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Cannabinoid receptor-mediated apoptosis induced by *R*(+)-methanandamide and Win55,212 is associated with ceramide accumulation and p38 activation in Mantle Cell Lymphoma

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Running title:

CB_1 and CB_2 mediate apoptosis via ceramide and p38 in MCL

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Abbreviations: MCL, mantle cell lymphoma; CB_1 and CB_2, cannabinoid receptors type 1 and 2; *R*(+)-MA, *R*(+)-methanandamide; Win55, Win55,212-2; FB1, fumonisin B1; L-CS, L-cycloserine; MAPK, mitogen-activated protein kinase; JNK, JUN amino-terminal kinase; FCS, foetal calf serum; B-PLL, B-prolymphocytic leukemia; HPLC, High Performance Liquid Chromatography; MS, Mass Spectrometry; ESI/MS/MS, electrospray ionization mass spectrometry; SPLs, sphingolipids, IS, internal standard; Pi, lipid phosphate; dh, dihydro, $DiOC_6$, 3,3'-dihexyloxacarbocyanine iodide; z-VAD-FMK, Z-Val-Ala-Asp(Ome)-CH_2F

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ABSTRACT

We have recently shown that cannabinoids induce growth inhibition and apoptosis in mantle cell lymphoma (MCL), a malignant B-cell lymphoma that expresses high levels of cannabinoid receptors type 1 and 2 (CB_1 and CB_2). In the current study, the role of each receptor and the signal transduction triggered by receptor ligation were investigated. Induction of apoptosis following treatment with the synthetic agonists *R*(+)-methanandamide (*R*(+)-MA) and Win55,212-2 (Win55) was dependent on both cannabinoid receptors, as pre-treatment with SR141716A and SR144528, specific antagonists to CB_1 and CB_2, respectively, abrogated caspase-3 activity. Preincubation with the inhibitors SB203580 and SB202190 showed that phosphorylation of MAPK p38 was implicated in the signal transduction leading to apoptosis. Treatment with *R*(+)-MA and Win55 was associated with accumulation of ceramide, and pharmacological inhibition of ceramide synthesis *de novo* prevented both p38 activation and mitochondria depolarization assessed by binding of 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$). In contrast, the pan-caspase inhibitor Z-Val-Ala-Asp(Ome)-CH_2F (z-VAD-FMK) did not protect the mitochondrial integrity. Collectively, these results suggest that concurrent ligation of CB_1 and CB_2 with either *R*(+)-MA or Win55 induces apoptosis via a sequence of events in MCL cells: accumulation of ceramide, phosphorylation of p38, depolarization of the mitochondrial membrane and caspase activation. While induction of apoptosis was observed in both MCL cell lines and primary MCL, normal B-cells remained unaffected. The present data suggest that targeting CB_1/ CB_2 may have therapeutic potential for the treatment of mantle cell lymphoma.

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INTRODUCTION

MCL is a malignant B-cell lymphoma with an aggressive course and generally a poor clinical outcome. MCL tumors respond to chemotherapy, but the remissions are short and the median survival is only 3 years. Using gene expression profiling to identify potential new therapeutic targets in MCL, we recently reported overexpression of the $G_{i/o}$-protein coupled receptors CB_1 and CB_2 (Islam et al., 2003). Under normal conditions, the CB_1 receptor is predominantly expressed in the central nervous system, while CB_2 is mainly expressed in the immune system. In addition to their roles in neuromodulation and immune function, the cannabinoid receptors participate in the regulation of cell survival (Guzman et al., 2001a). Endogenous as well as natural and synthetic cannabinoids act as agonists to the receptors (Howlett et al., 2002), and have been shown to induce growth inhibition *in vitro* and tumor regression *in vivo* in a variety of cell types (Guzman, 2003). Both cell-cycle arrest and apoptosis have been reported to contribute to cannabinoid receptor mediated growth inhibition (Guzman, 2003). The cellular signal transduction induced by cannabinoids is complex and depends both on the cellular context and on the kind and duration of the stimulus. Cannabinoids can modulate mitogen-activated protein kinases (MAPKs) that are generally believed to play opposing roles in the control of cell growth (Wada and Penninger, 2004). Both cannabinoid-induced attenuation (Ellert-Miklaszewska et al., 2005) and activation (Galve-Roperh et al., 2000) of ERK signaling, which is normally associated with cell survival, have been reported to mediate apoptosis via inhibition of the AKT/ PKB survival pathway (Ellert-Miklaszewska et al., 2005; Gomez del Pulgar et al., 2002). Activation of the stress-activated kinases JUN amino-terminal kinase (JNK) and p38 MAPK in response to cannabinoid receptor ligation has also been reported (Derkinderen et al., 2001; Liu et al., 2000; Rueda et al., 2000). In some

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cases, cannabinoids have shown to induce accumulation of ceramide (Derkinderen et al., 2001; Velasco et al., 2005), a sphingolipid that acts as a second messenger (Pettus et al., 2002). Increased ceramide levels in response to treatment with cannabinoids can be caused by either sphingomyelin breakdown (Sanchez et al., 1998) or by *de novo* ceramide synthesis (Galve-Roperh et al., 2000; Gomez del Pulgar et al., 2002). The *de novo* pathway is activated in response to TNF and several chemotherapeutic agents, and has been suggested to play a critical role in the regulation of apoptosis (Pettus et al., 2002). In glioma (Galve-Roperh et al., 2000; Gomez del Pulgar et al., 2002) and prostate tumor cells (Mimeault et al., 2003) treated with cannabinoids, *de novo* ceramide accumulation has been shown to precede apoptosis.

We have previously observed that treatment with cannabinoid receptor agonists leads to growth inhibition and induction of apoptosis in both primary MCL cells and in the MCL cell line Rec-1 (Flygare et al., 2005). The objective of the present investigation was to clarify the role of each receptor, and to delineate the signaling pathway leading to apoptosis in MCL following treatment with the endocannabinoid analog R(+)-MA and the synthetic cannabinoid Win55. In MCL cell-lines and primary MCL cells, induction of apoptosis following treatment with R(+)-MA and Win55 was dependent on concurrent ligation of CB_1 and CB_2, while normal B-cells remained unaffected. A sequential connection between signaling events was mapped: cannabinoid receptor ligation was followed by ceramide synthesis prior to p38 activation, which preceded a drop in the mitochondrial membrane potential and, finally, caspase activation. Given the low expression of CB_1 in normal immune cells, our results suggest that cannabinoid receptor ligands could be developed as novel therapeutic agents for the treatment of MCL.

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MATERIALS AND METHODS

Ethical permission.

This study was approved by the Ethics committee at Karolinska Institutet (Etikkommitté syd).

Reagents.

SR141716A and SR144528 were kind gifts from Sanofi-Reserche (Montpellier, France). R(+)-methanandamide was purchased from Tocris-Cookson (UK). Win 55,212-2 mesylate, Fumonisin B1, L-cycloserine and Z-Val-Ala-Asp(Ome)-CH_2F were purchased from Sigma Aldrich (St Louis, MO). SB202190 and SB203580 were purchased from Calbiochem Inc. (San Diego,CA), and 3,3'-dihexyloxacarbocyanine iodide from Molecular Probes (Eugene,OR). The reagents were dissolved to 10 mM in DMSO or EtOH (R(+)-MA) and further diluted in culture medium on the day of the experiment.

Tumor tissue and controls.

