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1. Introduction

1.1. Cannabinoids

Cannabinoids are the unique active compounds of the marijuana plant *Cannabis sativa* L. (Velasco et al., 2012). Δ^9 -Tetrahydrocannabinidiol (Δ^9 -THC) was the first cannabinoid isolated by Gaoni and Mechoulam (1964) from *Cannabis* and is also the main psychotropic plant cannabinoid (Howlett et al., 2002). Other phytocannabinoids are (-)- Δ^8 -THC, (-)-trans- Δ^9 -Tetrahydrocannabinidiol (Δ^9 -THCV), cannabigerol, cannabinol and (-)-Cannabidiol (Pertwee, 2008), with cannabinol and (-)-Cannabidiol not exerting any psychotropic effect (Mackie & Stella, 2006). Today ~70 cannabinoids produced by *Cannabis sativa* L. are known (Velasco et al., 2012). Since cannabinoids are hydrophobic, they were believed to exert their psychotropic and physiologic effects via direct insertion through the cell membrane and into the cell (Guzmán, 2003). In the late eighties, however, the development and labelling of THC analogues led to the discovery of a specific ligand system. (Di Marzo et al., 2004). In 1990, the first cannabinoid receptor (CNR1 = Cannabinoid receptor 1) was cloned, followed three years later by the second specific receptor (CNR2 = Cannabinoid receptor 2) (Pertwee, 2008; Di Marzo et al., 2004). Cannabinoids binding as ligands to the two receptors can be, based on their structure and pharmacologic activity, classified into different groups (Howlett et al., 2002). Those cannabinoids that act as agonists on the cannabinoid receptors are assigned to four groups: The classical and the nonclassical cannabinoids, both deriving structurally from Δ^9 -THC (Howlett et al., 2002), the aminoalkylindoles, and the eicosanoids (Howlett et al., 2002). The classical cannabinoids refer to the natural phytocannabinoids and their synthetic analogues (e.g. HU210) (Howlett et al., 2002). The nonclassical cannabinoids differ from the classical cannabinoids by lacking the dihydropyran ring. The aminoalkylindoles differ structurally from but have pharmacological properties similar to THC. The well known (R)-(+)-WIN55212 belongs to this group (Howlett et al., 2002). The fourth group of ligands acting as agonists are the eicosanoids. All endogenous cannabinoid receptor agonists, called endocannabinoids, belong to this group, of which anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the most intensely investigated ones (Howlett et al., 2002). The psychotropic effects induced by Δ^9 -THC are achieved by mimicking endocannabinoids (Guzmán, 2003).

In addition to receptor agonists, which stimulate cellular response (Mackie, 2008) there are cannabinoids that act as antagonists or inverse agonists on the cannabinoid receptors, like for instance diarylpyrazoles (Howlett et al., 2002). The binding of an antagonist prevents agonists from receptor binding by interacting either with the active or the inactive receptor,

the inverse agonist binds to the inactive state of the receptor and leads to a decrease in basal receptor signalling (Mackie, 2008).

1.2. Cannabinoid receptors

The cannabinoid receptors received their name since they respond to cannabinoid drugs (Howlett et al., 2002). The two receptors are seven-transmembrane $G_{i/o}$ protein coupled receptors (Mackie and Stella, 2006). Whereas both have a high affinity for G_i , CNR1 has a higher affinity for G_o than CNR2 (McAllister and Glass, 2002). In addition, data exist indicating that CNR1 also binds to G_s (Demuth and Molleman, 2006). Both receptors show 68% amino sequence homology within the transmembrane domain and 44% throughout the whole protein (Jean-Gilles et al., 2010). The amino acid (AA) length is 472AA for CNR1 and 360AA for CNR2 (Howlett et al., 2002). Thus, the predicted size for CNR1 is 53kDa (Daaka et al., 1996) and 38kDa for CNR2 (Small-Howard et al., 2005).

