Cannabinoid receptor ligands mediate growth inhibition and cell death in mantle cell lymphoma

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Abstract **We have earlier reported overexpression of the central and peripheral cannabinoid receptors CB1 and CB2 in mantle cell lymphoma (MCL), a B cell non-Hodgkin lymphoma. In this study, treatment with cannabinoid receptor ligands caused a decrease in viability of MCL cells, while control cells lacking CB1 were not affected. Interestingly, equipotent doses of the CB1 antagonist SR141716A and the CB1/CB2 agonist anandamide inflicted additive negative effects on viability. Moreover, treatment with the CB1/CB2 agonist Win-55,212-2 caused a decrease in long-term growth of MCL cells in culture. Induction of apoptosis, as measured by FACS/Annexin V–FITC, contributed to the growth suppressive effect of Win-55,212-2. Our data suggest that cannabinoid receptors may be considered as potential therapeutic targets in MCL.**

Keywords: Mantle cell lymphoma; Cannabinoid receptor; Viability; Growth; Apoptosis

1. Introduction

Mantle cell lymphoma (MCL) is an essentially incurable non-Hodgkin lymphoma of B cell type that is characterized by short remissions and poor prognosis [1]. Recently, highly increased expression of the central cannabinoid receptor type 1 (CB1) and moderately increased expression of cannabinoid receptor type 2 (CB2) was reported in MCL [2,3]. In brain, CB1 mediates the effect of delta9-tetrahydrocannabinol, the active metabolite of cannabis. In contrast to CB2, CB1 is normally expressed at low levels in immune cells, rev. in [4]. Most naturally occurring ligands, the so-called endocannabinoids, act on both receptors [4]. Several studies have shown that both natural and synthetic cannabinoids may induce growth arrest and apoptosis in cell lines of widely different origin (rev. in [5]) [6]. In contrast, other reports have suggested that low doses of cannabinoids may actually enhance proliferation of malignant and normal cells [7,8]. Ligation of CB1 receptors might also affect non-malignant immunocompetent cells, either directly or via accessory cells. Cannabinoids have been shown to modulate both T cell responses [9] and the production of cytokines in macrophages and dendritic cells [10]. Not only agonists to cannabinoid receptors, but also antagonists alone have recently been reported to inhibit growth [11] or induce apoptosis in cancer cells [12,13]. In addition to their role as antagonists, these substances have been reported to block the constitutive, low level signalling through CB-receptors, thereby acting as inverse agonists, rev. in [18]. In this study, we have investigated the effects of treatment with cannabinoid receptor agonists and antagonists on viability and growth of MCL cells. Our results show that cannabinoid receptor ligands mediate inhibition of growth and survival of MCL cells, and that these effects in part are mediated by induction of apoptosis.

2. Materials and methods

2.1. Reagents

DMEM, RPMI-1640, fetal bovine serum and gentamicin were purchased from Life Technologies, Invitrogen Corporation (Grand Island, NY), rabbit polyclonal antibody to CB1 (ab3559) from Abcam (United Kingdom), anti-rabbit Ig horseradish peroxidase linked antibody from Amersham Biosciences (United Kingdom), Cy3 conjugated donkey-anti-rabbit antiserum from Jackson ImmunoResearch (West grove, PA), PVDF membrane from Millipore (Bedford, MA), Supersignal® West Pico chemiluminescent substrate from Pierce Biotechnology (Rockford, IL), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay from Roche Diagnostics (Germany), Annexin V kit from Caltag Laboratories (Burlingame, CA) DMSO, G418, formaldehyde, BSA, Saponin, p8340, G418, 4′,6-diamidino-2-phenylindole (DAPI), (R)-(+)-Win-55,212-2 (2,3 dihydro)-5-methyl-3 [(morpholinyl)-methyl]pyrollo (1,2,3 de)-1,4-benzoxazinyl]-[1-naphthaleny]methanone and arachidonic acid *N*-(hydroxyethyl)amide (anandamide) from Sigma–Aldrich (St. Louis, MO), AM 251 from Tocris (Avonmouth, Bristol, United Kingdom), SR141716A was a generous gift from Sanofi-Reserche (Montpellier, France). The CB1/CB2 ligands were dissolved to 10 mM in DMSO or 70% ethanol and further diluted in culture medium at the day of the experiment.

