Action of CB1 and CB2 antagonists/inverse agonists on mantle cell lymphoma

Daniel Chui 2011
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Department of Laboratory Medicine, Division of Pathology
Karolinska University Hospital, Huddinge, Sweden

Head supervisor
Agata Wasik, PhD
Karolinska University
Huddinge, Stockholm

Co-supervisor
Birgitta Sander, PhD,MD,Professor
Karolinska University
Huddinge, Stockholm

Examiner
Sven Hamp, Senior Lecturer
Mälardalens Högskola
Eskilstuna/Västerås

Abstract

In this study, the effects of antagonists to the cannabinoid receptors in MCL cell lines were studied. Results presented in this study show that signalling through cannabinoid receptor with antagonists such as SR141716, SR144528 decreases cell viability but hemopressin when analyzing with XTT. The decrease in cell viability by SR141716 is caused by apoptosis triggered after 5 hours of treatment. The CB1 expression was confirmed in all MCL cell lines tested via western blotting but the expression of CB2 and GPR55 – another receptor to which SR141716 has affinity - was not confirmed due to lack of reliable antibodies. Specific agonist to GPR55 – LPI (l-α-lysophosphatidylinositol) showed different response compared to SR141716 which suggests that the effect seen by SR141716 was not induced through GPR55. The effect induced by CB1/CB2 agonist AEA is shown to be neither through CB1 or CB2 alone but possibly on another receptor yet to be described.

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Abbreviation list

Δ^9-THC	Δ^9-tetrahydrocannabinol
2-AG	Arachidonoyl glycerol
AEA	Anandamide
CB	Cannabinoid receptor
DMSO	Dimethyl sulfoxide
FBS	Fetal Bovine Serum
Hp	Hemopressin
HRP	Horse Radish Peroxide
LPI	l-α-lysophosphatidylinositol
MCL	Mantle Cell Lymphoma
PARP	Poly (ADP-ribose) polymerase
SR1	SR141716
SR2	SR144528
TVPR1	Vannilloid type-1

Introduction

1. Background

Cannabis, marijuana and hashish are some well known names of products of the Cannabis Sativa plant. This special plant has its extraordinary effect on the human body and it is known to be used by mankind already in the third millennium B.C for rituals or medical purposes for instance[1]. The active substance Δ9-tetrahydrocannabinol (Δ9-THC) in Cannabis Sativa was discovered 1964 and from there more studies and researches have been done on this remarkable substance. In modern time the THC is classified as a ligand which interacts with the cannabinoid receptors in the human body. More cannabinoids and interacting receptors have been identified and also some of its effect since THC was first described. The mankind has been using cannabinoids in a medical aspect in ancient times and in modern time science has confirmed that the cannabinoids do have potency for treatment of diseases and disorders. Cannabinoids are already widely used as treatment in various diseases and disorders today and one of the types of disease where the cannabinoid have shown to have a medical importance is cancer [2]. Cannabinoids have shown to have different effect on different type of cancers. Two of the studied effect is growth arrest or killing of cells through programmed cell death in some types of cancers [3]. In this study the focus is on one of the B-cell lymphomas, known as the mantle cell lymphoma where the effects of the ligands to the cannabinoid receptors in these cancer cells are studied.

2. B-cells and the lymphatic system

B-cells, also known as B lymphocytes are one kind of white blood cells that has an important role in the adaptive immune response system. When a B-cell surface receptor encounters and matches to an antigen the B-cell produces more of that specific receptor and releases it and more receptors will bind to those antigens. Those receptors are called antibodies when released and helps other cleaning cells to recognize antigens for disposal. Antibody producing B-cells are known as plasma B-cells and some of them will develop into memory B-cells which has data stored about the specific antigen it encountered. The memory B-cells has a longer lifespan and will react faster and produce antibodies specific to that kind of antigen on next encounter. The development of plasma cells and memory B cells are activated by helper T-cells. Immature B-cells are produced within the bone marrow in mammals and have many different steps before differentiating into mature B-cells [4].

High concentration of B-cells and T-cells can be found in the lymph nodes in the human body. Lymph nodes are small organs throughout the body which functions as filter for harmful substances and these nodes are connected together in the lymphatic system. This system is further linked to most of the major organs like the colon, blood system etc to help maintain an environment free from harmful antigens. The lymphatic system has also other functions in the body except cleansing it from antigens such as absorbing and transferring fatty acids and removing interstitial fluid from tissues. Through these connections, cancer cells may go into a phase known as metastasis. Metastasis is when cancer cells relocate themselves in another part of the body; in this case it is through the lymphatic system. These lymph nodes can accumulate cancer and become a site for secondary tumor growth.

3. Mantle cell lymphoma

Mantle cell lymphoma is a malignant non-Hodgkin b-cell lymphoma. The cancer cells are developed in the mantle area of the lymph nodes[5] and in the spleen which gives this disease its name. A schematic figure of development of MCL is shown in figure 1 below.

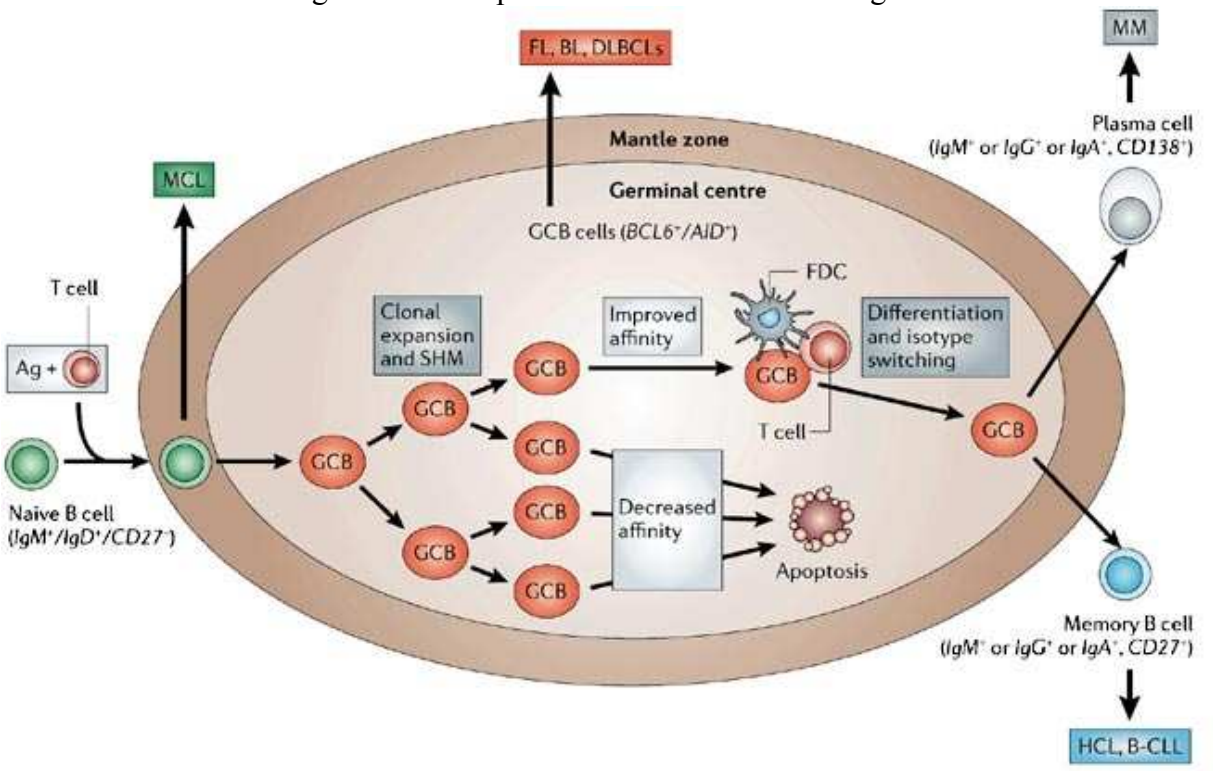


Figure 1. Schematic figure of development of naïve b-cell to various kinds of lymphomas. *MCL development of naïve b-cell in the mantle zone is shown in green. Adapted from E.Tiacci in Nature Reviews Cancer 6, June 2006.*

8% of all lymphoma cases are MCL and its median survival is 5-7 years [6]. It is a difficult disease to treat and the treatments are immunotherapy and chemotherapy which has become more efficient through studies and researches. The genetic hallmark of MCL is the chromosomal translocation of t(11;14)(q13;q32) that leads to over expression of cyclin D1 which is not expressed in normal lymphocytes[6].

Cyclin D1 regulates the cell cycle and acts on the G1-S transition [7]. Studies of the cyclin D1 indicates that it is interfering with several key processes such as metabolism and proliferation in B-cells and contributes to malignancies [7].

The MCL disease can be divided into two different groups, the classical MCL and blastoid MCL. The blastoid MCL is more aggressive and proliferates faster compared with the classical MCL due to more genetic alterations that alters the proliferation of cells.

Studies have shown that MCL cells have overexpression of cannabinoid receptors when compared to normal lymphocytes[8].The two cannabinoid receptors over expressed are known as cannabinoid receptor 1 and cannabinoid receptor 2 (CB1 and CB2). Due to the up regulation of the cannabinoid receptors, effect of cannabinoid treatment is only seen in cancer cells which make cannabinoids an interesting candidate for treatment of MCL.