CNR1 is mainly expressed in the brain but also in peripheral nerve endings and extra neural sites, for example the spleen (Guzmán, 2003). In addition leukocytes were reported to express CNR1 (Mackie and Stella, 2006). CNR2 is mainly expressed in leucocytes. It is also called “peripheral” cannabinoid receptor due its abundance in thymus, tonsils, bone marrow and spleen and even retina (Croxford and Yamamura, 2005). Within peripheral blood mononuclear cells (PBMC), B lymphocytes show the highest CNR2 expression, the rank order being B lymphocytes, natural killer cells, monocytes/macrophages, neutrophils, CD8+ T lymphocytes, and CD4+ T lymphocytes (Mackie and Stella, 2006).

Besides the two cannabinoid receptors, additional receptors were described to bind endocannabinoids. These include the transient receptor potential cation channel subfamily V member 1 (TRPV1), orphan G-protein coupled receptor (GPR) 55, GPR119 and GPR18 (Velasco et al., 2012). TRPV1 is normally activated by heat or capsaicin, which is the active component of chilli peppers. The endogenous cannabinoid anandamide also was shown to bind to this receptor (Demuth and Molleman, 2006). Beside these receptors, cannabinoids also influence ligand-gated ion channels like the Serotonin (5-HT)₃ receptor and Nicotinic acetylcholine receptors (nAChRs) (Demuth and Molleman, 2006).

1.3. The endocannabinoid system

Together with the endocannabinoid ligands, e.g. AEA and 2-AG, the two cannabinoid receptors constitute the endocannabinoid system (Guzmán, 2003). The first discovered endocannabinoid, AEA, is produced on demand by enzymatic cleavage of membrane lipid

precursors (Di Marzo et al., 2004) and inactivated by fatty acid amide hydrolase (FAAH) (Mackie and Stella, 2006). 2-AG is synthesized via phospholipase C (PLC) and diacylglycerol lipase (DGL) pathway and inactivated by monoacylglycerol lipase (MGL) (Mackie and Stella, 2006).

Three additional endocannabinoids are proposed today: 2-arachidonyl-glycerol ether (2-AGE), *O*-arachidonoyl-ethanolamine (virodamine) and *N*-arachidonoyl-dopamine (NADA) (De Petrocellis et al., 2004). All seem to be synthesized by different pathways, thus endocannabinoids constitute diverse compounds with different sites of action.

One of the roles of the endocannabinoid system is modulation of neurotransmitter release (McAllister and Glass, 2002). In this case, endocannabinoids bind to CNR1, which is located on a presynaptic neuron and coupled to Ca^{2+} and K^{+} channels. This leads to depolarisation of the neuron membrane and inhibition of further neurotransmitter release (Guzmán, 2003). By this, endocannabinoids control physiological functions like movement, learning, cognition, appetite and emesis (Croxford and Yamamura, 2005) and seem to play a protective role by antagonizing central nervous system (CNS) excitotoxicity and neuroinflammation (Jean-Gilles et al., 2010). In addition, the endocannabinoid system controls pain and functions in immune system modulation (Croxford and Yamamura, 2005). Regarding immune modulation, the injection of THC into mice resulted in suppression of cell-mediated immunity T helper cell 1 and an increase of T helper cell 2 answer (Klein et al., 2003).

Furthermore, endocannabinoids might play an important role in spermatogenesis, since a special isoform of CNR2 is predominantly expressed in human testis (Liu et al., 2009).