2.2. Tumor tissue

Tumor tissues from diagnostic biopsies from 15 patients with MCL were investigated by morphology, flow cytometry and by IHC for cyclin D1. In all cases t(11;14) translocation was confirmed by FISH

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Abbreviations: MCL, mantle cell lymphoma; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; Win-55,212-2, (R)-(+)-Win-55,212-2 (2,3 dihydro)-5-methyl-3 [(morpholinyl)-methyl]pyrollo (1,2,3 de)-1,4-benzoxazinyl]-[1-naphthaleny]methanone; anandamide, arachidonic acid *N*-(hydroxyethyl)amide; DAPI, 4′,6-diamidino-2-phenylindole; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PI, propidium iodide

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analysis. Control tissue: Biopsies from reactive lymphoid tissue ($n = 8$). This study was approved by the Ethics Committee at Karolinska Institutet (Etikkommitté Syd).

2.3. Cell culture

Single cell suspensions from biopsies were frozen in DMSO and stored at −120 °C. At the day of the experiment, the cells were thawn and cultured at 1×10^6/ml in RPMI-1640. The EBV transformed MCL cell line Rec-1, the plasma cell leukaemia cell line SK-MM-2, the AtT20 cell line transfected with cDNA for the rat CB1 receptor (AtT20-CB1) and AtT20-wt were obtained and treated as described [3]. The breast cancer cell line MCF7 was cultured in DMEM. Cell cultures were maintained in medium supplemented with 50 μg/ml gentamicin and 10% fetal calf serum. Experiments were performed at the indicated serum concentrations.

2.4. RNA isolation, oligonucleotide array hybridization and gene expression analysis

Total RNA was prepared and hybridizations were performed using U133A arrays (Affymetrix, Inc, Santa Clara, CA) as described in [3]. The data were analyzed using Affymetrix Micro array Suite version 5.0. Comparative analyses were performed using Affymetrix Datamining Tool version 3.0.

2.5. Western blotting

Cell extracts were prepared using ice-cold lysis buffer (50 mM HEPES, 500 mM NaCl, 0.05% Tween 20, 0.1% Triton X-100) to which protease inhibitor cocktail p8340 had been added. Proteins (40 μg/lane) were resolved by 10% SDS–PAGE and transferred onto PVDF membrane, which was incubated with a rabbit polyclonal antibody to CB1. Antibody binding was detected by enhanced chemiluminescence using Supersignal® West Pico chemiluminescent substrate.

2.6. Cell viability

Viability of cells treated with CB1/CB2 ligands dissolved to 10 mM in DMSO or 70% ethanol (anandamide) was determined by cell counting (trypan blue exclusion) or using the MTT assay according to the manufacturer's instructions. Absorbance was measured at 570 nm and reported as optical density (OD). Percent viability is calculated as 100 * OD treated cells (mean of three individually treated wells)/OD vehicle-treated cells (mean of three individually treated wells).

2.7. Cell growth

0.75 million Rec-1 cells/ml were cultured in medium containing 10% fetal calf serum. Indicated doses of Win-55,212-2 or SR141716A were added to duplicate cultures. An equal concentration of DMSO was maintained in each flask. The cells were either counted after three days or every second day. In the latter case, 0.75 million cells/ml were seeded again and treated as described above. The theoretical total number of cells deriving from the original flask was calculated every second day until the end of the experiment at day eight.

2.8. Apoptosis and flow cytometric analysis

Rec-1 cells growing exponentially in 10% serum were treated with 10 μM Win-55,212-2 and harvested 24 h after the treatment. The cells were labeled with Annexin V–FITC and propidium iodide (PI) using the Annexin V kit 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).

2.9. Statistical analysis

Cell viability and growth was compared using Kruskal–Wallis test between vehicle-treated cells and cells treated with CB1/CB2 ligands. *P*-values smaller than 0.05 were considered significant. The software Statistica (StatSoft Scandinavia AB, Tulsa, OK) was used.

3. Results and discussion

3.1. Expression of CB1 and CB2 in primary MCL and in cell lines

Results of expression analysis of CB1 and CB2 in the present and earlier studies are summarized in Table 1. Western blotting (Fig. 1) showed high levels of CB1 protein in Rec-1, while no expression was detectable in SK-MM-2.

3.2. Effect of CB1/CB2 ligands on viability of primary MCL cells and cell lines

To investigate the effect of cannabinoid receptor ligands on viability, freshly isolated MCL cells were cultured in the presence of the endocannabinoid anandamide or the synthetic high-efficacy cannabinoid Win-55,212-2. Both substances are non-selective cannabinoid receptor ligands with affinity for both CB1 and CB2, rev. in [4]. Five μM of either agent decreased cell viability measured as trypan blue exclusion 24 h after treatment (Fig. 2A). The effect was more pronounced at lower serum concentrations, as expected from earlier studies [14,15]. Therefore, the next experiment using the CB1 antagonist SR141716A was performed at 0.5% serum. SR141716A alone reduced the viability of MCL cells isolated from tumor biopsies of two patients (L144 and L102) (Fig. 2B). Thus, ligation with both agonists to CB1/CB2 and an antagonist to CB1 caused decreased viability in primary MCL cells.

Earlier studies in breast cancer [16] and prostate cancer cell lines [6] have shown that antiproliferative and growth inhibitory effects of anandamide could be antagonized by SR141617A. However, potentiation of anandamide-mediated cell death by preincubation with these antagonists has also been reported [17]. In view of the conflicting observations, we wanted to investigate the effects of combined treatment with SR141716A and anandamide in MCL cells. In these studies, the MCL cell line Rec-1 was used. Only at a low dose (0.5 μM) of SR141716A a slight antagonizing effect was observed in Rec-1 cells treated with 5 μM of anandamide (Fig. 3A). Instead, an additive negative effect on viability was observed after treatment with equipotent doses (5 μM)

Table 1
Expression of CB1 and CB2 and in primary MCL and cell lines as described in [3][a], present publication[b] (gene expression array signal value for CB1 in Rec-1 was 124 and in SK-MM was an absent call; signal value for CB2 in Rec-1 was 83 and in SK-MM was 46), [20][c] and [21][d]

Cell lines	CB1		CB2
	RNA	Protein	RNA
Primary MCL	+[a]	+[a]	+[a]
Rec-1	+[b]	+[b]	+[b]
SK-MM	−[b]	−[a]	+[b]
AtT20-CB1		+++[a,c]	
AtT20-wt		+[b]	
MCF7	+[d]	+[d]	+[d]

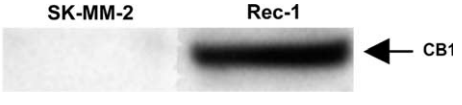

Fig. 1. Protein expression of CB1 in SK-MM-2 and Rec-1. Western blot analysis of CB1 protein expression in whole cell extracts of the plasma cell line SK-MM-2 (left) and the MCL cell line Rec-1 (right).