4. Endocannabinoid system

The cannabinoid receptors 1 and 2 belong to the seven-transmembrane G-protein coupled receptor family. CB1 is mainly expressed in the brain while CB2 is mainly expressed in the immune system. The similarity of sequence between the two receptor is 44% [9]. Different ligand interactions with these receptors can activate different pathways that regulate some of the human mind and senses. Some known effects of cannabinoids are affecting metabolism, pain sensation, cardiovascular and immune functions as well as memory and mood by signaling through cannabinoid receptors [2,10]. Some known effects of cannabinoids on lymphocytes are affecting cell survival, apoptosis, cytokines suppression and cytokine release.

a. Other possible interacting receptors

The GPR55 receptor was first described in 1999[14] and is expressed in tissues such as breast adipose tissues, testis, spleen, and several regions of the brain [14]. Recent studies have shown that Rimonabant that interacts with cannabinoid receptors also interacts with the GPR55 receptor as an agonist [15] like the endogenous lysophospholipid l-α-lysophosphatidylinositol (LPI) [16] that does not have affinity to cannabinoid receptors. The GPR55 has a sequence similarity of 13.5% to the CB1 and 14.4% to the CB2 receptor [17]. Although this orphan G-coupled receptor [18] has shown to interact with a substance binds to a cannabinoid receptor it is not yet classified as a cannabinoid receptor [16].

The transient receptor potential channels of the vannilloid type-1(TVPR1) have also been proven to interact with substances that interact with cannabinoid receptors. The endocannabinoid Anandamide induces apoptosis through this receptor by increasing the calcium levels which decreases the mitochondrial potential which leading to release of cytochrome c that activates caspases in U937 cell lines (histocytic lymphoma) [19]. The pathway can be seen in figure 2.

b. Ligands

Cannabinoids are distinguished by its origin into three subgroups, endocannabinoids, natural cannabinoids and synthetic cannabinoids. Endocannabinoids are derived from membrane lipids [11] such as Anandamide (AEA), the first described endocannabinoid or 2-Arachidonoyl glycerol (2-AG) [12]. Natural cannabinoids are found in plants, an example of that's the active substance Δ9- THC. Examples of synthetic cannabinoids are WIN 55,212-2 or HU-210 [13] which are synthetically made within labs.

Cannabinoids binds to the same active area at the receptors but can act differently and induce different pathways. Agonists bind and induce an effect while antagonist binds and blocks the active area without inducing an agonistic effect. Inverse agonists act as the antagonist but it also reverses the effect of the agonist and might inhibit a constitutive basal activity while blocking the binding site. Some cannabinoids may even have several roles; it might act as an agonist but change to act as an antagonist depending on the concentration and the competition of binding to the receptor.

c. Hemopressin

Hemopressin (PVNFKFLSH), first discovered in the mid 2000, is another CB1 selective cannabinoid and it acts as an antagonist on low doses and as an inverse agonist on higher doses [11]. Hemopressin is different from other ligands because it is the first found cannabinoid in a peptide form instead of lipid form. This peptide is an endocannabinoid which is a small part of the alpha chain subunit in the hemoglobin complex. Hemopressin is a 9 amino acid long peptide but there are also two known isoforms which are N-terminally extended by two or three amino acids [20]. The two isoforms are RVD-Hpα and VD-Hpα which both act as agonists to CB1 [20]. These agonistic peptide endocannabinoids induces a release of calcium from intracellular stores more rapidly through a different pathway compared with the lipid agonistic endocannabinoids [21]. Using peptides for medical treatment instead of lipid are more convenient since peptides can be administrated through different various ways like injection, orally etc that is not possible with lipids. This is why hemopressin is highlighted as a potential treatment for cancer.

5. Cell death pathways

Apoptosis is programmed cell death and can be induced by extrinsic or intrinsic pathways. Figure 2 shows different pathways that lead to apoptosis. Signaling through a death receptor is an extrinsic pathway and it can accumulate ceramide in the cell and induce cytochrome c release which activates caspases [22]. In apoptosis caspases denaturizes the cell in many well organized steps from inside without releasing cytoplasm into the environment, PARP cleavage is one of those steps [23].

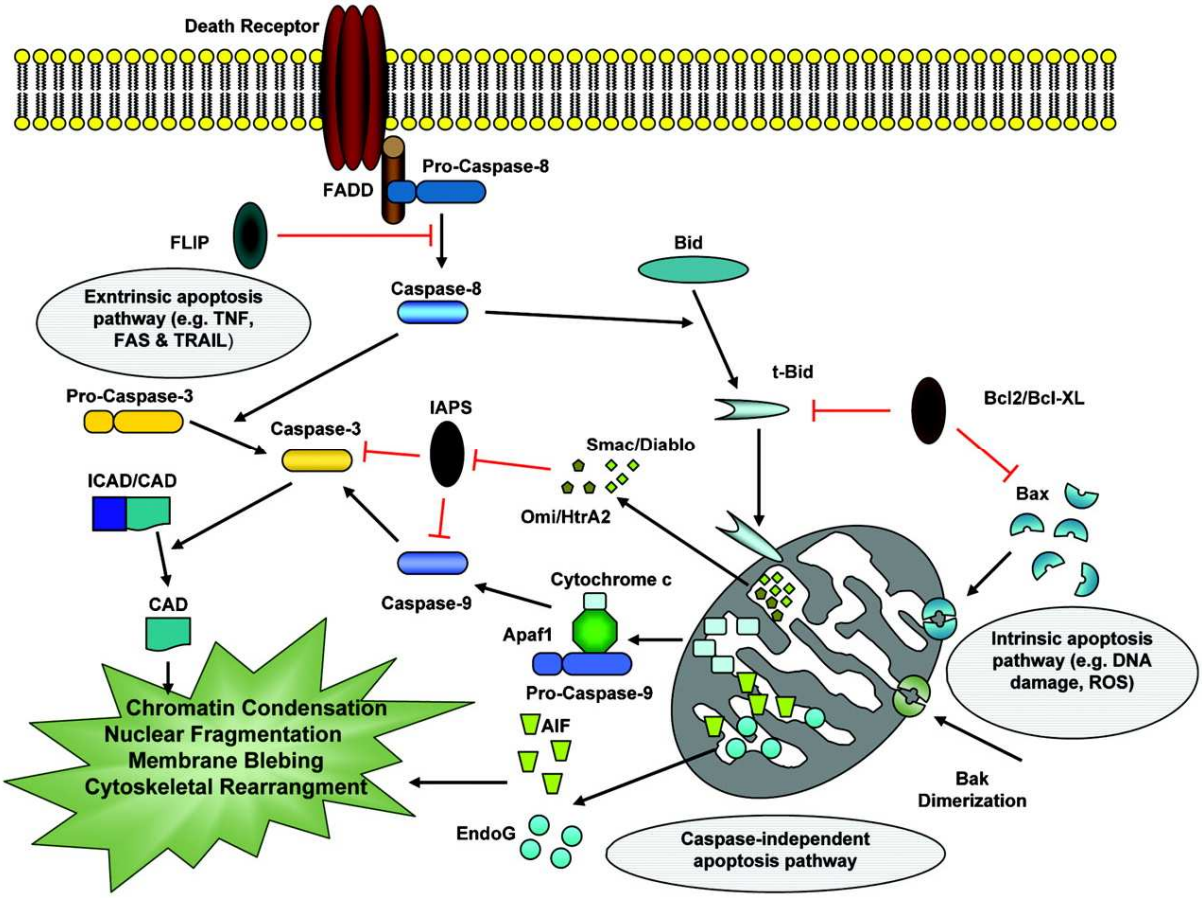


Figure 2. Extrinsic and intrinsic pathways to apoptosis of cell. *Adapted from S.Ghavami in Journal of Medical Genetics Volume 46 issue 8 2009.*

Autophagy is another cell survival/death mechanism where no activation of caspase is found. The cell forms vesicles and encapsulates organelles or unwanted protein and fuses the vesicle with lysosomes to break the content to amino acids. The amino acids are then released back into the cytoplasm where it can be formed into other proteins or organelles that the cell has more use of. Autophagy is a process which can occur during starving of cells [24].

Aim

The general aim was to study the effects of antagonists to the up regulated cannabinoid receptors CB1 and CB2 in MCL cells. More specifically, the aims were to determine the impact on cell death induced by CB1 antagonists: SR141716 and hemopressin and CB2 antagonist SR144528 as well as the type of cell death occuring MCL cell lines after treatment with CB1 and CB2 antagonists. The study was intended to help the process of understanding the role and potential of ligands to the cannabinoid receptors as a treatment of cancer.

Materials and Methods

1. Reagents

DMEM Dulbeccos Modified Eagle Medium (Invitrogen), RPMI 1640+GlutaMAX^{T1-1}(Invitrogen), Fixer and Replenisher (Kodak Readymatic), Developer and replenisher (Kodak Readymatic), Anandamide (Tocris Bio Science) 5mg diluted to 14.39µM in DMSO, Hemopressin (Tocris Bio Science) 1mg diluted to 10µM in DMSO, Cell Proliferation kit II XTT Labeling reagent (Roche), Cell Proliferation kit II Electron Coupling reagent (Roche), Peroxidase substrate kit Vector^{R}SG SK4700 (Vector Laboratories Inc), Western lightning plus-ECL Enhanced Chemiluminescence substrate (Perkin Elmer Inc), 10%, 12% Bis-Tris Gel1.0mmX12 well (NuPAGE Invitrogen), BCA Protein Assay Reagant A (Pierce^{R}), BCA Protein Assay Reagant B (Pierce^{R}), MES SDS Running buffer (20x) (NuPAGE Invitrogen), 10x Tris/Glycine buffer (Biorad laboratories Inc), Precision Plus ProteinStandard (Biorad), RIPA buffer (Sigma).