1.4. Cannabinoid signalling

Binding of cannabinoids to the cannabinoid receptors leads to signal transduction via the $\text{G}_{i/o}$ pathway (Demuth and Molleman, 2006), resulting in inhibition of adenylyl cyclase and therefore in a decrease of cAMP (Demuth and Molleman, 2006). Without the binding of cAMP to the regulatory subunits of protein kinase-A (PKA), the catalytic subunits of this holoenzym stay inactive (Almeida and Stratakis, 2011). The decrease in PKA activity seems to have stimulatory effect on the mitogen-activated protein kinase (MAPK) pathway (Demuth and Molleman, 2006). This downstream signalling by activation of MAPK includes extracellular signal regulated kinase (ERK), p38 and c-jun-N-terminal kinase (JNK) (McAllister and Glass, 2002). The activation of the MAPK signalling by CNR appears to occur by activation of PI3K-Akt pathway (McAllister and Glass, 2002). Activation of phosphatidylinositol 3-kinase (PI3K) phosphorylates and thereby activates the MAP kinase kinase (RAF), which in turn activates MAP kinase kinase (MEK), leading to activation

of MAP kinase and finally phosphorylation of cytoplasmic or nuclear proteins (Demuth and Molleman, 2006). Also, sphingomyelin hydrolysis and the release of ceramide after activation of RAF have been reported (Demuth and Molleman, 2006).

Under certain conditions, CNR1 activation can lead to activation of G_s instead of $G_{i/o}$ (McAllister and Glass, 2002). The activation of G_s results in the stimulation of adenylyl cyclase, this leads to the production of cAMP, which acts as a second messenger in the cell (Hein et al., 2006). In addition, CNR1 and CNR2 signalling influence intracellular calcium concentrations. While CNR1 was reported to interact with ion channels (Demuth and Molleman, 2006), CNR2 apparently does so poorly (McAllister and Glass, 2002). Furthermore, cannabinoid receptor ligation activates PLC, which leads to the generation of diacylglycerol and inositol triphosphatase thus increasing calcium levels and resulting in the activation of protein kinase C (PKC). Besides the activation of transcription factors of the MAPK family, NF- κ B might be activated and nitric-oxide (NO) levels increased by activation of the cannabinoid receptor (Klein et al., 2003).

1.5. Clinical application of cannabinoids

The plant-derived cannabinoid Δ^9 -THC was introduced into the clinics in 1988 as dronabinol (Marinol) followed by one of its synthetic analogues, nabilone (Cesamet) (Pertwee, 2008; Di Marzo et al., 2004). Nabilone is used for the suppression of nausea and vomiting during chemotherapy, Marinol for stimulation of appetite in AIDS patients (Pertwee, 2008). Furthermore, nabilone and THC showed promising results in the alleviation of tics in Tourette syndrome (Di Marzo et al., 2004). A cannabis extract, nabiximols (Sativex), has been approved in Canada and is tested in a Phase III clinical study against cancer-associated pain (Velasco et al., 2012). In addition, Sativex is available for the relief of central neuropathic pain and spasticity associated with multiple sclerosis (Karst et al., 2010).

Also, (-)-Cannabidiol is a potential candidate for treatment of rheumatoid arthritis, since it was shown that this phytocannabinoid could suppress progression in collagen-induced arthritis in an animal model (Malfait et al., 2000). Rimonabant (SR141716A), a CNR1 antagonist and inverse agonist, was used in treating obese persons and was also successful in doubling the abstinence rate in smokers (Di Marzo et al., 2004). Dexanabilon (HU-2111), a synthetic cannabinoid, is tested as neuro protective compound in cases of severe head injury (Di Marzo et al., 2004).

1.6. Role of cannabinoids in cancer

Cannabinoids have been reported to block angiogenesis, invasion and metastasis, inhibit cancer cell proliferation and induce cancer cell death by apoptosis (Velasco et al., 2012; Guzmán, 2003).