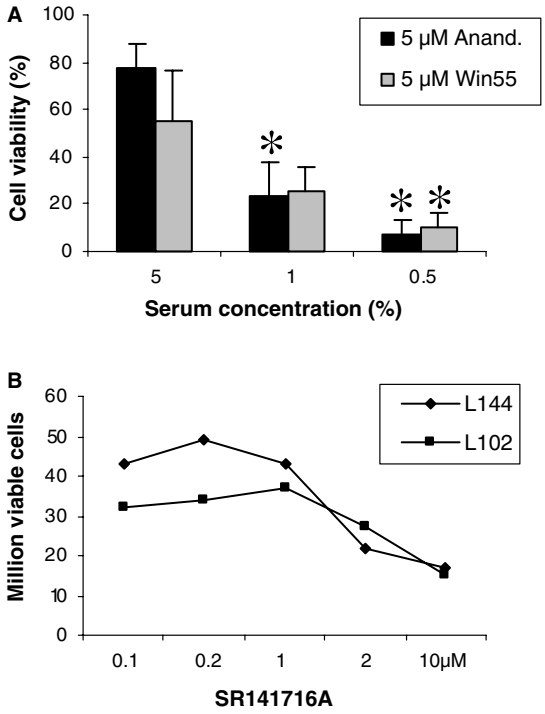

Fig. 2. Viability of MCL cells in response to treatment with CB1/CB2 ligands. (A) Primary MCL cells freshly isolated from a diagnostic tumor biopsy treated with anandamide (5 μM) or Win-55,212-2 (5 μM) in the presence of the indicated serum concentrations. Cell viability was measured 24 h after treatment using trypan blue exclusion. Viability of treated cells is expressed as percent of vehicle-treated control and based on the mean value of four individual treatments. Error bars indicate standard deviations. *, $P < 0.05$ compared to control for anandamide at 1% and 0.5% serum and for Win-55,212-2 at 0.5% serum, Kruskal–Wallis test. (B) Titration of the CB1 antagonist SR141716A on primary MCL cells from tumor biopsies of two patients (L144 and L102). The cells were treated and cultured in the presence of 0.5% serum. Cell viability was measured 24 h after treatment using trypan blue exclusion. Viable cells $\times 10^6$ are shown.

of SR141716A and Win-55,212-2 (Fig. 3A). In contrast, the earlier described antagonizing effect of SR141716A [16] was confirmed in the breast cancer cell line MCF7 (Fig. 3B). SR141716A has, in addition to its antagonizing effect, been shown to act as an inverse agonist to both CB1 and CB2 [18]. Such a role could possibly explain our observed additive effect of combined treatment with SR141716A and anandamide or Win-55,212-2 in MCL.

In order to elucidate the role of CB1 as a mediator of the observed effects, additional studies of cell viability were performed using SK-MM-2, a cell line of B cell origin that does not express CB1 (Fig. 1), as a control. SK-MM-2 harbors a Cyclin D1 t(11;14) translocation similar to that observed in Rec-1. The experiments were performed at 10% serum, a concentration that allows long-term studies of cell growth in culture. Forty-eight hours after treatment with 10 μM of anandamide, cell viability was significantly reduced in Rec-1 cells, whereas SK-MM-2 cells remained unaffected (Fig. 4A). Similarly, a selective reduction of viability in response to anandamide was observed in AtT20 cells transfected with CB1 [19] (Fig. 4B). These results suggest that CB1 is involved in the viability reduction induced by anandamide in MCL cells, although a role of CB2 cannot be excluded.

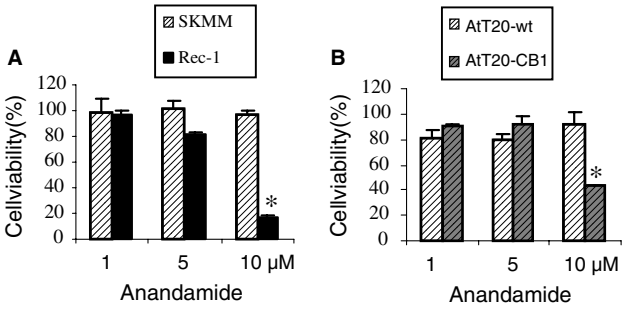

Fig. 4. Viability of cell lines with different CB1 expression in response to CB1/CB2 ligands. (A) Rec-1 cells with high CB1 expression and SK-MM-2 with no detectable CB1 expression. (B) AtT20-CB1 with high ectopic CB1 expression and AtT20-wt with low endogenous CB1 expression. The cells were cultured in the presence of 10% serum and treated with the CB1/CB2 agonist anandamide. The MTT assay was performed 48 h after treatment. Viability of treated cells is expressed as percent of vehicle-treated control and based on the mean value of three individual treatments. Error bars indicate standard deviations. *, $P < 0.05$ compared with control, Kruskal–Wallis test.

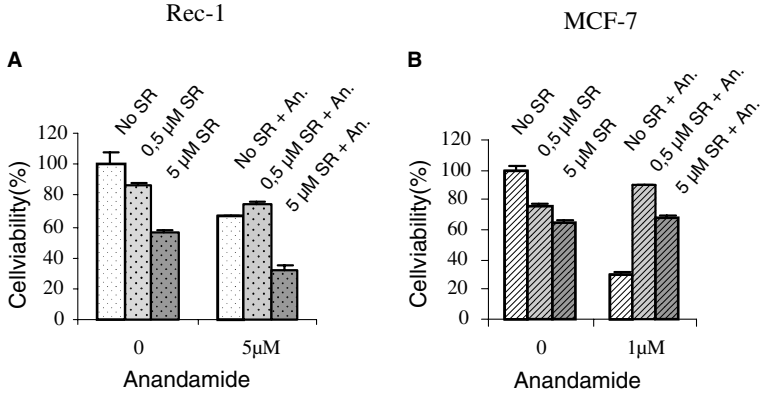

Fig. 3. Effect of the CB1 receptor antagonist SR141716A on reduction of viability induced by the CB1/CB2 agonist anandamide. Cells were preincubated with vehicle (white bars), 0.5 μM of SR141716A (light grey bars) or 5 μM of SR141716A (dark grey bars) for 15 min prior to treatment with 5 μM (Rec-1) or 1 μM (MCF7) of anandamide for 24 h. The MTT assay was employed to assess viability, which is expressed as percent of vehicle-treated control and based on the mean value of three individual treatments. Error bars indicate standard deviations. (A) Rec-1 cells cultured in 1% serum were treated with 0 or 5 μM of the CB1/CB2 agonist anandamide and increasing concentrations of the CB1 antagonist SR141716A. (B) MCF7 cells cultured in 1% serum were treated with 0 or 1 μM of the CB1/CB2 agonist anandamide and increasing concentrations of the CB1 antagonist SR141716A.

3.3. Effect of Win-55,212-2 on growth of Rec-1 cells

Next, the effect of treatment with a CB1/CB2 agonist or a CB1 antagonist on long-term growth of Rec-1 cells was studied. Win-55,212-2, which is a more stable agonist than anandamide, was chosen for this experiment. Previously reported short-term studies at low serum concentration have suggested pro-proliferative effects of treatment with CB1/CB2 agonists in the nanomolar range [7,8,11]. However, when Rec-1 cells were cultured in the presence of Win-55,212-2 for 3 days, 10 and 100 nM of the agonist caused a slight growth inhibition (Fig. 5A). When growth was studied for 8 days, 1 μM of Win-55,212-2 caused a considerable decrease in the accumulated number of cells at the end of the period. A similar reduction in growth was observed in response to 10 μM of SR141716A (Fig. 5B). Thus, at 10% serum, a low dose of the CB1/CB2 agonist Win-55,2121-2 caused substantial growth suppression in Rec-1 cells, while a 10-fold higher dose of the CB1 antagonist SR141716A was required for similar effects.

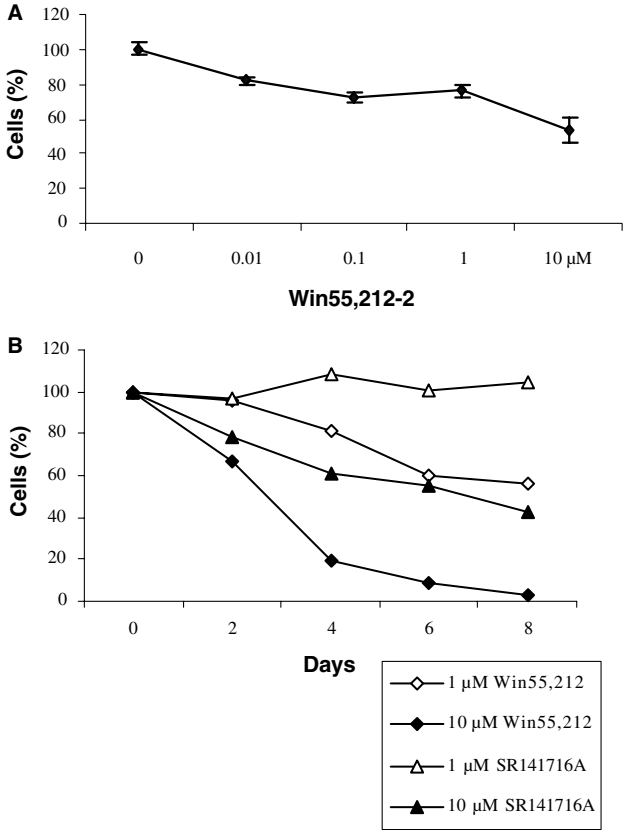

Fig. 5. Effects of the CB1/CB2 agonist Win-55,212-2 on growth of Rec-1 cells. (A) Rec-1 cells cultured in the presence of 10% serum and treated with the indicated concentrations of Win-55,212-2 for three days. Viability of treated cells is expressed as percent of vehicle-treated control and based on the mean value of two individually treated cultures. Error bars indicate highest and lowest value. (B) Long-term growth of Rec-1 cells cultured in the presence of 10% serum and treated with the indicated doses of Win-55,212-2 or the CB1 antagonist SR141716A. The percentage of the accumulated number of treated cells versus the accumulated number of control cells during eight days of culturing in the presence of 10% serum is shown. The accumulated growth is based on the mean value of two individually treated cultures. For the sake of clarity, error bars are omitted in the graph.

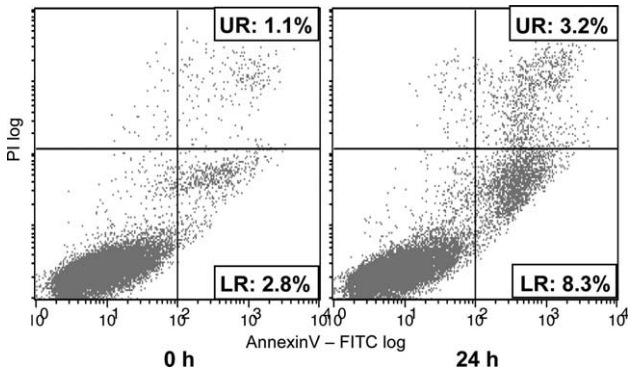

Fig. 6. Induction of apoptosis in Rec-1 following treatment with Win-55,212-2. Flow cytometry analysis of Annexin V–FITC and PI 24 h after treatment of Rec-1 cells with 10 μM Win-55,212-2 in the presence of 10% serum. One representative cytogram showing early apoptotic cells that bind to Annexin V–FITC but exclude PI, lower right-hand quadrant (LR), and late apoptotic/necrotic cells that bind to both Annexin V–FITC and PI, upper right-hand quadrant (UR). The experiment was performed in duplicate.

3.4. Effect of Win-55,212-2 on apoptosis of Rec-1 cells

To determine whether treatment with Win-55,212-2 at 10% serum would lead to induction of apoptosis in Rec-1, the cells were labeled with Annexin V and PI and analyzed by flow cytometry. Twenty-four hours after treatment with 10 μM of Win-55,212-2, the fraction of early apoptotic cells was 8.3% as compared to 2.8% in the control culture (Fig. 6). The sum of the fractions of early (8.3%) and late (3.2%) apoptotic/necrotic cells was 11.5% in the Win-55,212-2 treated culture versus 3.9% (2.8% + 1.1%) in the control culture. Our results show that apoptosis accounts for part of the decreased cell growth observed in Rec-1 cells treated with Win-55,212-2. However, other factors, e.g., alterations in cell cycle progression, might contribute to the growth inhibition.

In conclusion, we have found that cannabinoid receptor ligands induce decreased viability, growth suppression and cell death by apoptosis in MCL cells, which express high levels of the CB1 receptor and moderate levels of CB2. The current results in vitro suggest that CB1/CB2 ligands should be considered as agents for the treatment of MCL. In vivo confirmation of the growth-suppressive effects of cannabinoids on MCL cells and investigations of possible effects on non-malignant immunocompetent cells will further elucidate the potential of cannabinoid receptors as targets of therapy in mantle cell lymphoma.

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