2. Instruments

Power wave HT (BioTek), Spectra max 250 (GMI), Hera cell incubator (Heraeus), Microscopy (Zeiss West Germany), labofuge 300 (Heraesus), Icematic F90 Electronic, Yellow line RS10 basic, Electronic balance FX-200 (AND), Semi-Phor (Hoefer Scientific Instruments San Francisco), Power Pac 1000 (Biorad), Hypercassette^{TM} (Amersham Biosciences), Amersham hyperfilm^{TM}ECL (GE Healthcare), VWR Galaxy mini (Grant).

3. Softwares

Excel 2007 (Microsoft), Origin pro 8 version 8E (OriginLab), Softmax (Softmax), Gen 5 (BioTek).

4. Cell culture

MCL cell lines Granta519, JeKo, JVM2 and neuroblastoma cell line SHSY5Y were obtained from Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ; Germany), MCL cell line Rec1 was a kind gift from Dr. Christian Bastard, Ronan, France. All cells were cultured in RPMI 1640+GlutaMAX^{TI-1} medium except SHSY5Y which was cultured in DMEM medium supplemented with Gentamicin (50µg/ml) and 10% fetal bovine serum (FBS) in a cell culture incubator at standard conditions (37°C, 5% CO2 and high humidity). Cells were passaged twice per week (Monday and Thursday) to $0.5x10^{6}$/ml. Cells were always passaged in 10% FBS in RPMI two days before experiments were commenced and a day before the experiment cells were starved with 1% FBS in RPMI.

5. Freezing and thawing of cells

Cells were kept in cryovials containing serum with 5% DMSO in a freezer at -70°C overnight and transferred to -135°C the day after for longer storage when freezing cells.
Cells were thawed in warm water and cultured in 20% FBS in RPMI on the day of thawing. Within two days after thawing the cells, they were cultured to 10% FBS in RPMI by gradually decreasing the serum concentration.

6. Viability assay

For cell culture, MCL cells that grow in cell suspension (Granta519, Rec1, JeKo and JVM2) were counted in the Burker chamber and the cell viability was assessed by trypan blue exclusion (positive for dead cells).
The effect of drugs on MCL cell lines was assessed by XTT assay according to the manufacturer's protocol. In short, MCL cells at final cell density of $0{,}25x10^6$/ml were seeded in 96-well plates with drugs at the chosen concentration range with a final volume of 100µl and were incubated for 48 hours. A volume of 50µl of XTT solution mixed with electron coupling reagent at 51:1 ratio was added to each well and incubated for additional 4 hours before reading with spectrophotometer at 470nm with 650nm as reference wavelength. The final incubation time of cells was 52 hours. Prior to reading, the plate were brought to room temperature and shaken for 5 seconds by setting it up with the program of the spectrophotometer. Analyses of the plate readout were done with Gen5, and the graph analyses in Excel and Origin Pro 8 software.

7. Protein lysate preparation and protein concentration estimation

$5x10^6$ cells of each cell lines were collected and washed with PBS. The cell pellets were stored at -20°C until the preparation of the protein lysates were to be done. The protein lysates were prepared by adding 60µl RIPA buffer (Sigma Aldrich) supplemented with proteases inhibitors cocktail III (Sigma Aldrich) at 1:1000. Protein concentration estimation was done using BCA assay according to the manufacturer's protocol. Bovine serum albumin (BSA) at five different concentrations prepared by double dilution ranging from 2mg/ml to 0.125mg/ml was used as standards. Working reagent was added to standard or samples with a ratio of 110:10 on a 96 well micro plate in triplets and incubated for 30 minutes in a dry incubator at 37°C before reading with spectrophotometer at 562nm wavelength. Analyses of the plate were done with Softmax software.

8. Western blotting

50µg proteins with loading buffer were incubated at 95°C for 4 minutes prior to gel electrophoresis. Electrophoresis was done using precast Tris acetate gels (Invitrogen) with MES running buffer at 200V constant voltage. For determination of protein size, the Precision Plus Protein standards from Bio-Rad were used. Proteins were transferred onto PVDF membranes using semi-dry transfer system (Hoefer).Blocking was done using 10% milk in TBS for 1 hour and then incubated with primary antibodies in 5% milk in TBS overnight at 4°C. Stronger primary antibodies such as β-actin and LC3 required shorter incubation (1 hour and 2 hours at RT). Concentrations of primary antibodies used were as follows: anti-CB1 (in-house made, raised against aa sequence within N-terminus) 1:1000, anti-CB2 (Abcam) 1:200, anti-CB2 (in-house made) 1:1000, anti β-actin (Sigma Aldrich) 1:5000, anti caspase 3 (Cell Signalling) 1:1000, anti PARP (Cell Signalling) 1:2500 , anti GPR55 (Abcam, nr 1 (ab12700) and nr 2 (ab41515)) 1:1000 and 1:2000. Then the membranes were incubated with horseradish peroxidase conjugated secondary anti-rabbit or anti-mouse antibodies (both from GE Healthcare) at 1:5000 in 5% milk in TBS at RT for 2 hours (45min for β-actin).Blots were wet developed using developer and fixer from Kodak. As a loading control β-actin was used. Before re-probing blots with anti HRP signal was blocked using SG solution kit from VectorLabs. Membranes were incubated with SG solution (3 drops of SG substrate and 3 drops of H_2O_2 were added *per* 15mls of PBS) for 30 minutes at RT, then the reaction was stopped by rinsing membranes twice under the tap water.

Results

1. CB1 & CB2 receptor expression

To analyze the cannabinoid receptor expression in mantle cell lymphoma cell lines, cell lysates were probed with anti-CB1 and anti-CB2 in western blot. Expression of CB1 is shown in figure 3 below and expression of CB2 is shown in figure 4. The expected size of CB1 is 53kDa.

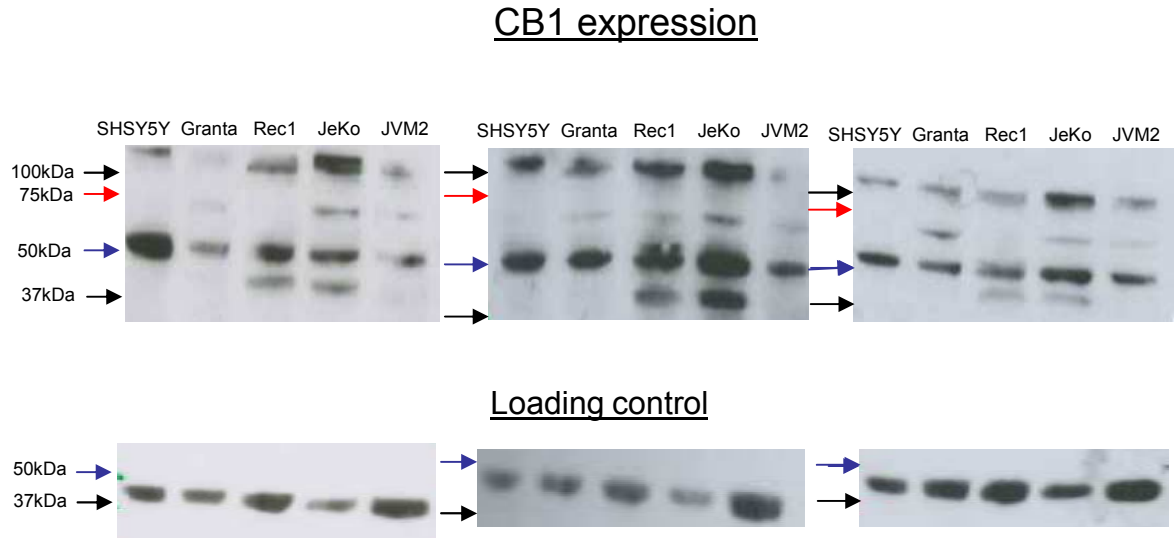


Figure 3. Blots of* CB1 *expression with loading control from three different experiments. *SHSY5Y was used as positive control for the four MCL cell lines. Multiple bands were detected in the MCL cell lines while the positive control showed only two bands at around 50kDa and above 100kDa*

Between 37kDa and 75kDa several bands were detected when probing with anti-CB1 antibody. The neuroblastoma cell lines SHSY5Y was used as a positive control for CB1 expression. Anti-CB1 antibody used detected two bands: at around 50kDa and above 100kDa in SHYSY5Y and all MCL cell lines tested. However, JVM2 cells had in all repeats of experiment the lowest expression levels of proteins detected with anti-CB1 antibody. Several bands were detected in some MCL cell lines that were not expressed in the positive control. In Rec1 and JeKo cells, an extra band between 37kDa and 50kDa was detected. Also, occasionally another extra band between 50kDa-75kDa was detected in Granta519 and JeKo cell line.

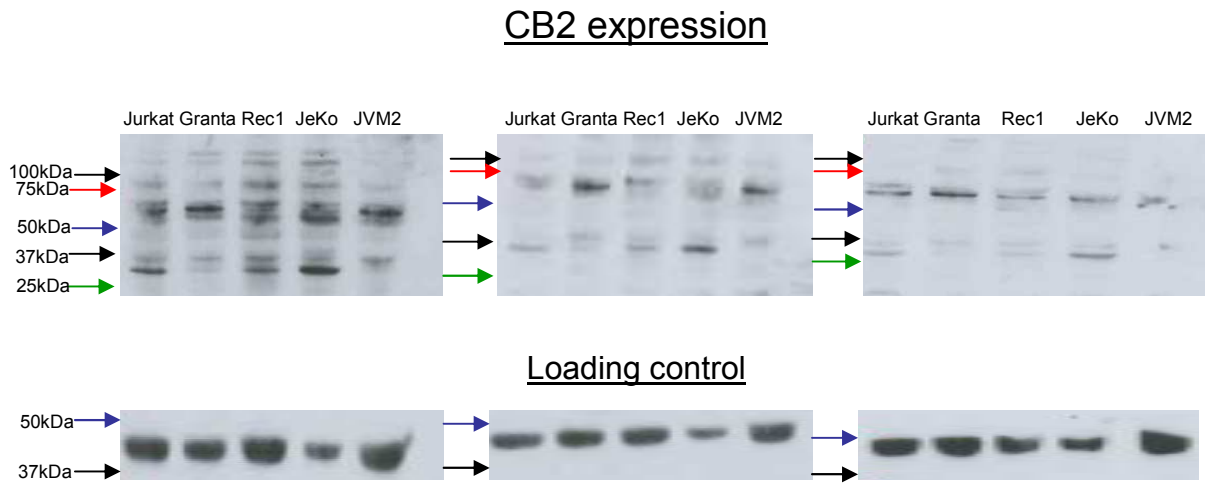


Figure 4. Blots of* CB2 *expression with loading control form three different experiments. *Unspecific and unclear bands detected when probed with CB2 antibodies.*

When probing with anti-CB2 antibody bands were detected between 25kDa and 75kDa. The expected size is 40kDa . Jurkat was used as a positive control for CB2 expression. Anti-CB2 antibody used detected two possible bands: the first one between 25kDa and 37kDa and the second one between 50kDa and 75kDa in Jurkat. Probing with anti-CB2 antibody from Abcam did not give any clear and consistent results so another anti-CB2 antibody, N18 (in-house designed) was further used. The blots of N18 compared with the antibodies from Abcam are shown in figure 5 below.

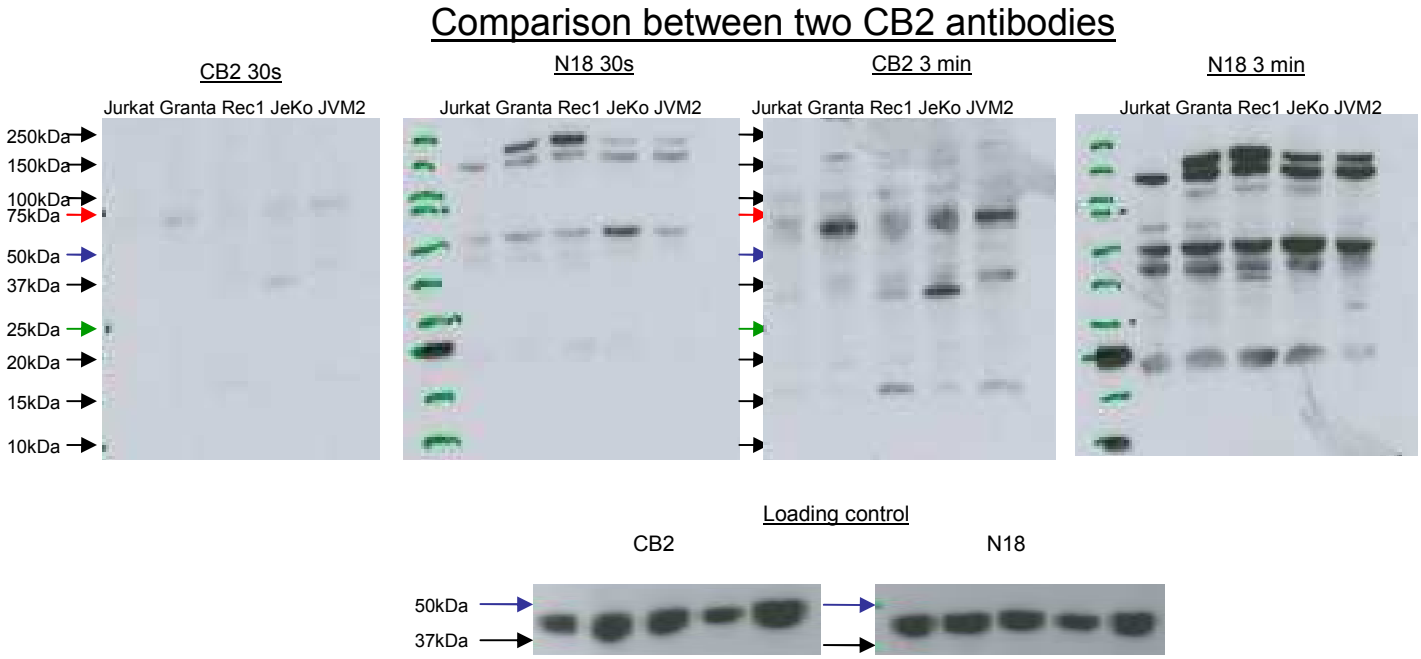


Figure 5. Comparison between the two anti-*CB2 *antibodies with β-actin as a loading control; anti-*CB2 *from Abcam and anti-*CB_2 *N-18). *The two first blots were exposed for 30 seconds while the other two blots were exposed for 3 minutes.*

Comparison between the Abcam (CB2) and the N-18 antibodies showed that the N-18 antibody detects more bands and bands could even be found within the ladder. Between 37kDa and 50kDa, double bands were detected in the blot with the N-18 antibody.

2. CB1 and CB2 antagonists' effects on cell viability

XTT viability assays were used to analyse viability of MCL cell lines treated with the selective CB1 antagonist/inverse agonist hemopressin at the range of concentration 100µM-0,78µM, obtained by double dilution of the peptide. Cell viability assessed by XTT assay reflects the state of mitochondria and thus it is referred in the text to as mitochondrial activity assay alternatively to cell viability assay. The effect is shown in figure 6 below.

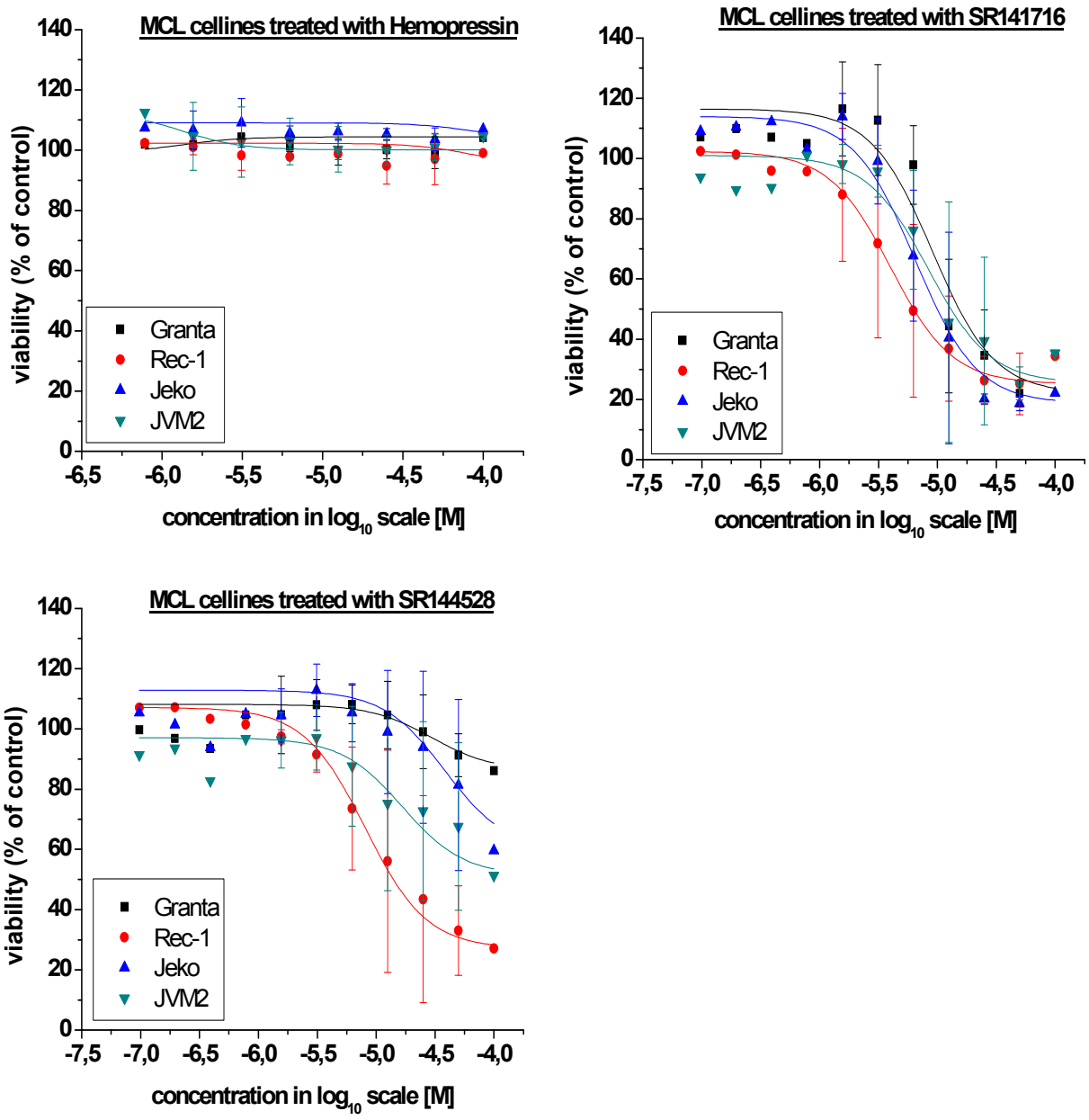


Figure 6. Effect of different CB1 antagonists (hemopressin and SR141716) and CB2 antagonist (SR144528) on cell viability in MCL cell lines. *Y-axis represents cell viability in percentage and x-axis represents the concentration of antagonist in log_{10} scale [M].*

Treatment with hemopressin did not show any detectable changes in mitochondrial activity in any of the MCL cell lines.

Two similar cell viability assays were done with the two other antagonists, which are selective to CB1 (SR141716) and CB2 (SR144258). The titration range was 100µM-0.098µM, obtained by double dilution of the drugs. The effects on mitochondrial activity by the two antagonists are shown in figure 6 and the EC_{50} values are shown in Table 1.

The viability assay showed decreased mitochondrial activity for all MCL cell lines when treated with SR141716 but Rec1 seemed to be a little bit more sensitive to SR141716. The maximum effect was reached with 50µM of the drug. The mitochondrial activity decreased to a level of around 30% of the control cells.

Treatment with SR144258 resulted in the decrease of mitochondrial activity of the MCL cell lines to different extents. Granta519 was least affected by the drug while Rec1 was the most sensitive to SR144258 cell line. Due to the higher resistance of Granta519 and JeKo, the EC_{50} values could not be determined since the maximum effect of the cannabinoid was not within the range of titrated drug.

3. Agonists effect on cell viability

A viability assay of the nonselective agonist to CB1 and CB2 AEA in a concentration range of 20µM-0.3125µM was done to see the effect of AEA on mitochondrial activity. The effect on mitochondrial activity is shown in figure 7 and the EC_{50} value is shown in Table 1.

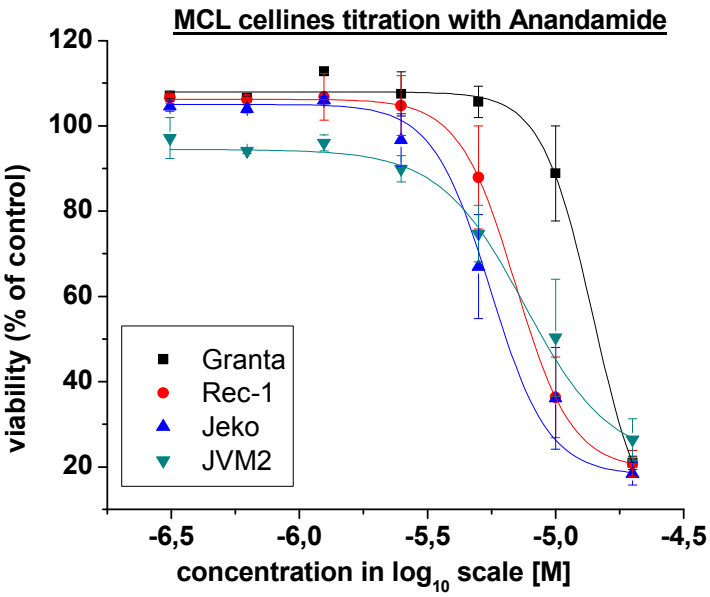


Figure 7.Graph of non selective agonist effect on cell viability in MCL cell lines.
Y-axis represents cell viability and x-axis represents the concentration of antagonist in log_{10} scale [M].

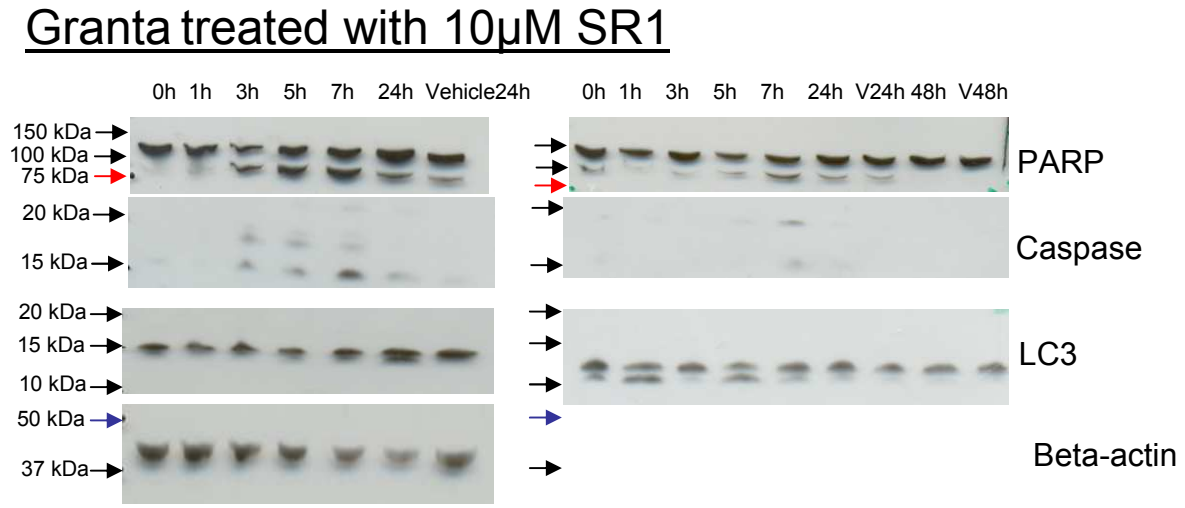
The Granta 519 cell line was more resistant to AEA treatment than the other cell linesand required a higher dose of the drug to give the same change in mitochondrial activity as for the other MCL cell lines. The slopes in the graph drop abruptly to 20% when MCL cell lines were treated with 20µM of AEA.

Table 1. EC_{50} values for different cannabinoids on MCL cell lines calculated from the graphs in figure 6 and 7. *No decrease in mitochondrial activity was seen in the treatment of all MCL cell lines with hemopressin therefore the EC_{50} value were not determined. The EC_{50} values of Granta519 and JeKo with treatment of SR144528 could not be determined since the maximum effect of the drug was not within the titration range.*

	Granta 519 EC_{50} value [M]	Rec1 EC_{50} value [M]	JeKo EC_{50} value [M]	JVM2 EC_{50} value [M]
Hemopressin treated	-	-	-	-
SR141716 treated	9.05950E-06	4.00965E-06	6.48089E-06	8.40096E-06
SR144528 treated	Not determined	8.07217E-06	Not determined	1.66648E-05
Anandamide treated	1.40248E-05	6.97826E-06	5.57269E-06	7.58321E-06

4. Detection of apoptosis and autophagy

Detection of apoptosis and autophagy was done to verify which type of programmed cell death is responsible for SR141716 concentration-dependent decrease of viability of MCL cell lines. Cells were treated with 10µM SR141716 and collected at 1hr, 3hr, 5hr, 7hr and 24hrs after treatment. Western blots were done on these samples to determine the time of treatment when the possible cell death processes were induced. Blots were probed with specific antibodies against PARP, active caspase-3 and of LC3. Detection of active caspase-3 and PARP cleavage, induced by active caspase-3 was done in order to track possible apoptosis, whereas detection of LC3 in two different forms: LC3I and LC3II, the latter characteristic for autophagosome formation, was performed to track the possible autophagy. The blots of the MCL cell lines treated with 10µM SR141716 in time are shown in figure 8 below.