Angiogenesis is inhibited by blocking the activation of the vascular endothelial growth factor (VEGF) pathway (Velasco et al., 2012). By acting on matrix metalloproteinase 2 (MMP2) and its inhibitor, tissue inhibitor of matrix metalloproteinases 1 (TIMP1), cannabinoids inhibit adhesion, migration and therefore invasiveness of cancer (Velasco et al., 2012). Cell death is induced by *de novo* synthesis of ceramide which induces endoplasmatic reticulum (ER) stress leading to Akt inhibition of autophagy and intrinsic mitochondrial apoptosis (Velasco et al., 2012). Inhibition of Akt also leads to the activation of p27 and p21 resulting, via cyclin-dependent kinase (CDK) and retinoblastoma (RB), in cell cycle arrest and thus in apoptosis (Velasco et al., 2012). Through activation of the MAPK pathway, JNK induces apoptosis and p38 anti-proliferative effects (McAllister and Glass, 2002). Under normal conditions, activation of ERK leads to cell proliferation; however, prolonged ERK activation induced by cannabinoids mediates cell cycle arrest and cell death (Guzmán, 2003).

Thus, tumour cell proliferation decreased in melanoma cells by the cannabinoids WIN-55,212-2 and JWH133 *in vivo*. Furthermore, incubation with THC or WIN-55,212-2 resulted in a dose dependent anti-proliferative effect on melanoma cells but not on a non-tumorigenic line of melanocytes (Blázquez et al., 2006). It was shown in melanoma cells that inhibition of Akt contributes to the anti-proliferative effect of cannabinoids, whereas ERK, JNK, and p38 were not significantly involved (Blázquez et al., 2006).

In a human prostate cancer cell line (PC-3), R-(+)-Methanandamide was reported to exert an anti-proliferative effect and to induce dose dependent decrease in cell viability (Olea-Herrero et al., 2009). These effects were induced by *de novo* synthesis of ceramide, increase of JNK phosphorylation, and decrease in Akt phosphorylation (Olea-Herrero et al., 2009). The incubation of acute lymphoblastic leukemia (ALL) primary cells with THC at a concentration of 5µM resulted in a significant reduction of viability (McKallip et al., 2002). Cell lines of mantle cell lymphoma (MCL) and the chronic lymphocytic leukemia cell lines MEC-1 and MEC-2 showed apoptosis and cell death after incubation with R-(+)-Methanandamide (Gustafsson et al., 2008). This effect was abrogated by treatment with the CNR1 and CNR2 antagonists SR141716 and SR144528 (Gustafsson et al., 2008). Besides the cytotoxic effect of cannabinoids, it was found that the CNR1 and CNR2 are higher expressed in the majority of non-Hodgkins lymphomas than in control tissues (Gustafsson et al., 2008).

1.7. Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is a monoclonal proliferation of B cells (Hallek et al., 2008) belonging to the non-Hodgkin lymphomas (Müller-Hermelink et al., 2008) and the most prevalent leukemia of adults in Western countries (Müller-Hermelink et al., 2008).

Incidence rate is 2-6 cases per 100.000 persons per year, increasing with age (Müller-Hermelink et al., 2008). At the mean age at diagnosis, 65, the incidence rate reaches 12,8/100.000 with males being more frequently affected than females (ratio of 1,5-2:1) (Müller-Hermelink et al., 2008). Concerning the inheritability of CLL, it has the highest genetic predisposition of all hematologic neoplasias (Müller-Hermelink et al., 2008). The overall risk is 2-7 times increased in first degree relatives of CLL patients (Müller-Hermelink et al., 2008). CLL is diagnosed when there is an increase in clonal B cells with at least 5×10^9 B lymphocytes per litre peripheral blood (Hallek et al., 2008). Further clinical parameters are: Lactate dehydrogenase (LDH) elevation, bone marrow (BM) infiltration, lymphocyte doubling time (LDT), CD23, β_2 -microglobulin and thymidine kinase (TK) (Mozaheb et al., 2012).

Symptoms of this disease are fatigue, enlargement of spleen and liver, lymphadenopathy, infections, and autoimmune haemolytic anaemia (Müller-Hermelink et al., 2008).

For clinical staging two systems are established. In North America, mainly the Rai classification system is used (Sagatys and Zhang, 2012). It includes five stages with lymphocytosis, anemia and thrombocytopenia as parameters (Sagatys and Zhang, 2012). In Europe, the Binet staging system is more common (Sagatys and Zhang, 2012). This system distinguishes three stages with haemoglobin level, platelet count and number of involved areas as discriminators (Sagatys and Zhang, 2012). The clinical course of the disease is heterogeneous (Malavasi et al., 2011). Of unclear reasons, patients of the same stage progress differently (Mozaheb et al., 2012). To better discriminate between cases and for better stratification of patients, more refined prognostic markers are needed.

A number of prognostic markers have been established so far. An important marker is the mutation status of the immunoglobulin heavy chain (IGH) V gene for which an unmutated status predicts a worse disease course (Müller-Hermelink et al., 2008). In more than 80% of all CLL cases cytogenetic lesion can be found (Hallek et al., 2008). The most common lesions are: deletion at 13q14.1, trisomy of chromosome 12, deletions at the long arms of chromosomes 11 or 6 and/or the short arm of chromosome 17 (Hallek et al., 2008). A worse prognosis is given for the chromosomal lesions del11q22-23, del17p and del6p (Müller-Hermelink et al., 2008). The alteration of the p53 gene is not only associated with a poor prognosis but also with resistance to treatment and is a consequence of the deletion on chromosome 17p (Sturm et al., 2003).

Furthermore the expression of zeta-chain associated protein kinase-70 (ZAP-70) and the CD38 surface protein convey worse prognosis (Müller-Hermelink et al., 2008).

CLL cells are small, mature lymphocytes with a narrow border of cytoplasm and a nucleus with partially aggregated chromatin (Hallek et al., 2008). On the surface, CLL cells co-express CD5 and B-cell surface antigens CD19, CD20 and CD23, while immunoglobulin (Ig), CD20, and CD79b show lower expression compared to normal B cells (Hallek et al., 2008). CLL cells accumulate in lymphoid organs, BM and peripheral blood (Granziero et al., 2001). Circulating CLL cells are long-lived and arrested in G0 early G1 phase of cell cycle (Granziero et al., 2001), but CLL is not a static disease (Messmer et al., 2005). Deuterium, a nonradioactive isotope that labels newly synthesized DNA of dividing cells, was administered to nineteen CLL patients (Messmer et al., 2005). It could be shown that CLL has slow but steady dynamics, with a birth rate of leukemic cells in CLL patients between 0,1% to over 1% of the clone per day (Messmer et al., 2005). As proliferation hotspots for CLL cells specific micro-environmental structures were reported (Damle et al., 2010): These structures (pseudofollicles or proliferation centres), mainly consisting of CLL cells, T cells, and follicular dendritic cells, are found in lymph nodes (LN) and BM (Damle et al., 2010).

CLL cells seem to be antigen experienced (Chiorazzi and Ferrarini, 2011) raising questions regarding the origin of CLL cells. One hypothesis postulates a two cell origin for mutated and unmutated IGHV CLL cells, respectively (Chiorazzi and Ferrarini, 2011). In this model Ig unmutated CLL cells derive from pre-germinal center B cells and Ig mutated CLL cells derive from cells that passed the germinal center (GC) (Rosenwald et al., 2001). According to another model, both M and UM CLL cells derive from marginal zone B cells (Chiorazzi and Ferrarini, 2011). In a study using Lymphochip cDNA microarrays it could be shown that B cell activation genes are differently expressed between Ig mutated and Ig unmated CLL cells, although both subgroups share a characteristic gene expression signature (Rosenwald et al., 2001), thus favouring the one cell of origin theory. Comparing gene expression profiles of CLL cells with normal B cell subpopulations, CLL cells appeared to be more closely related to memory B cells (post GC) than to pre-GC B cells, CD5+ B cells, germinal centre centroblasts and centrocytes (Klein et al., 2001). Still, CLL cells differ from memory B cells in the expression of genes encoding for functions in proliferation, adhesion, cytokinesis and apoptosis (Klein et al., 2001). