-way ANOVA followed by a Fisher's test). *Panel B*: Effects of either selective CB1 (AM251; 1 μM) or CB2 (AM630; 1 μM) receptor antagonists at graded concentrations upon cell viability in COLO38 and OCM-1 human melanoma cells incubated 96 h in vitro with WIN55212-2. WIN55212-2 was used at graded

concentrations. Vertical bars are means ± SE; *p<0.05 compared to untreated cells (CTRL) (one-way ANOVA followed by a Fisher's test).

**Figure 7**. *Panel A:* Reverse transcriptase PCR for VR1 vanilloid receptor mRNAs in different human melanoma cells. *Panels B and C:* Respectively, concentration, or time related effect of the VR1 agonist capsaicin upon cell viability in COLO38 and OCM-1 human melanoma cells. Vertical bars are SE of means; *p<0.05 compared to untreated cells (CTRL) (one-way ANOVA followed by a Fisher's test). *Panel D:* Effects of the selective VR1 receptor antagonists SB366791 (20 nM) upon cell viability in COLO38 and OCM-1 human melanoma cells incubated 96h in vitro. WIN55212-2 was used at graded concentrations. Vertical bars are means ± SE; *p<0.05 compared to untreated cells (CTRL) (one-way ANOVA followed by a Fisher's test).

**Figure 8**. Time related effect the membrane lipid raft disruptor methyl-ß-cyclodestrin (MCD; 1 mM) upon cell viability in COLO38 and OCM-1 human melanoma cells incubated in vitro with the mixed CB1/CB2 agonist WIN55212-2 (5 μM). Vertical bars are means ± SE

of means; *p<0.05 compared to untreated cells (CTRL) (one-way ANOVA followed by a Fisher's test).

**Figure 9.** *Panel A:* Time-related effects of the mixed CB1/CB2 agonist WIN55212-2 (5 μM), either alone or combined with the membrane lipid raft disruptor methyl-ß-cyclodestrin (MCD) on the activity of inducer caspases 8 and 9 in the COLO38 human melanoma cell line. Vertical bars are means ± SE of means; *p<0.05 compared to untreated cells (CTRL) (one-way ANOVA followed by a Fisher's test). *Panel B:* Time-related effects of the mixed CB1/CB2 agonist WIN55212-2 (5 μM), either alone or combined with the membrane lipid raft disruptor methyl-ß-cyclodestrin (MCD) on the activity of effector caspases 3 and 7 in the COLO38 human melanoma cell line. Vertical bars are means ± SE of means; *p<0.05 compared to untreated cells (CTRL) (one-way ANOVA followed by a Fisher's test).

**Figure 10.** *Panel A:* Time-related effects of the mixed CB1/CB2 agonist WIN55212-2 (5 μM), either alone or combined with the membrane lipid raft disruptor methyl-ß-cyclodestrin (MCD) on the phopshorylation of the ERK (p42/44) in the COLO38 human

melanoma cell line. *Panel B:* Densitometric analysis: vertical bars are means ± SE of means; *$p<0.05$ compared to untreated cells (CTRL) (one-way ANOVA followed by a Fisher's test).

# FIGURES

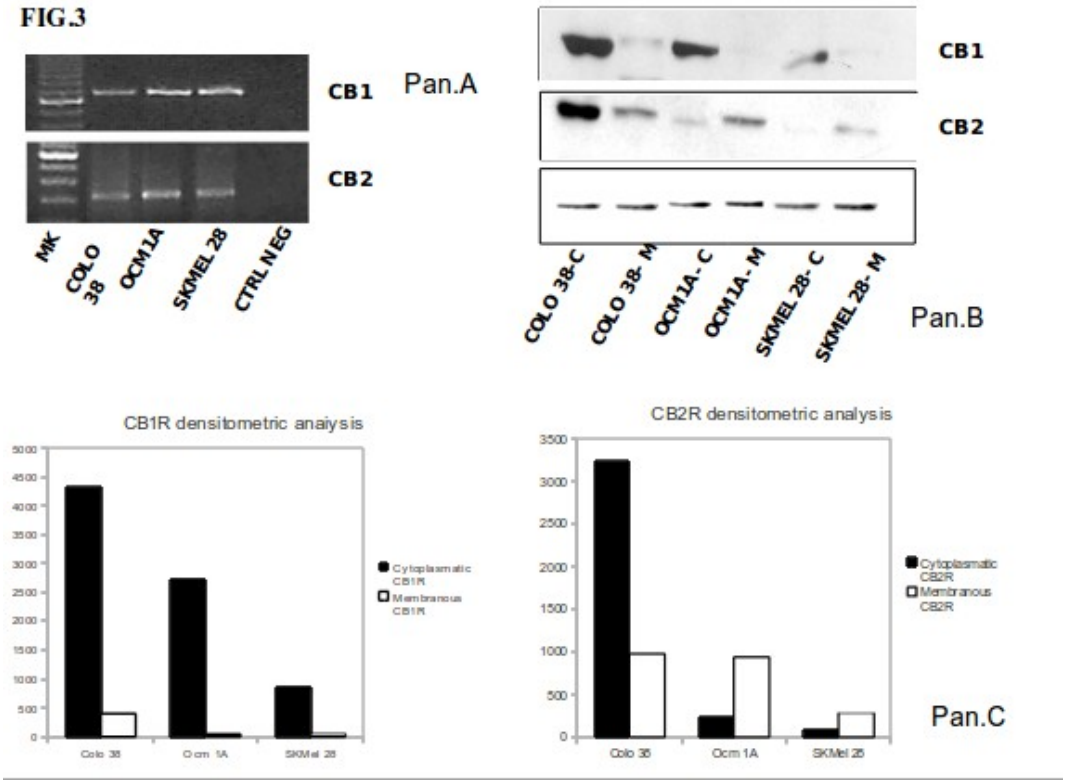


FIG.3
CB1
CB2
MK
COLO 38
OCM1A
SKMEL 28
CTRL NEG
Pan.A
CB1
CB2
COLO 38-C
COLO 38- M
OCM1A- C
OCM1A- M
SKMEL 28- C
SKMEL 28- M
Pan.B
CB1R densitometric anaiysis
Cytoplasmatic CB1R
Membranous CB1R
Colo 38
Ocm 1A
SKMel 28
CB2R densitometric analysis
Cytoplasmatic CB2R
Membranous CB2R
Colo 38
Ocm 1A
SKMel 28
Pan.C