Tumor tissue from MCL was obtained from viability frozen patient samples with more than 90% purity of tumor B-cells. Three samples (L718, L1547 and L1686) were collected from tonsil, lymph node and peripheral blood, respectively, from three MCL patients. In all instances the MCL diagnosis was based on typical morphology, immune phenotype, expression of cyclin D1 protein and/ or t(11;14)(q13;q32) assessed by FISH. Controls were B lymphocytes isolated from peripheral blood or from nonmalignant tonsil tissue. MCL and tonsil tissue were homogenized using Medimachine, DAKO (Aarhus,Denmark), resuspended and washed in PBS.

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B-lymphocyte separation.

Human peripheral blood lymphocytes were separated from blood cell concentrates of healthy blood donors by density centrifugation on Ficoll paque gradient. Erythrocytes were lyzed in ammonium chloride lysis buffer for 8 min. The cells were resuspended in RPMI 1640 medium and stored at 4°C overnight. Human B lymphocytes from tonsil tissue and blood were separated from T-cells by AutoMACS, using 160 µl CD3+ microbeads to $8,7*10^6$ cells. The B-cells separated from buffy coat were stained with PE-Cy5 anti-human CD19 (BD Biosciences Pharmingen, (San Jose, CA) for 15 min at RT, in the dark. Cells were washed and sorted for CD19+ in FACS Vantage SE/Diva sorter with excitation wavelength 488 nm.

Cell lines.

The MCL cell line Rec-1, carrying the t(11;14)(q13;q32) translocation (Rimokh et al., 1994) was a kind gift from Dr. Christian Bastard, Ronan, France. Rec-1 and the myelomonocytic cell line U937 cells were maintained in RPMI 1640 medium supplemented with 2 mM L- glutamine and 10% foetal calf serum (FCS) and 50µg/ml gentamicin under standard conditions (humidified atmosphere, 95% air, 5% CO_2, 37°C). The EBV negative MCL cell line JeKo (Jeon et al., 1998) and the JVM-2 cell line generated from a B-prolymphocytic leukemia (B-PLL) with t(11;14)(q13;q32) (Melo et al., 1986) were kept under the same conditions except for supplementation with 20% FCS. Several studies have suggested that B-PLL is not a specific disease entity, but a blastic variant of a different lymphoma. Most cases carrying the t(11;14) translocation correspond to blastoid variants of MCL (Ruchlemer et al., 2004). Prior to performing the experiments, cells were serum starved overnight.

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Apoptosis and Flow Cytometric Analysis.

Cells were serum starved overnight, treated with 10 µM of Win55 or R(+)-MA and harvested 4 h after the treatment. The cells were labeled with Annexin V–PE and 7AAD using the Annexin V-PE Apoptosis Detection kit (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's instructions. Subsequent flowcytometric analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA). Fluorescence data were displayed as dot plots using the Cell Quest software (Becton Dickinson, Franklin Lakes, NJ).

Caspase-3 assay.

DEVD-dependent caspase activity was measured using a Caspase-3/CPP32 Fluorimetric Assay (MCL International, Massachusetts, USA) according to the manufacturer's instructions. The assay is based on fluorimetric detection of cleavage of the substrate DEVD-AFC. Uncleaved substrate emits blue light (λmax: 400 nm), whereas free AFC emits yellow-green light fluorescence (λmax: 505 nm). In brief, cells from cell lines were serum starved overnight, whereas primary MCL and tonsil tissue cells were used directly after preparation. Cells were pre-treated with inhibitors for 30 min or 1h (see Figure legends) prior to incubation with 10 µM of R(+)-MA or Win55 for 30 min. Cells were then harvested, lyzed and incubated with buffer containing DEVD-AFC for 2 hrs at 37°C. Emission was measured using a fluorimeter at 400 nm excitation and 505 nm emission.

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Enzyme Immunometric phosphorylation assays.

The amount of phosphorylated p38 and total p38 in the cells was measured using pp38 and p38 enzyme immunoassays, (Assay Designs Inc., AnnArbor, Michigan) according to manufacturer's instructions. In brief, Rec-1 cells were serum starved overnight, pre-treated with inhibitors for 30 min or 1h (see Figure legends) prior to incubation with 10 µM of R(+)-MA or Win55 for 30 min. Thereafter, the cells were washed twice with PBS and lysed in RIPA buffer (Assay Designs Inc.) containing protease inhibitor cocktail p8340 (Sigma-Aldrich). The cell lysate was allowed to bind to the enzyme immunoassay plate for 1 hr. The microtiterplate was incubated with phospho specific antibodies against phosphorylated p38 or against total p38 for 1 hr on a plate shaker, followed by washing and incubation with secondary conjugate solution for 30 min. Thereafter, TMB substrate was added for 30 min. Adding stop solution terminated the reaction, and the emission was determined at 450 nm. The ratio of phosphorylated/ total p38 MAPK was then calculated, and results were expressed as a percentage of the ratio calculated for unstimulated control cells.

Ceramide analysis.

High Performance Liquid Chromatography (HPLC)- Tandem Mass Spectrometry (MS) was performed at the Lipidomics Core, Medical University of South Carolina, Charleston, SC 29425, USA, as described in (Bielawski et al., 2006, Methods, in press). Briefly, Rec-1 cells were serum starved overnight, whereas B lymphocytes from tonsil tissue were used directly after preparation. The cells were incubated with 10 µM of R(+)-MA or Win55 for 4 h. Thereafter, simultaneous electrospray ionization mass spectrometry (ESI/MS/MS) analysis of sphingoid bases, sphingoid base 1-phosphates and ceramides was performed on a Thermo Finnigan TSQ 7000

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triple quadrupole mass spectrometer operating in a multiple reaction monitoring positive ionization mode. Cells were fortified with internal standards (ISs), extracted into a one-phase neutral organic solvent system, and analyzed by a HP1100/TSQ 7000 LC/MS system. Qualitative analysis of sphingolipids (SPLs) was performed by a Parent Ion scan of a common fragment ion characteristic for a particular class of SPLs. Quantitative analysis was based on calibration curves generated by spiking an artificial matrix with known amounts of target synthetic standards and an equal amount of IS. The calibration curves were constructed by plotting the peak area ratios of analyte to the respective IS against concentration using a linear regression model. SPL levels were normalized to total lipid phosphate (Pi).

Mitochondrial transmembrane potential.

3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) is a strong cationic dye that binds to undamaged mitochondria with intact membrane potential. To evaluate changes in the mitochondrial transmembrane potential, Rec-1 cells were serum starved overnight and pre-treated with inhibitors for 30 min or 1h (see Figure legend) prior to incubation with 10 µM of R(+)-MA or Win55 for 30 min. After washing and resuspension in RPMI 1640 medium without FCS and phenol red, cells were labelled with $DiOC_6$ (40 nM) for 15 min at 37°C. Thereafter, cells were washed twice with PBS and analyzed directly on a flow cytometer using excitation and emission wavelengths of 495 and 525 nm respectively.

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Statistical Analysis.

Caspase-3 activity and p38 phosphorylation were evaluated using Kruskal-Wallis test between control cells and treated cells. P-values are presented in figure legends. The software Statistica (Statsoft AB, Tulsa, OK) was used.

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RESULTS

Induction of apoptosis by R(+)-MA and Win55.

Initially, we examined whether exposure of the MCL cell lines Rec-1, JeKo and JVM-2 to R(+)-MA, which displays higher affinity for CB_1 than for CB_2 (Howlett et al., 2002) or Win55, which binds to CB_1 and CB_2 with similar affinities (Howlett et al., 2002), would lead to induction of apoptosis. Cell death was detected by flow cytometry analysis of cells labelled with Annexin-PE and 7AAD. 10 µM of R(+)-MA or Win55 caused apoptosis and necrosis to a varying extent in the different MCL cell lines, while the control cell line U937, which does not express CB_1 or CB_2 (Maccarrone et al., 2000), remained unaffected (Fig 1).

To assess the individual contributions of the CB1 and CB2 receptors to the apoptotic response to treatment with R(+)-MA and Win55, studies with inhibitors were performed. Cells were pre-treated with SR141716A or SR144528, specific inhibitors of CB1 and CB2 respectively, followed by exposure to 10 µM of R(+)-MA or Win55. Caspase-3 activity was measured using a quantitative assay. 10 nM of SR141716A or SR144528 abrogated the caspase-3 activity observed in the MCL cell lines (Fig.2a) and in primary MCL samples (Fig.2c) treated without inhibitor. Thus, ligation of both CB1 and CB2 seems to be required for the induction of apoptosis in MCL cells. In contrast, the control cell line U937 (Fig. 2b) and normal B lymphocytes from tonsil tissue (Fig. 2d) or buffycoat (Fig. 2e) were only marginally affected by treatment with the agonists.

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Cannabinoid receptors mediate p38 phosphorylation followed by caspase-3 activation.

MAPK signaling has earlier been shown to mediate apoptosis induced by cannabinoids in different cell types (Ellert-Miklaszewska et al., 2005; Howlett and Mukhopadhyay, 2000; Wada and Penninger, 2004). Therefore, the potential role of MAPK signaling in the induction of apoptosis in the MCL cell line Rec-1 was investigated. Pre-treatment of Rec-1 with 10 µM of the p38 inhibitors SB202190 or SB203580 prior to incubation with 10 µM of R(+)-MA or Win55 caused a substantial decrease in caspase-3 activity compared to the samples treated without inhibitors (Fig. 3a). In contrast, the JNK inhibitor SP500125 and the p44/42 inhibitor UO126 were less effective (data not shown).

Activation of p38 in response to treatment with R(+)-MA or Win55 was confirmed by ELISAs specific for phospho p38 and total p38. Pre-treatment with 10 nM of SR141716A or SR144528 for 30 min prior to incubation with 10 µM R(+)-MA or 10 µM Win55 caused an almost complete inhibition of the p38 phosphorylation observed in the samples treated without inhibitors (Fig. 3b). Thus, p38 phosphorylation and caspase-3 activation are mediated in a similar way by ligation of both the CB_1 and the CB_2 receptor.

Ceramide accumulation precedes p38- activation.

Recent studies have suggested that apoptosis induced by cannabinoids can be preceded by ceramide accumulation (Derkinderen et al., 2001). In view of these observations, ceramide formation in response to treatment with R(+)-MA and Win55 was studied in Rec-1 and normal B lymphocytes from tonsil. In order to measure levels of different ceramide subspecies ranging from C12 to C26 in fatty acid length,

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High Performance Liquid Chromatography-Tandem Mass Spectrometry was employed. In Rec-1, 10 µM of cannabinoid receptor agonists induced a 2.3-fold (R+(-)MA) and 1.5-fold (Win55) increase in both C14 (Fig. 4a) and C16 (Fig. 4b). During *de novo* ceramide synthesis, dihydroceramide (dh) species serve as precursors to ceramide species (Pettus et al., 2002). dhC14 and dhC16, the dihydroceramide counterparts of C14 and C16, both increased 5-fold and >3-fold following treatment with R+(-)MA and Win55, respectively, in Rec-1 (Fig 4c and d). Increased levels of dhC18, dhC20, dhC24, dhC26:1 and of dhSphingosine were also observed in Rec-1 (see supplementary information). Generally, the induction of ceramide and dihydroceramide was less pronounced in B lymphocytes from tonsil as compared to Rec-1 (Fig 4 a-d and supplementary information). Increased levels of dihydroceramide in response to the cannabinoid receptor agonists in MCL suggest that the *de novo* ceramide synthesis pathway was activated.

To determine whether ceramide accumulation occurs upstream or downstream of p38 activation, we next examined the effects of pre-treatment with inhibitors of *de novo* ceramide synthesis on p38 activation. Fumonisin B1 (FB1) and L-cycloserine (L-CS), inhibitors of dihydroceramide synthase and of ketosphinganine synthase, respectively, were used. As shown in Fig. 5, 25 µM of the inhibitors prevented p38 phosphorylation induced by 10 µM of R(+)-MA or Win55 in Rec-1. Thus, *de novo* ceramide accumulation induced by R(+)-MA or Win55 is followed by p38 phosphorylation in MCL.

Ceramide accumulation causes changes in the mitochondrial membrane potential upstream of caspase activation.

Ceramide accumulation is associated with mitochondrial loss of function, release of

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cytochrome C and caspase activation (Pettus et al., 2002). Mitochondrial damage can be assessed biochemically by measuring $DiOC_6$, a cationic dye that is released when the mitochondrial membrane potential is damaged. A clear decrease in the binding of $DiOC_6$ was observed in Rec-1 cells treated with 10 μM of Win55 or R(+)-MA (Fig. 6, column 1). Pre-treatment with 10 nM SR141716A or 10 nM SR144528 (Fig 6, column 2 and 3) protected the mitochondrial integrity. Moreover, the drop in $DiOC_6$ binding was inhibited of FB1 (Fig 6, column 4) or SB202190 (Fig 6, column 5), suggesting that ceramide accumulation and p38 activation precede loss of mitochondrial membrane potential. In contrast, the pan-caspase inhibitor z-VAD-FMK did not protect against mitochondrial loss of function (Fig. 6, column 6), indicating that caspases are activated downstream of mitochondrial depolarization in MCL cells.

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DISCUSSION

Various kinds of cannabinoids have been shown to induce apoptosis in human leukemia and lymphoma cell lines via CB_2, the cannabinoid receptor normally expressed in the immune system (McKallip et al., 2002a). We have recently demonstrated that cannabinoid receptor ligands induce growth reduction and apoptosis in MCL (Flygare et al., 2005), a B-cell lymphoma that expresses high levels of both CB_1 and CB_2 (Islam et al., 2003). In the current study, we examined the role of each receptor, and investigated involvement of MAPK signaling and ceramide accumulation in the induction of apoptosis by two synthetic cannabinoids. Together, our data suggest that targeting CB_1 and CB_2 receptors may constitute a novel approach to treating MCL.

Initially, we showed that both R(+)-MA and Win55 induced apoptosis in MCL. The effect was more pronounced following treatment with the CB_1 selective agonist R(+)-MA as compared to the mixed CB_1/CB_2 agonist Win55. Programmed cell death in response to Win55 treatment has earlier been observed in skin cancer (Casanova et al., 2003) and prostate cancer cells (Sarfaraz et al., 2005) that express both CB_1 and CB_2. R(+)-MA has recently been shown to induce apoptosis in a cannabinoid receptor independent manner in glioma cells (Hinz et al., 2004). In MCL, caspase-3 activation induced by either R(+)-MA or Win55 was completely reversed by preincubation with 10 nM of SR141716A or SR144528. At this dose, the inhibitors act merely as neutral antagonists to CB_1 and CB_2, respectively, whereas roles as inverse agonists have been suggested at higher doses (Pertwee, 2005). Therefore, our

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current data suggest that R(+)-MA and Win55, irrespective of differences in CB_1/CB_2 selectivity, induce caspase-3 mediated apoptosis in MCL cells via ligation of both cannabinoid receptors. A similar requirement of ligation of both CB_1 and CB_2 has been observed following treatment of dendritic cells with Δ^9-tetrahydrocannabinol (THC) (Do et al., 2004) .

The major part of the studies on growth inhibition in response to cannabinoids have been performed in neuronal cells, where modulation of ERK and AKT/ PKB have been implicated in the signaling leading to apoptosis (Guzman, 2003). In contrast, we found that caspase-3 activity in MCL cells was to a large extent dependent on phosphorylation of p38, a MAPK whose activation can promote either apoptosis or survival depending on cell type (Wada and Penninger, 2004). Activation of this kinase prior to apoptosis induced by IgM cross-linking has earlier been reported in B-cells from patients with B-chronic lymphocytic leukemia (Nedellec et al., 2005) .

Recent studies have shown that the CB_1 receptor is coupled to the generation of ceramide, reviewed in (Guzman et al., 2001b). R(+)-MA and Win55 have been shown to induce ceramide accumulation in neuronal cells (Galve-Roperh et al., 2000; Ramer et al., 2003). Our results have shown that either agent induced accumulation of C14 and C16 ceramide and their respective dihydroceramide counterparts in Rec-1. In accordance with the effects of the agonists on induction of apoptosis (Fig. 1 upper row), R(+)-MA was more potent than Win55. The fold changes were similar to those observed during B cell receptor triggered cell death in Ramos cells (Kroesen et el., 2001). Induction of dihydroceramide synthesis suggests that the *de novo* ceramide synthesis pathway was activated. Moreover, pharmacological inhibition of *de novo* ceramide synthesis inhibited phosphorylation of p38 and changes in mitochondrial

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$\Delta\psi$. In contrast to ceramides C14 and C16, no induction of longer chain ceramides was observed. However, increased levels of longer chain dihydroceramides (dhC18-dhC26:1) indicate that changes in the corresponding ceramides may be observed at later time points.

Ceramide formation has been reported to induce both caspase-dependent and independent apoptosis (Zhao et al., 2004), and can either precede caspase activation or occur in between initiator caspases and effector caspases (Kroesen et al., 2001; Tepper et al., 1999) . The pan caspase inhibitor z-VAD-FMK did not affect the drop in mitochondrial $\Delta\psi$ caused by R(+)-MA and Win55 in Rec-1. Thus, ceramide accumulation precedes loss in mitochondrial $\Delta\psi$, which is placed upstream of caspase activation in MCL cells. In accordance with earlier studies in neuronal cells (Stoica et al., 2005), p38 inhibition blocked cannabinoid signaling upstream of the mitochondria in MCL. p38 may function both upstream and downstream of caspases, as reviewed in (Zarubin and Han, 2005). Taken together, the present observations suggest a signaling model in which concurrent ligation of CB_1 and CB_2 mediates cannabinoid-induced apoptosis via accumulation of ceramide, phosphorylation of p38, depolarization of the mitochondrial membrane and caspase activation (Fig. 7).

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Of great interest in the context of cancer therapy, cannabinoids have been shown to selectively suppress the growth of glioma, thyroid epithelioma and skin carcinoma cells as compared to their normal counterparts (Guzman, 2003). However, cannabinoid receptor mediated cell death has been observed in both normal and malignant immune cells (Do et al., 2004; Guzman, 2003; Guzman et al., 2002; McKallip et al., 2002b; Powles et al., 2005; Flygare et al., 2005). In the current study, R(+)-MA and Win55 induced apoptosis only in MCL cell lines and primary MCL, while normal B-cells remained unaffected.

In conclusion, our study demonstrates that the cannabinoid receptor agonists R(+)-MA and Win55 induce a sequence of signaling events leading to cell death of MCL cells. The requirement of ligation of both CB_1 and CB_2 raises the possibility that cannabinoids may be used to selectively target MCL cells to undergo apoptosis.

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FOOTNOTES:

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LEGENDS FOR FIGURES.

Fig. 1: Cannabinoids induce cell death in MCL cell lines. The MCL cell lines Rec-1, JeKo, JVM-2 and the control cell line U937 were serum starved overnight and treated with vehicle, 10 µM of R(+)-MA or Win55. Flow cytometry analysis of AnnexinV-PE and 7AAD was performed 4 h after treatment. Lower and upper right-hand quadrants show early apoptotic and late apoptotic/ necrotic cells, respectively. Representative results from three individual experiments are shown.

Fig. 2: Cannabinoids induce caspase-3 activity in MCL but not in normal B cells. The caspase-3 fluorimetric assay was performed, and fluorescence units are shown on the y-axis. One of two individually performed experiments is shown. *: $p<0.05$ when agonist treated cells with and without inhibitors were compared, Kruskal-Wallis analysis. *Role of CB_1 and CB_2 in cannabinoid-induced cell death in MCL cell lines. a)* Rec-1, JeKo and JVM-2 or *b)* control U937 cells were serum starved overnight and pre-treated with the CB_1 antagonist SR141716A (10 nM) or the CB_2 antagonist SR144528 (10 nM) for 30 min prior to incubation with vehicle, 10 µM of R+(-)MA (light grey) or Win55 (dark grey) for 30 min. 50 µM of camptothecin (black) was used as positive control. *Role of CB_1 and CB_2 in cannabinoid-induced cell death in primary MCL cells. c)* Primary MCL from three viability frozen patient samples and primary B lymphocytes from *d)* tonsil tissue from three patients or *e)* buffy coat from two healthy blood donors were pre-treated with the CB_1 antagonist SR141716A (10 nM) or the CB_2 antagonist SR144528 (10 nM) for 30 min prior to incubation with

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vehicle, 10 µM of R+(-)MA (light grey) or Win55 (dark grey) for 30 min. 50 µM of camptothecin (black) was used as positive control.

Fig. 3: Cannabinoid-induced p38 phosphorylation. One of two individually performed experiments is shown. *: $p<0.05$ when agonist treated cells with and without inhibitors were compared, Kruskal-Wallis analysis. *a) Role of p38 in caspase-3 activation*. Rec-1 cells were serum starved overnight and pre-treated with the p38 inhibitors SB293580 (10µM) or SB202190 (10µM) for 30 min prior to incubation with vehicle, 10 µM of R+(-)MA (light grey) or Win55 (dark grey) for 30 min. 50 µM of camptothecin (black) was used as positive control. The caspase-3 fluorimetric assay was performed, and fluorescence units are shown on the y-axis. *b) Contribution of CB_1 and CB_2 to p38 activation.* Rec-1 cells were serum starved and pre-treated with the CB_1 antagonist SR141716A (10 nM) or the CB_2 antagonist SR144528 (10 nM) for 30 min prior to treatment with 10 µM of R(+)-MA (light grey) or Win55 (dark grey) for 30 min. 3 µM of PMA (black) was used as a positive control. To assess p38 MAPK activity, both phosphorylated and total p38 MAPK immunoassays were performed. The ratio of phosphorylated/ total p38 MAPK was then calculated and results are expressed as a percentage of the ratio calculated for unstimulated control cells.

Fig. 4: Cannabinoid–induced accumulation of ceramide and dihydroceramide. Rec-1 (serum starved overnight) and B lymphocytes from tonsil were treated with vehicle, 10 µM of R+(-)MA (light grey) or Win55 (dark grey) for 4 h. Thereafter the cells were prepared for measurement of ceramide using High Performance Liquid Chromatography-Tandem Mass Spectrometry as described. The masses of the

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ceramide subspecies a) C14 and b) C16 and their corresponding dihydroceramides c) dhC14 and d) dhC16 were normalized to lipid phosphate (Pi).

Fig. 5: Cannabinoid-induced ceramide accumulation is required for p38 phosphorylation. Rec-1 cells were serum starved overnight and pre-treated with FB1 and L-CS for 1 h prior to treatment with vehicle, 10 µM of R+(-)MA (light grey) or Win55 (dark grey) for 30 min. 3 µM of PMA (black) was used as a positive control. To assess p38 MAPK activity, both phosphorylated and total p38 MAPK immunoassays were performed. The ratio of phosphorylated/ total p38 MAPK was then calculated and results are expressed as a percentage of the ratio calculated for unstimulated control cells. One of two individually performed experiments is shown. *: $p<0.05$ when agonist treated cells with and without inhibitors were compared.

Fig. 6: Cannabinoids induce changes in mitochondrial membrane potential downstream of ceramide accumulation and p38 activation but upstream of caspase activation. Rec-1 cells were serum starved overnight and pre-treated with SR141716A (10 nM), SR144528 (10 nM), FB1 (25 µM), SB202190 (10 µM) or z-VAD-FMK (50 µM) for 30 min prior to incubation with 10 µM of R(+)-MA or Win55 for 30 min. Thereafter, flow cytometric analysis of mitochondrial ΨΔ was performed using staining with DiOC6. Representative results from three individual experiments are shown.

Fig. 7. Proposed model of signaling events induced by the cannabinoid receptor agonists R(+)-MA or Win55 in MCL.

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Fig. 1

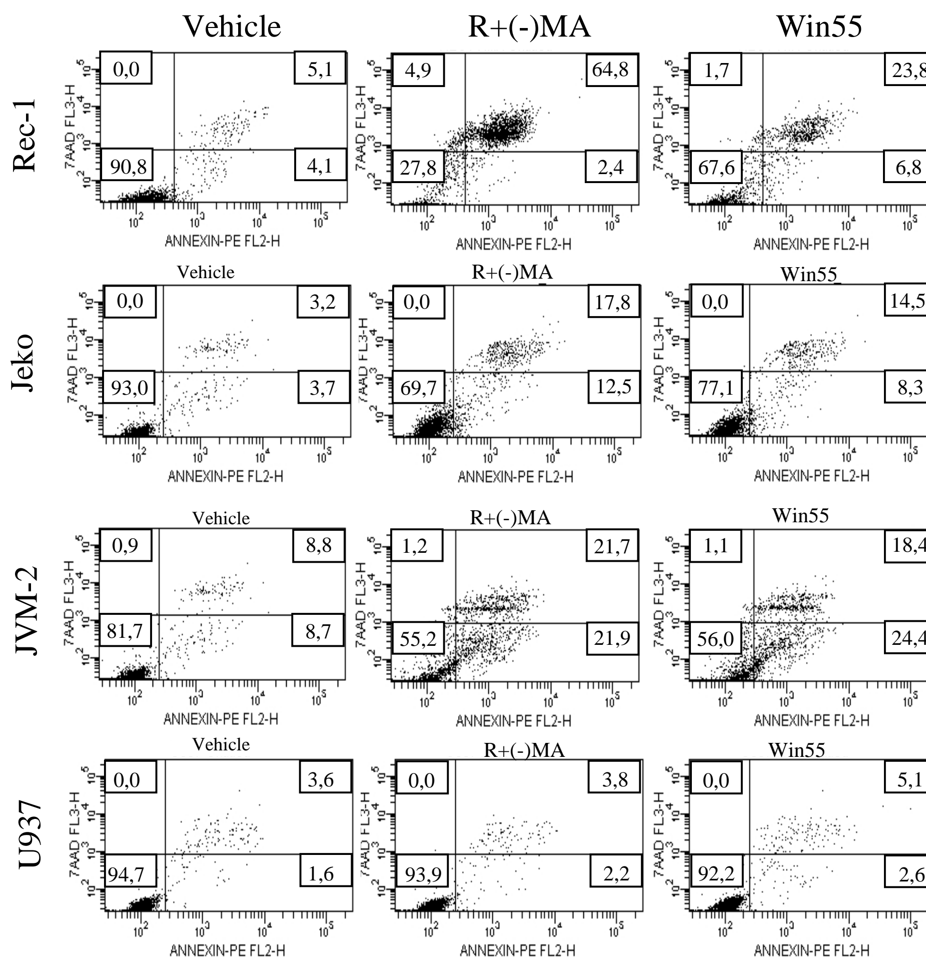

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Fig 2a

A

Rec-1 JeKo JVM-2

Fluorescence

2400
2000
1600
1200
800
400
0

Camptothecin - + - - - - - - - - - + - - - - - - - - - + - - - - - - - -
SR141716 - - + - - + - - + - - - + - - + - - + - - - + - - + - - + -
SR144528 - - - + - - + - - + - - - + - - + - - + - - - + - - + - - +
R+(-)MA - - - - + + + - - - - - - - + + + - - - - - - - + + + - - -
Win55 - - - - - - - + + + - - - - - - - + + + - - - - - - - + + +

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Fig. 2b

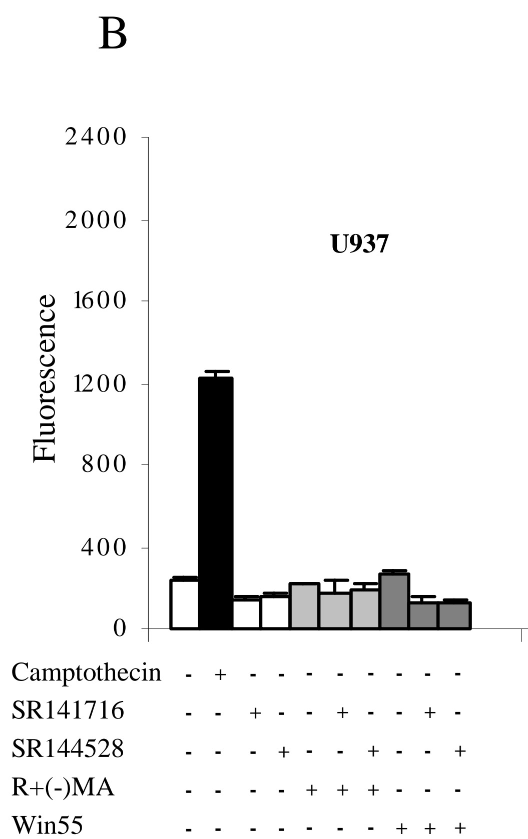

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Fig. 2c

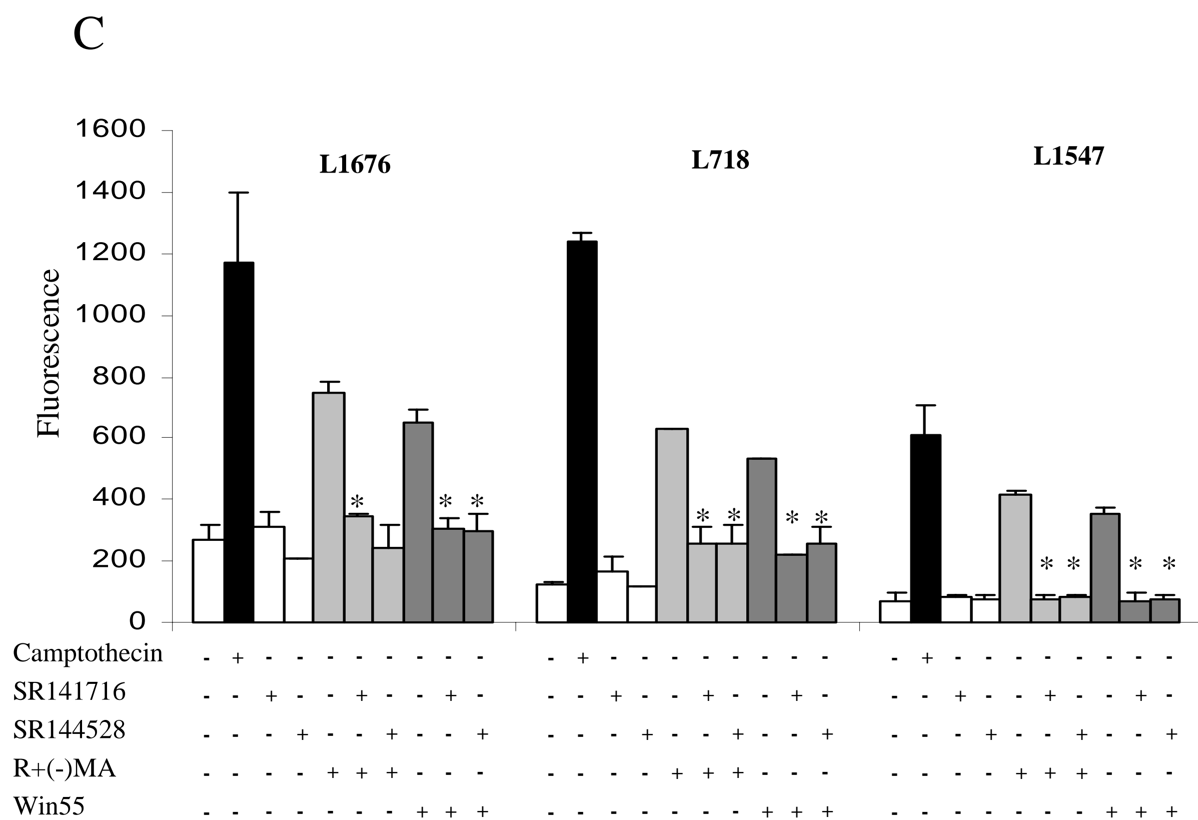

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Fig. 2d

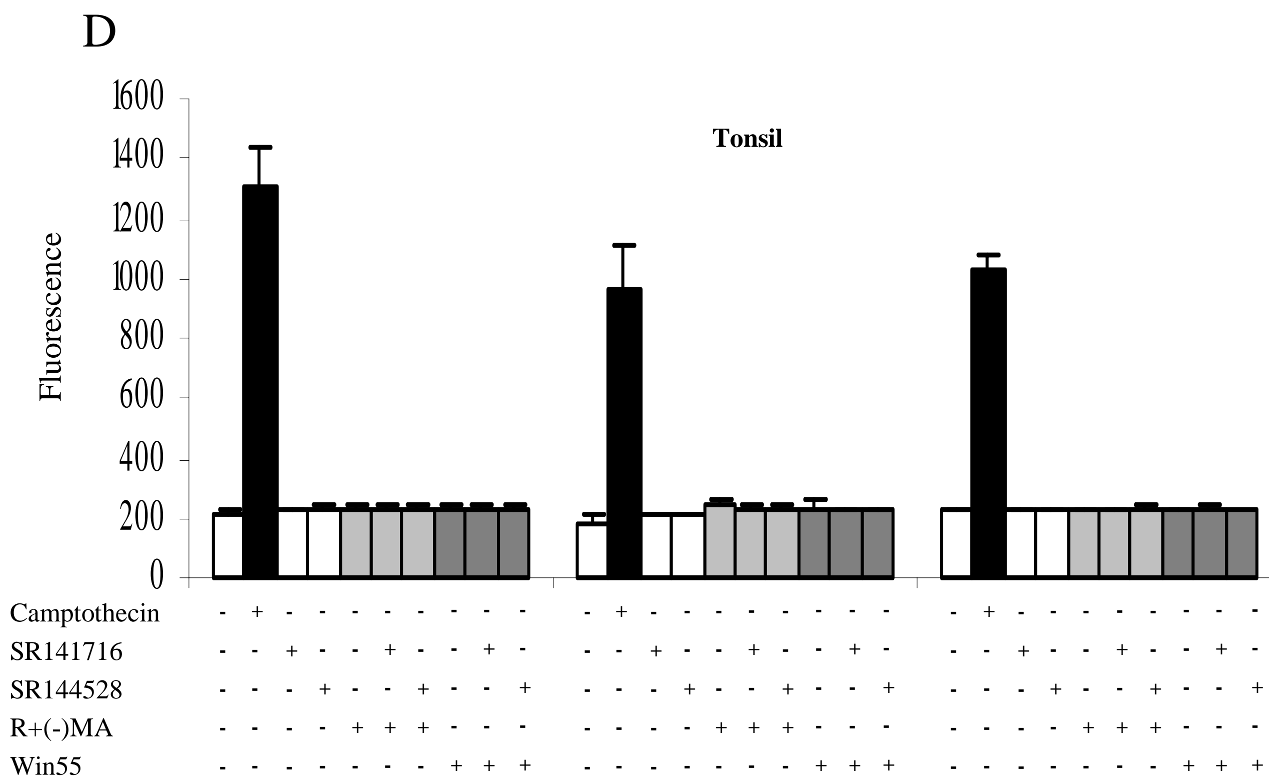

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Fig. 2e

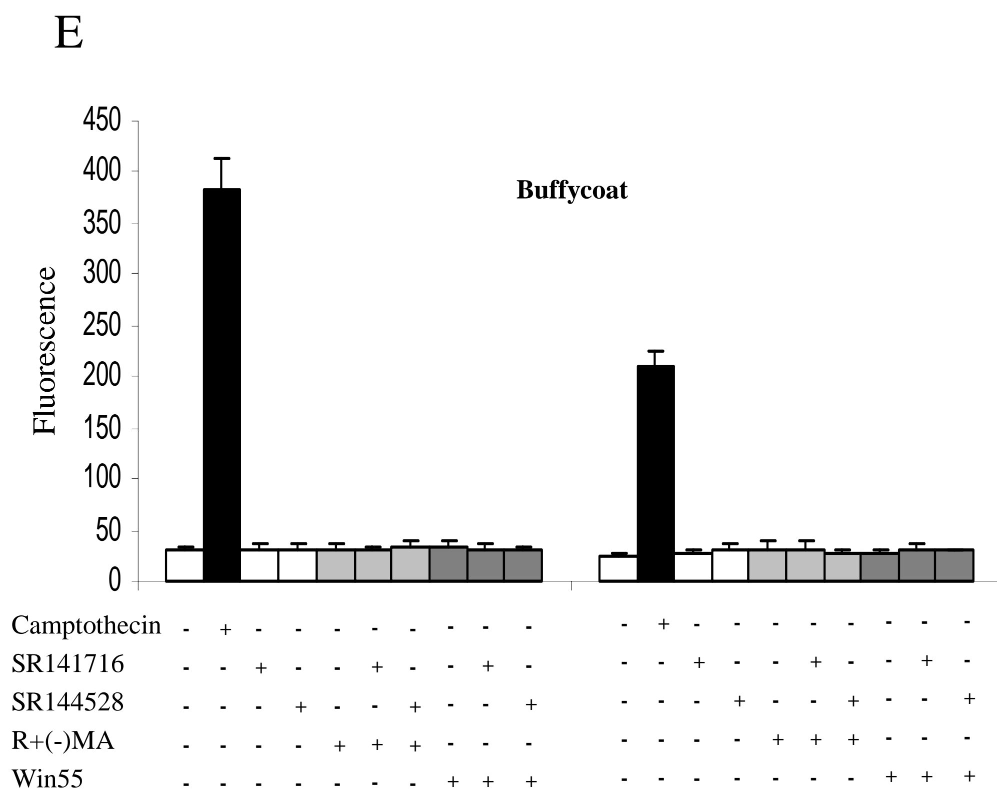

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Fig.3a

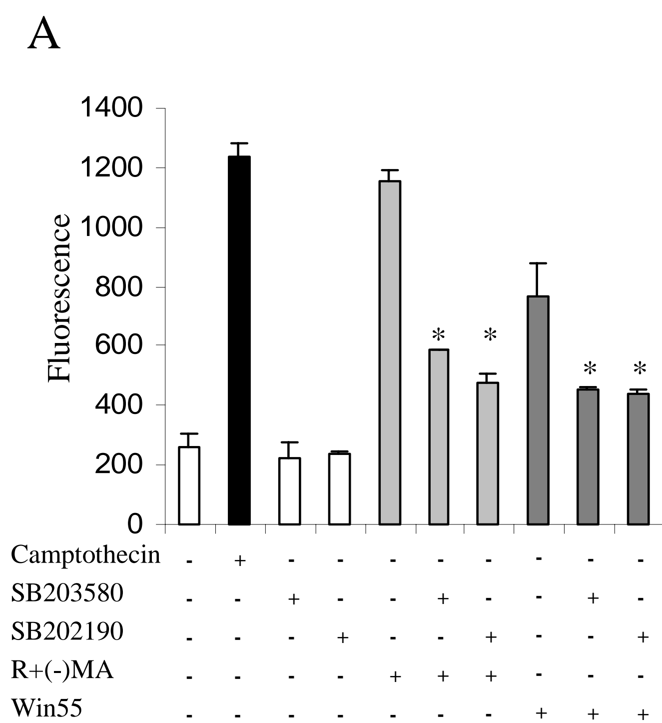

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Fig 3b

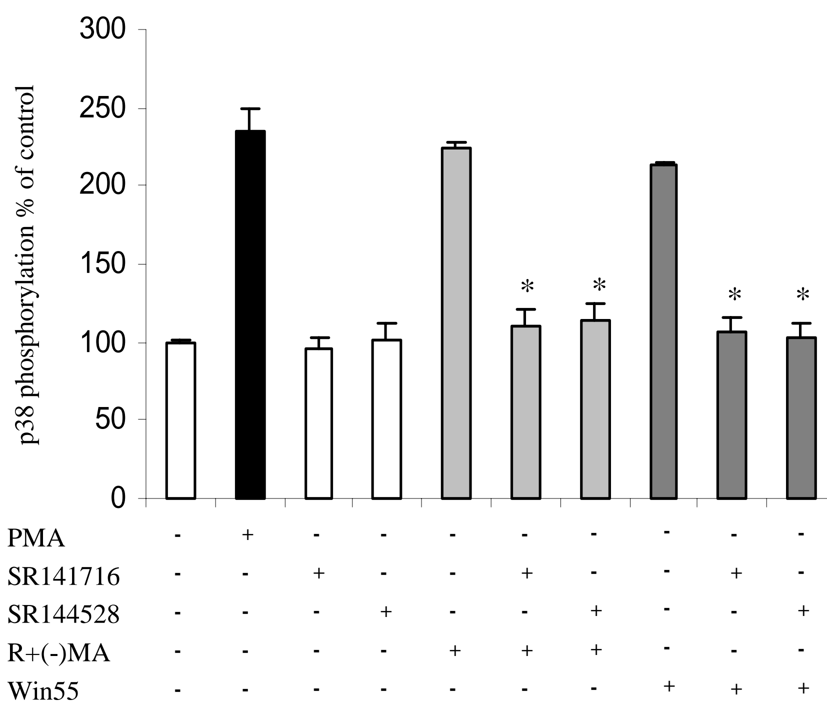

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Fig. 4

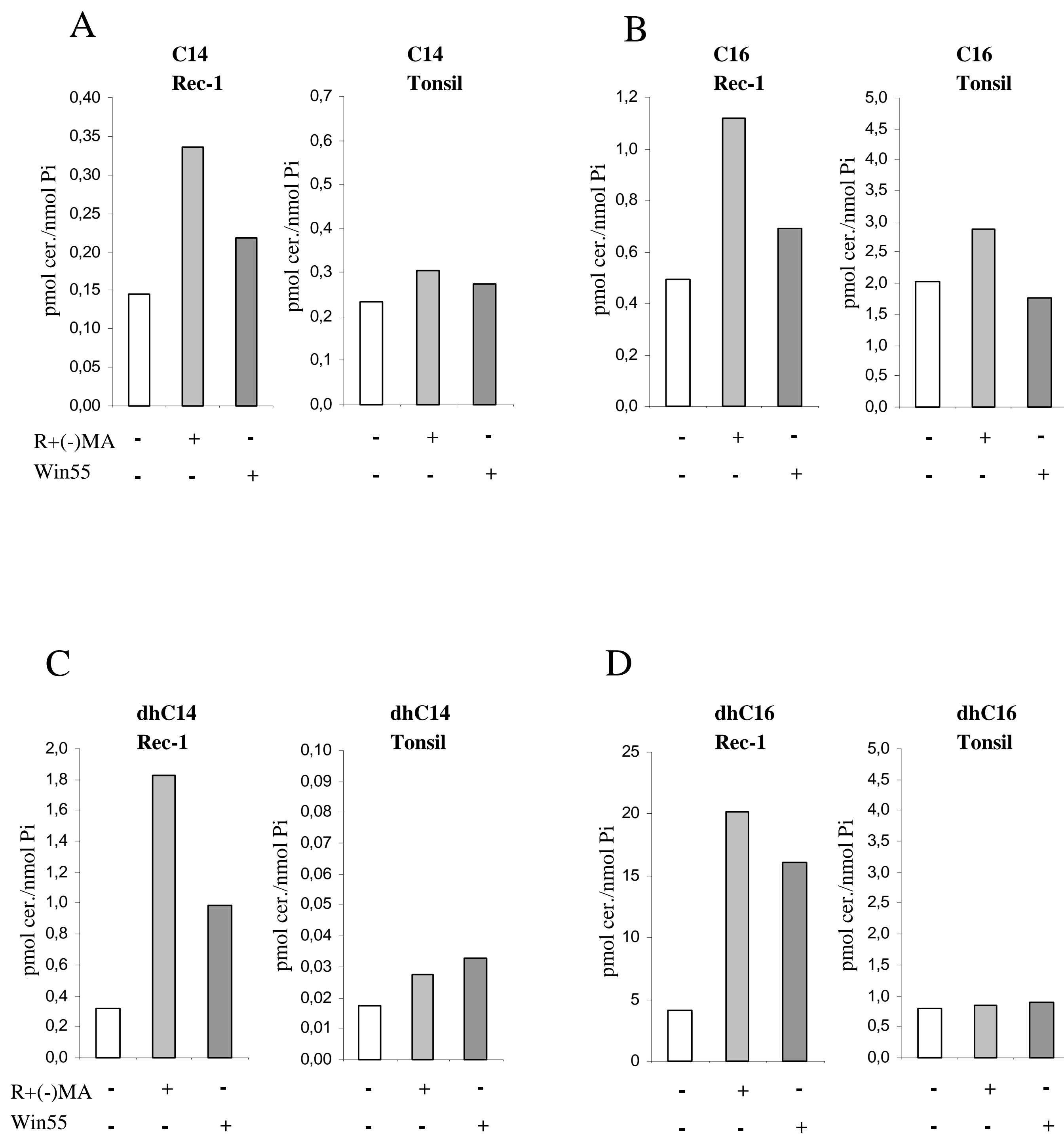

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Fig 5

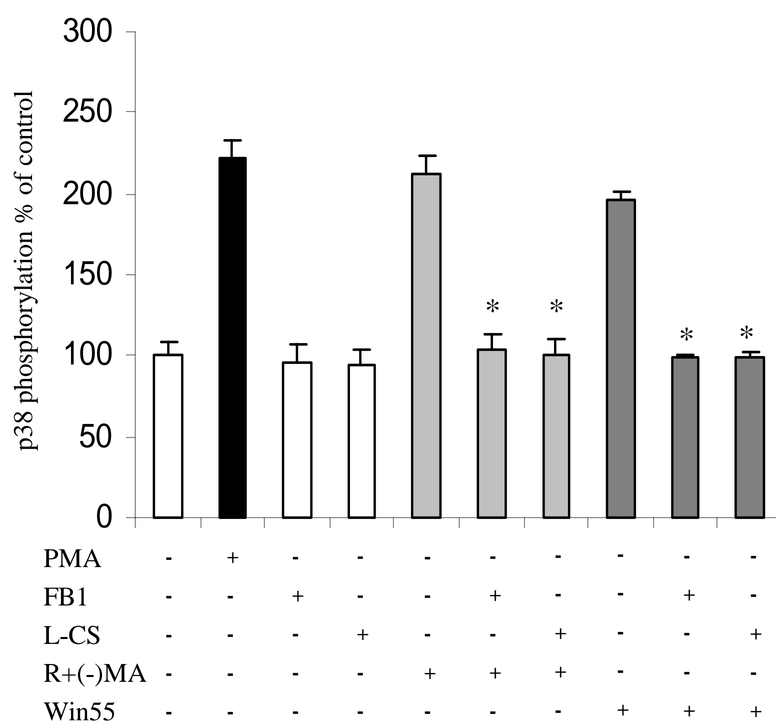

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Fig. 6

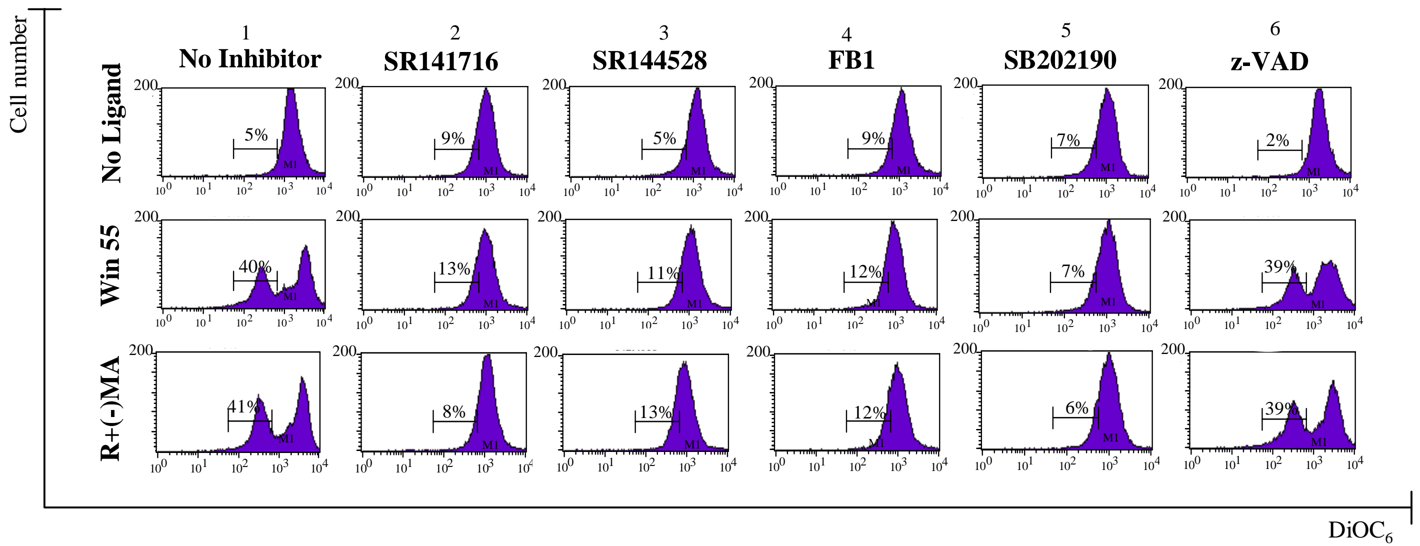

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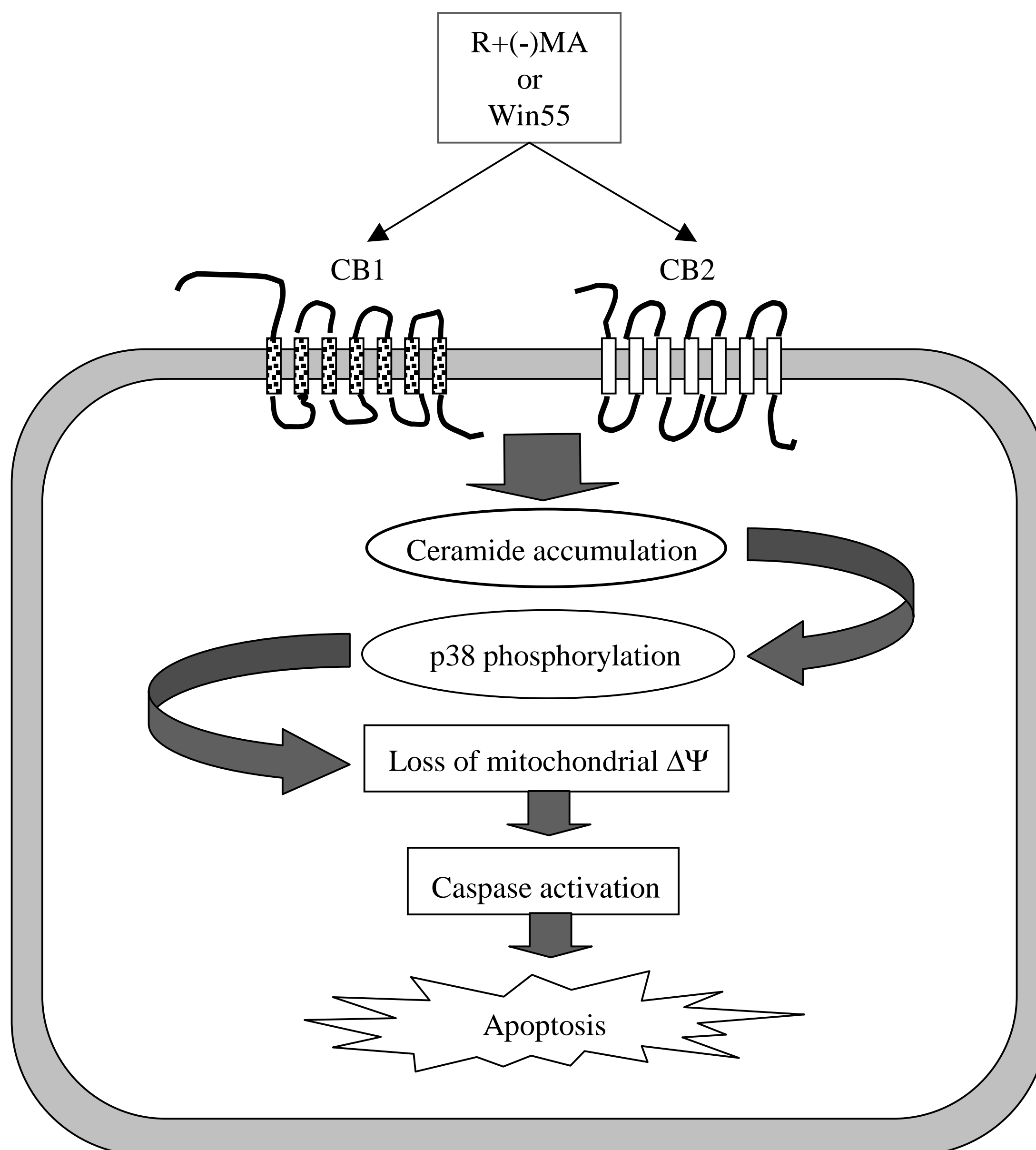
Fig. 7



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