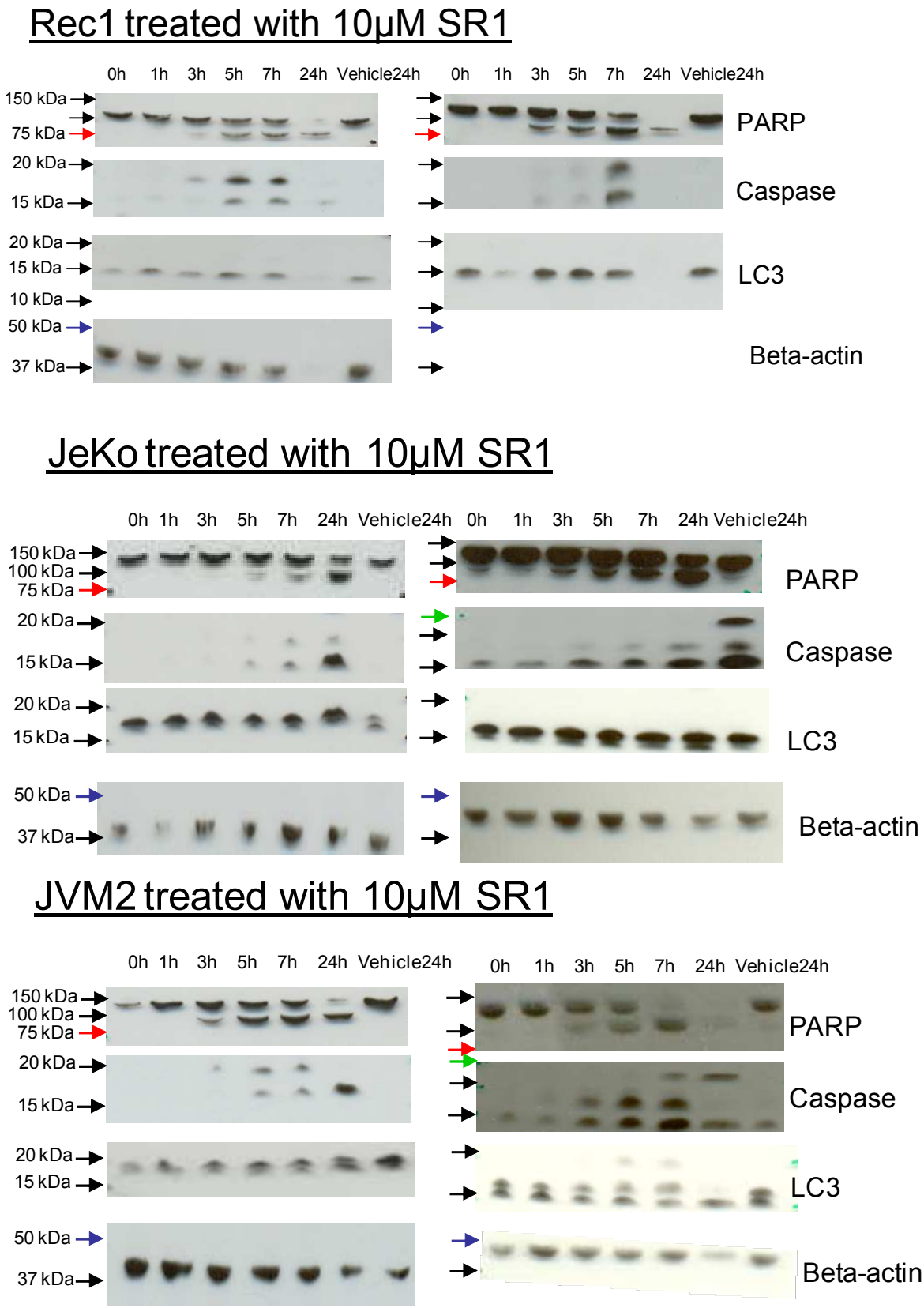


Figure 8. PARP, active caspase-3 and LC3 detection on MCL cell lines*. The figure shows representative blots out of two independent experiments. Vehicle refers to 24 hours treatment of cells with vehicle (DMSO).*

Western blot membranes probed with PARP antibody showed one or two bands between 75kDa and 150 kDa. In later time points after 24h the lower band, corresponding to the cleaved form of PARP, was more intense than the upper band and at 24hrs of treatment only the lower band could be detected in Rec1 and JVM2. Only the upper band was detected in the vehicle treated MCL cell lines except for Granta519 where two bands could be seen.

Probing with antibody against active caspase-3 gave raise to the bands between 15 and 20 kDa. Bands were only seen in later time points around 5-7hrs and 24hrs. The stronger bands were detected in the time point just before the one where most of the PARP were cleaved.

When probed with anti-LC3 antibody, one or two bands were detected near 15kDa in all time points for all of the MCL cell lines except Rec1 where only single band was detected. At time point 24h detection of LC3 was not possible in Rec1 cell line due to low protein concentration, probably caused by cell death, in the cell lysates.

5. GPR55 receptor expression

Studies have shown that SR141716 acts as an agonist to the GPR55 receptor [15]. In order to verify whether the observed effects of SR141716 on MCL cell lines were *via* GPR55, the expression of the GPR55 receptor in the MCL cell lines was investigated. Detection of GPR55 expression was done *via* western blot method with two different antibodies against GPR55 receptor. The blot of GPR55 expression in MCL cell lines is shown in figure 9 below.

Figure 9. GPR55 expression in MCL cell lines*. SHSY5Y cell line was used as positive control for GPR55.*

The estimated size of GPR55 is 37kDa between 20kDa and 37kDa two bands could be seen in positive control and in all of the MCL cell lines except Rec1 where only one band was detected.

Due to the double bands observed using this antibody, another antibody was tested. Two concentrations of the blocking peptide, was used to block the antibody as a pretreatment before probing. This was done to see how specific the antibody was. The blot is shown in figure 10 below.

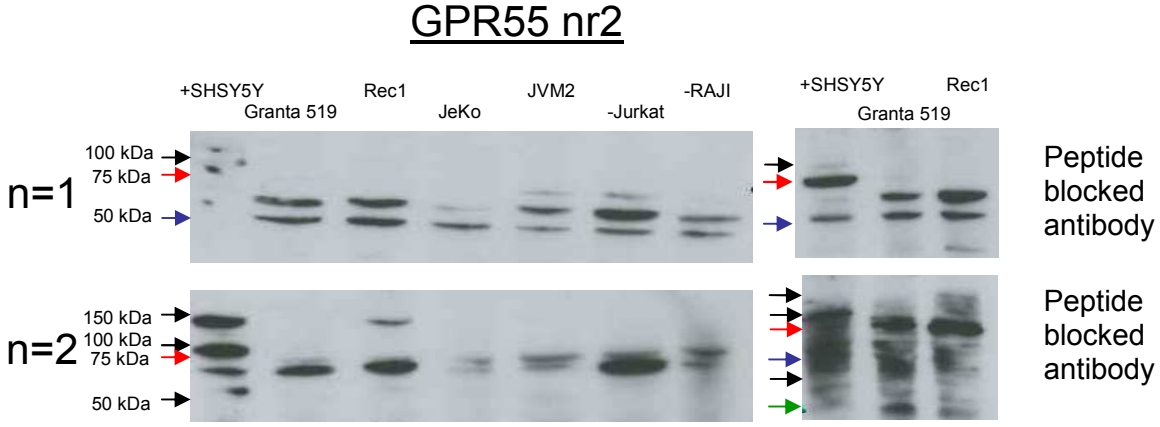


Figure 10. GPR55 expression in MCL cell lines with another antibody*. SHSY5Y cell line was used as positive control for the four MCL cell lines. On the left side antibodies were probed without pretreatment while on the right side antibodies were pretreated with the GPR55blocking peptide. The positive control of n=1 was not successful; it was smeared out on the gel electrophoresis. Antibodies pretreated with peptide on n=2 showed unclear bands.*

With the antibody number 2 several bands were detected between 50kDa and 150kDa in the positive control. Two bands between 50kDa and 75kDa were detected in both positive control and in the MCL cell lines.

Bands between 50kDa and 75kDa were detected when also probed with antibodies blocked with peptide in positive control, Granta 519 and Rec1.

Comparison between the two GPR55 antibodies showed inconsistent results.

6. GPR55 agonist effect on cell viability

Since the GPR55 expression could not be validated using WB and the commercially available antibody, the effect of a specific GPR55 agonist, l-α-lysophosphatidylinositol (LPI) on cell viability was done to see whether SR141716 agonistic properties towards GPR55 are responsible for MCL cell death. The change in cell viability caused by LPI on MCL cell lines is shown in figure 11 below.

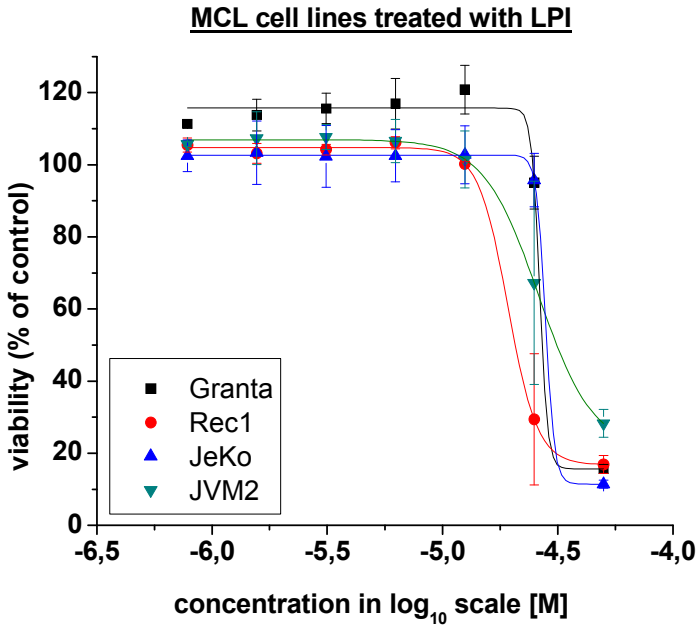


Figure 11. The GPR55 agonist LPI, effect on cell viability in MCL cell lines *.*
Y-axis represents cell viability and x-axis represents the concentration of antagonist in log_{10} scale [M].

The cell viability starts to decrease abruptly between 50µM and 12.5µM of LPI, decreasing to a cell viability around 10%.

7. Effect of Anandamide in cells pretreated with antagonists

To analyze if the selective CB1 antagonist SR141716 and hemopressin and the selective CB2 antagonist SR144528 could block the effect of CB1 and CB2 agonist, titration of AEA was done on cells pretreated with antagonists. The concentrations of antagonists were: 10µM for hemopressin, 0.5µM for both SR1 and SR2. The results of AEA effect on MCL cell lines when pretreated with antagonists is shown in the figure 12 below.