**FIG. 4**

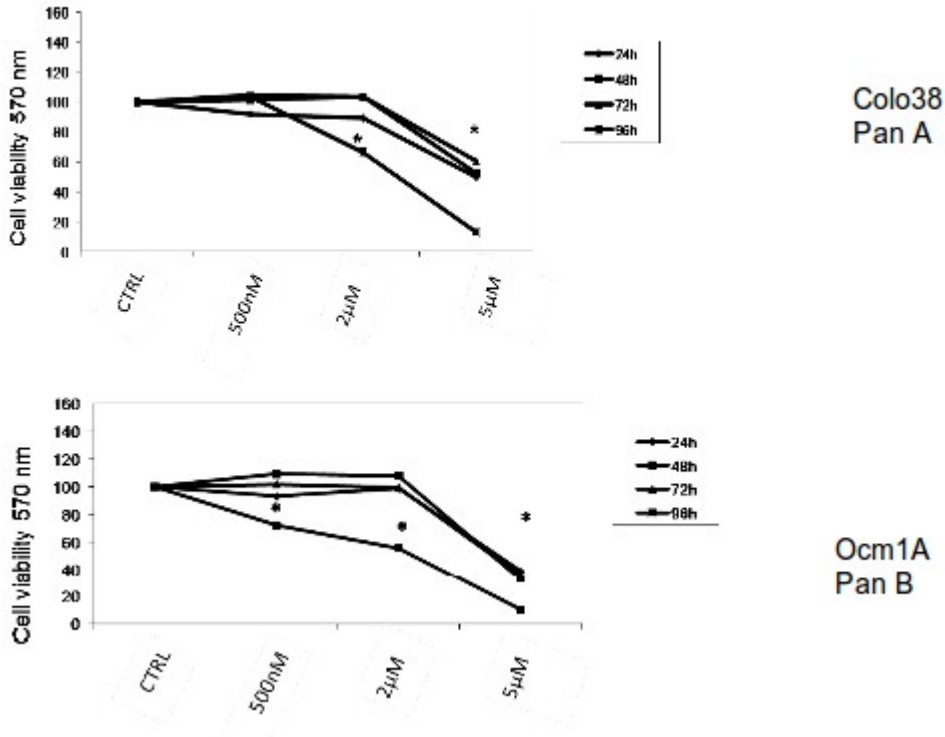

Cell viability 570 nm
160
140
120
100
80
60
40
20
0
CTRL
500nM
2μM
5μM
24h
48h
72h
96h
*
Colo38
Pan A
Cell viability 570 nm
160
140
120
100
80
60
40
20
0
CTRL
500nM
2μM
5μM
24h
48h
72h
88h
*
Ocm1A
Pan B

FIG. 5

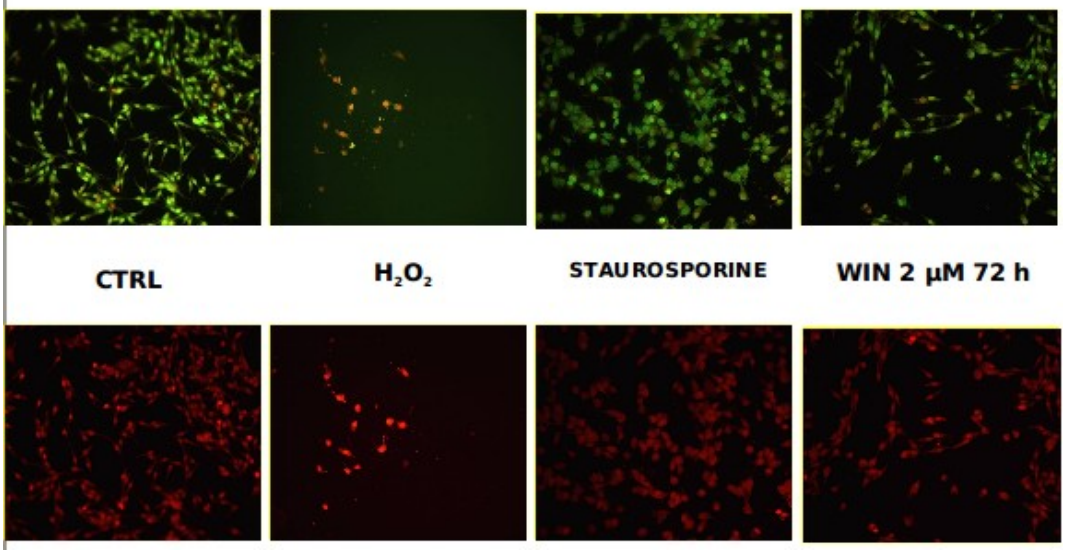
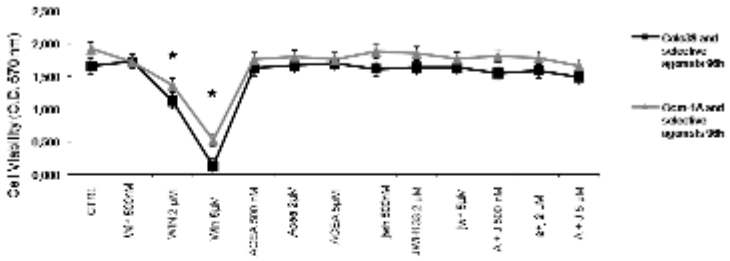


**FIG. 6**

Pan.A



Pan B

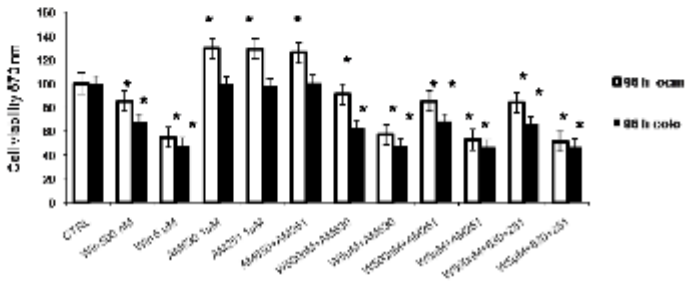


**FIG. 7**

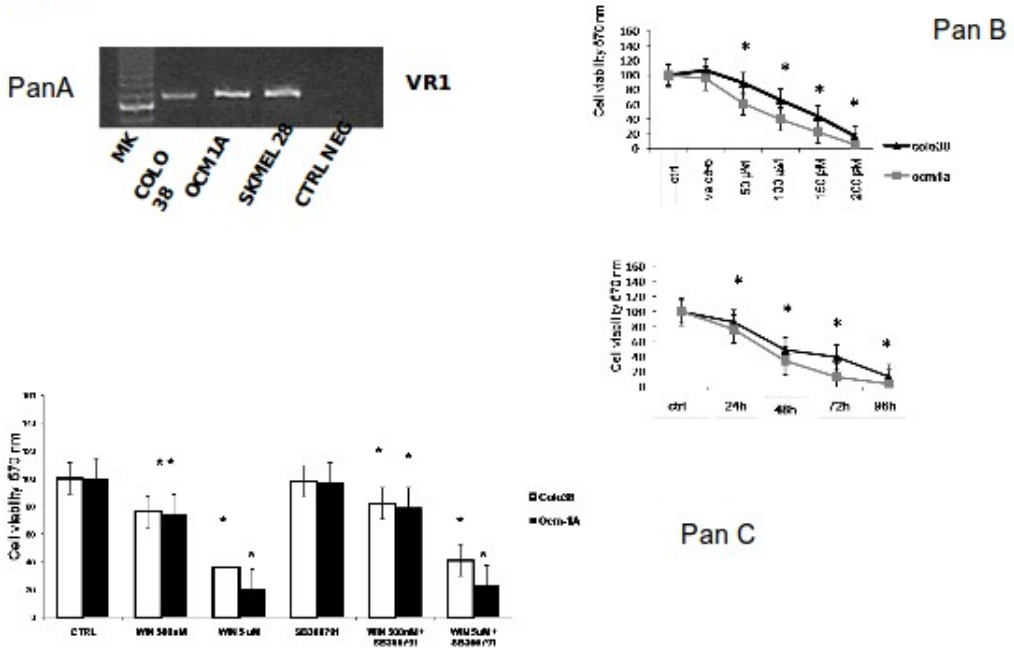

PanA
VR1
MK
COLO 38
OCM1A
SKMEL 28
CTRL NEG
Pan B
Cell viability 570 nm
ctrl
vehicle
50 μM
100 μM
150 μM
200 μM
colo38
ocm1a
ctrl
24h
48h
72h
96h
Cell viability 570 nm
CTRL
Colo38
Ocm-1A
Pan C

**FIG. 8**

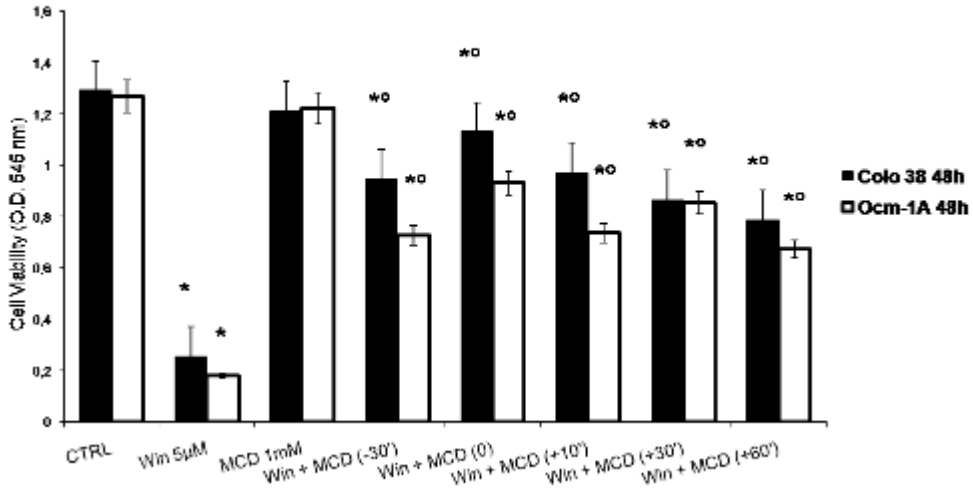
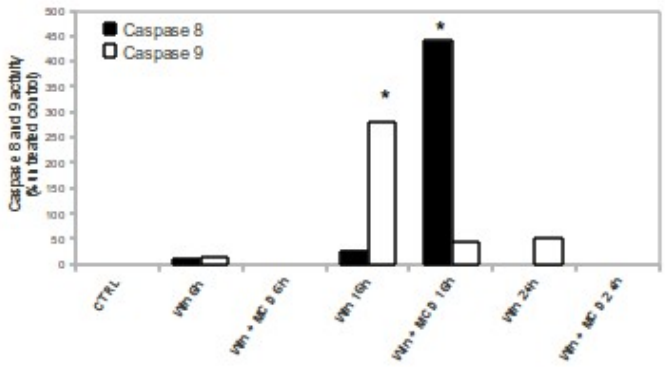
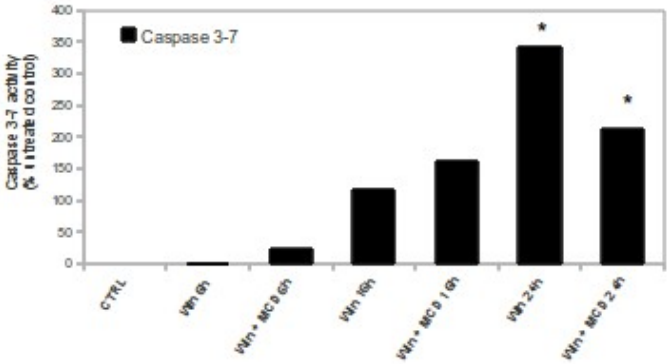

Cell Vability (O.D. 545 nm)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
CTRL
Wm 5µM
MCD 1mM
Wm + MCD (-30')
Wm + MCD (0)
Wm + MCD (+10')
Wm + MCD (+30')
Wm + MCD (+60')
Colo 38 48h
Ocm-1A 48h

FIG. 9

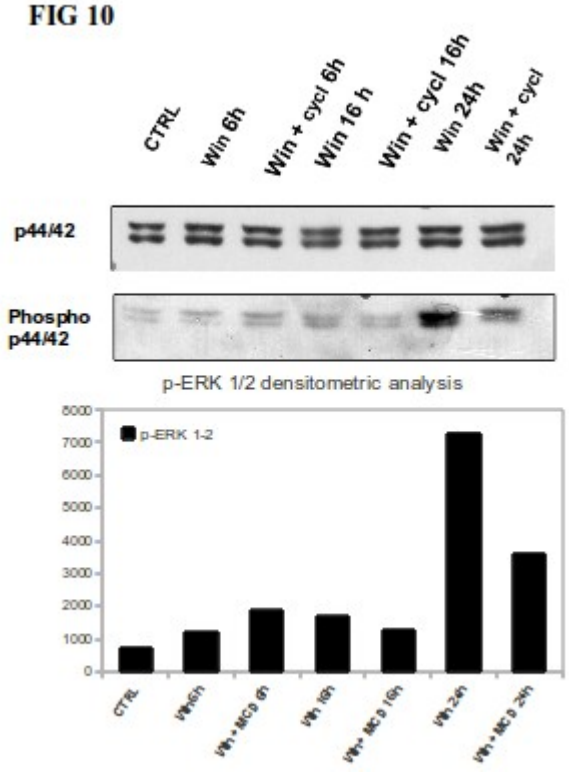



PanA




PanB

**FIG 10**


CTRL
Win 6h
Win + cycl 6h
Win 16 h
Win + cycl 16h
Win 24h
Win + cycl 24h
p44/42
Phospho p44/42
p-ERK 1/2 densitometric analysis
p-ERK 1-2
8000
7000
6000
5000
4000
3000
2000
1000
0

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