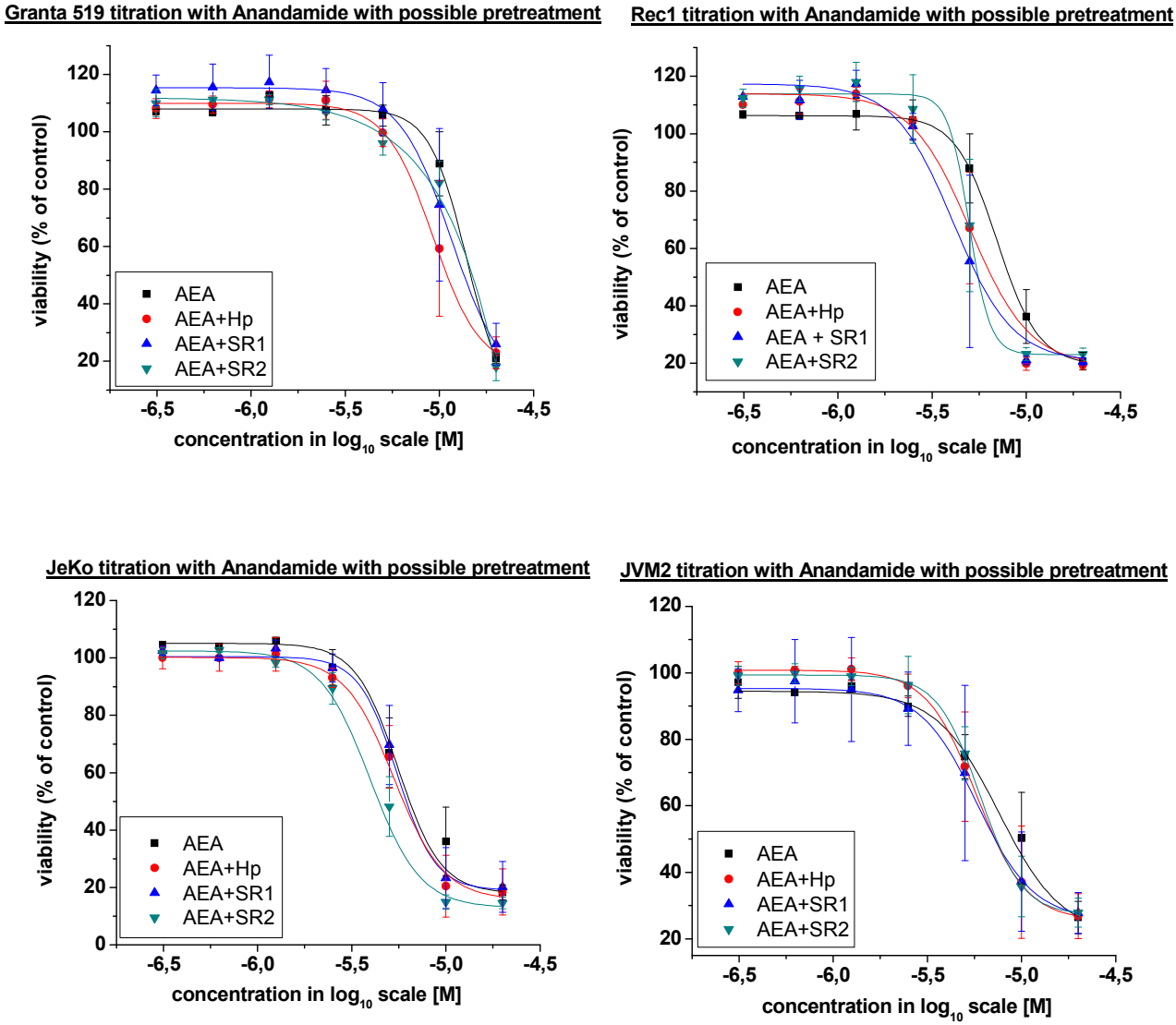


Figure 12. Anandamide effect on cell viability in MCL cell lines after pretreatment with antagonists*. Y-axis represents cell viability and x-axis represents the concentration of antagonist in log_{10} scale [M].*

None of the antagonists alone inhibited the effect of anandamide.

A control for this experiment was done to prove that the concentration of SR141716 used by itself did not induce programmed cell death in MCL cells. Blots of cells treated with SR141716 for 5 hours are shown in figure 13 below.

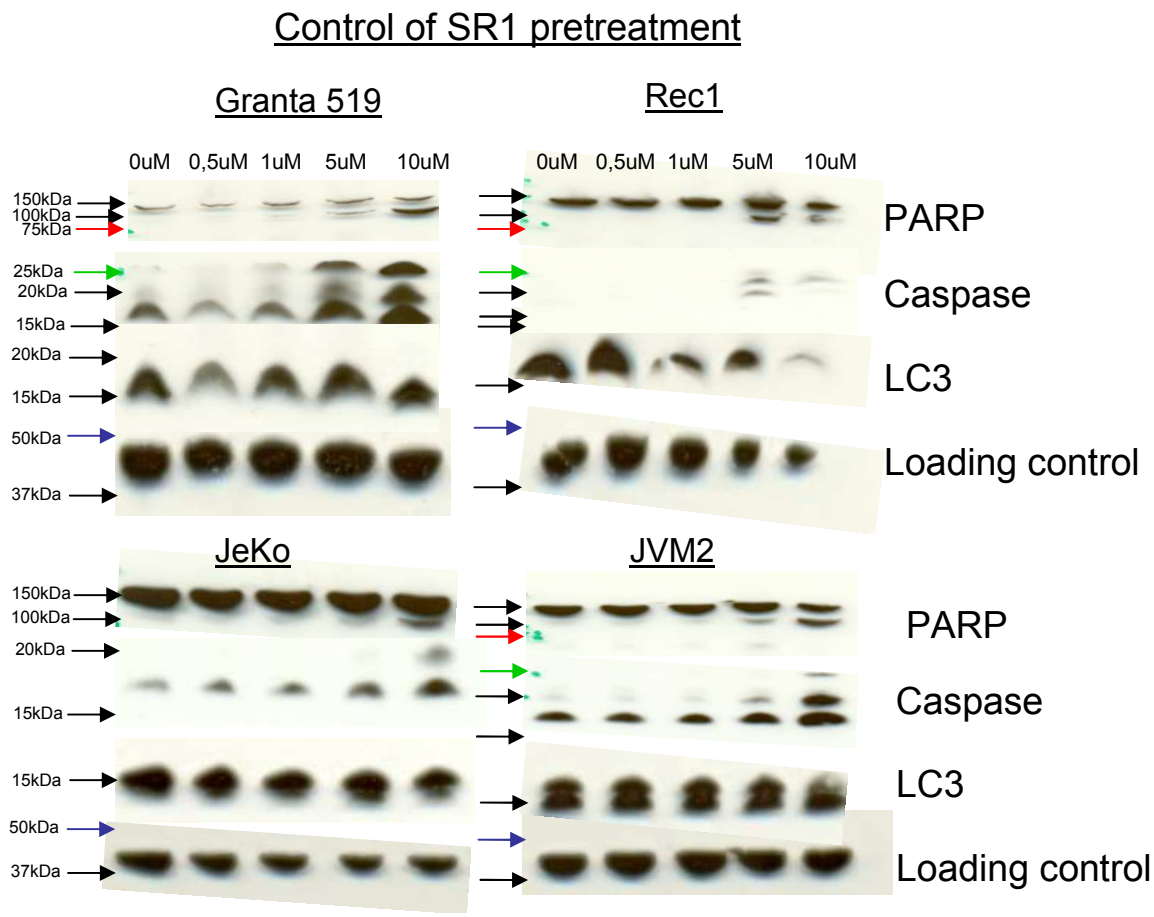


Figure 13. Control of SR1 pretreatment. *Blots shows PARP cleavage only in cell treated with 5µM and higher concentrations which proves that a concentration of 0.5µM does not induce any programmed cell death process.*

The blots show PARP cleavage and caspase activation in cells treated with 5µM or higher of SR141716. No PARP cleavage or caspase-3 activation could be seen in cells treated with lower concentration than 5µM.

8. Combined effect of CB1 and CB2 antagonists

Another cell viability assay was done using both SR141716 and SR144528 to see if they work in synergism or antagonism. The result of CB1 and CB2 combined effect is shown in figure 14 below.

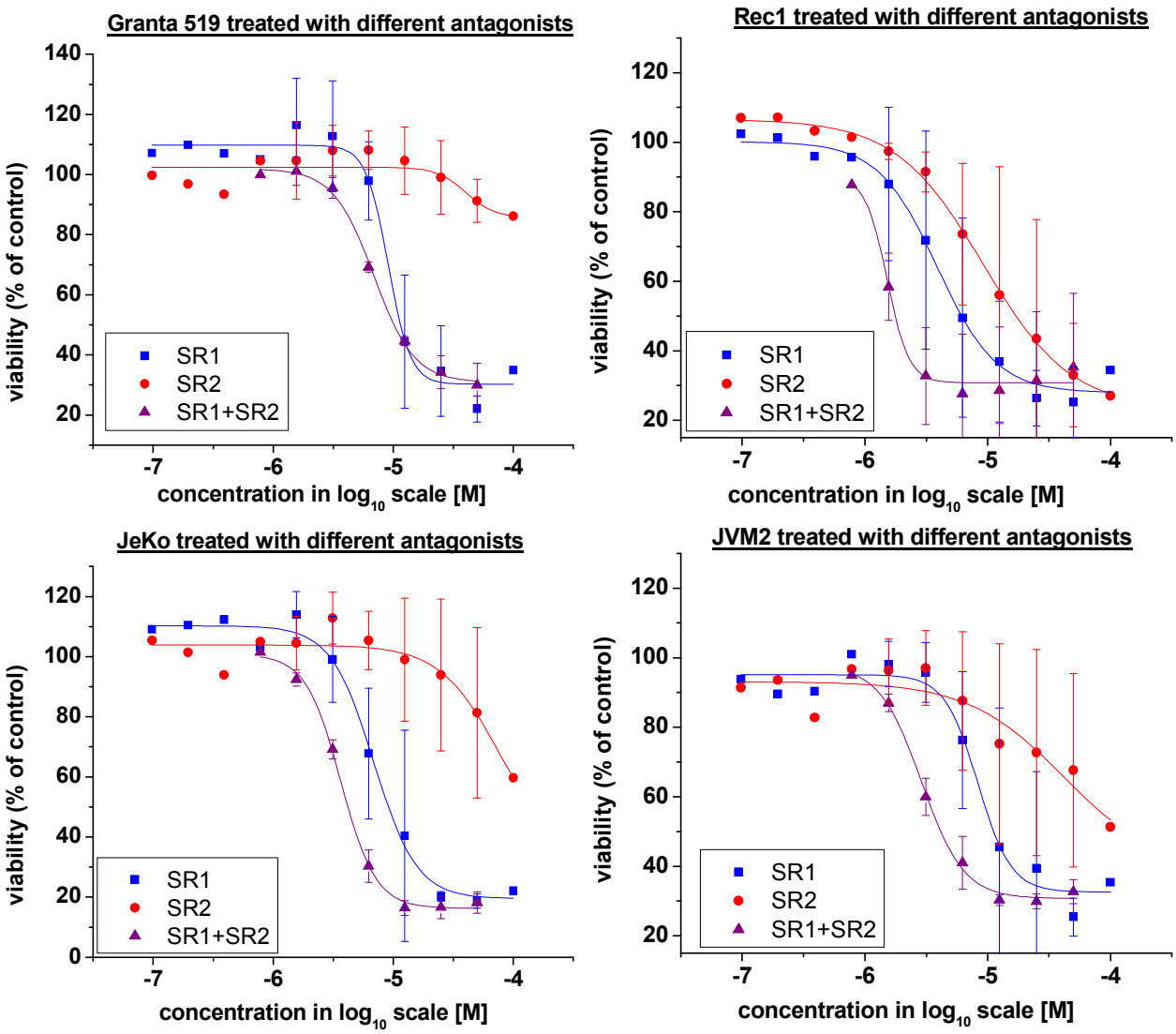


Figure 14. Graph of MCL cell lines titration with CB1/CB2 antagonists. *Y-axis represents cell viability and x-axis represents the concentration of antagonist in log_{10} scale [M].*

The antagonists seem to potentiate each other to different extents in different cell line. Potentiation is seen in all MCL cell lines but it is more pronounced in Rec1, JeKo and JVM2.

Discussion

The study was designed to investigate the possible effects of CB1 and CB2 antagonists/inverse agonists on MCL cell survival/cell death. For this, first the expression of CB1 and CB2 was investigated. The western blotting analysis of CB1 expression showed multiple bands between 37kDa and 75kDa. Human CB1 protein has an approximate size of 52kDa [25]. JVM2 have the lowest expression of the band at 50kDa that possibly represents the CB1 receptor. The bands at 75kDa could represent the glycosylated form of CB1 protein, since 3 potential glycosylation sites have been reported so far [25].

The western blotting analysis of CB2 expression in MCL cell lines were not conclusive. The size of the CB2 is 40kDa [26] and no bands are detected near the predicted size. The N18 antibody did detect the protein around the predicted size but there were multiple bands. This could be explained by the possible posttranslational modifications of the protein such as one possible glycosylation site and three posssible phosphorylation sites of the CB2 receptor [26]. However, the specificity of the antibody could also be questioned since bands were detected even within the ladder. Gustafsson *et al.* showed by quantitative PCR (qPCR) that the gene CNR2 encoding CB2 receptor is upregulated ~1.6-3.1 times in MCL compared to reactive tissue [27]. In future studies on CB2 protein expression in MCL cells, other, more specific, anti-CB2 antibody should ideally be used.

Using β-actin as loading control showed a repetitive pattern of unequal β-actin content between different MCL cell lines used in the study: JeKo had the lowest expression level, while JVM2 had the highest level of expression. This was confirmed by qPCR studies carried out within Birgitta Sander's research group (Xiao Wang, Stefan Almestrand, personal communication). The studies carried out by X. Wang and S. Almestrand indicate that JVM2 has the highest mRNA expression of β-actin and JeKo has the lowest mRNA expression which corresponds to the pattern from β-actin control. β-actin where therefore only used to compare loadings of same the cell lines.

Treatment with CB1 and CB2 antagonists decreased the mitochondrial activity in all MCL cell lines. SR141716 affected all of MCL cell lines similarly with EC_{50} ranges from 4μM to 9μM even though the CB1 expression levels seem to be lower in JVM2 compared to other MCL cell lines tested. Previous studies by Flygare *et al.* showed that 10μM, but not 1μM SR141716 decreases Rec1 cell viability after 2-4 days of treatment [28]. The study was done on cells cultured in 10% serum concentration while experiments in this study were done in 1% serum concentration. The effects was seen earlier in this study possibly due to the effect of lower serum concntrations. SR141716 has been shown to have affinity not only to CB1 but also to the GPR55 receptor [15]. That could be a possible explanation of the similar decrease seen in the cell lines even though they differ in CB1 expression. It cannot be excluded that there are yet other unknown receptors that has affinity to SR1. SR144528 decreases cell viability, measured by XTT assay, of Rec1 and JVM2 to a larger extent than it does for Granta519 or JeKo cells. The reason for this discrepancy remains unknown. One of the possible explanations would be different CB2 receptor level between these cells, however this could not be confirmed since approaches to estimate CB2 content in MCL cells by western blotting failed. The antagonistic effect of the peptide based cannabinoid hemopressin did not change the mitochondrial activity in the MCL cell lines when applied in a concentration range of 0.78μM-100 μM and when detecting with XTT. Possible explanation could be that hemopressin does not reach to the CB1 receptor. The cannabinoid receptor may not be on the cell surface but expressed intracellularily [29] so that peptides cannot get through the cell membrane as the lipophilic molecules such as SR141716 can. Another explanation could be that the effect seen with the other two antagonists SR141716 and SR144528 are effects

induced through another receptor that hemopressin does not have affinity to. A third possibility is that hemopressin induces other pathways than SR141716 does which does not affect the mitochondrial activity.

Treatment with the agonist AEA decreased cell viability in all of the MCL cell lines. Granta519 is least affected by AEA, whereas JeKo, Rec1 and JVM2 show a similar response. SR141716 caused cell death with similar EC50 values in all MCL cell lines tested, while SR144528 treatment of MCL resulted in the varied cell response between the cell lines. This suggests that the pathways leading to decreased cell viability by AEA, SR141716 or SR144528 are different since the pattern of the cell responses to different CB1/CB2 ligands is different.

Pretreatment with antagonists did not protect cells from AEA-induced cell death. Anandamide is either not killing cells through CB1/CB2 or another receptor is involved in the process. Future studies should involve possible expression of TVPR1 that has been reported to bind anandamide [19]. Other studies show that AEA can kill cancer cells *via* lipid rafts [30]. It would be interesting to verify in the future whether lipid rafts are involved in the observed process.

The combination of SR141716 and SR144528 potentiated each other. The potentiation was less in the Granta 519 cell line. The GPR55 expression study by western blot did not show consistent results. The size of the GPR55 receptor is estimated to be 37kDa and none of the antibodies used detected bands at predicted size. Probing with the antibody number 2 showed bands that were also found in the negative control, Jurkat and RAJI [14]. A possible explanation is that the antibodies used are not specific. Treatment with the GPR55 agonist LPI decreased the mitochondrial activity abruptly at 12.5μM-25μM which indicates nonreceptor specific effects. The effect seen after treatment with SR141716 is probably induced by another receptor than GPR55.

Detailed studies on the mode of action of SR141716 showed by XTT assay that cell death was apoptotic. SR141716 induces apoptosis after 5 hours when treating MCL cell lines with 10μM of the drug. This is confirmed with western blot where PARP cleavage and active caspase-3 were detected. Autophagy as detected by LC3 lipidation is also indicated to be involved in SR141716 treated MCL cell lines except in Rec1. The LC3 lipidation (LC3II) seen in cells treated with SR141716 does not necessary have to be an effect of the SR1141716 substance. Before setting up experiments cells were always starved overnight in 1% serum first, as this is the standard protocol when working with cannabinoids. The starvation could possibly activate autophagy. In Granta 519 there are indications of LC3 lipidation even in the vehicle treated and that could be induced by the starving effect. Recent studies also show that LC3 lipidation are found in Granta 519 cells that might be due to alternative to autophagy vacuoaltion processes (Agata Wasik, personal communication).

With this study the CB1 protein expression was found in the MCL cell lines but the CB2 protein expression could not able to be confirmed. Both treatment with agonists and antagonists decreased the cell viability. The decrease in cell viability with SR141716 is confirmed to be by apoptotic processes after 5 hours of treatment through another receptor than GPR55. The effect of the AEA is not is not through the CB1 or CB2 alone.

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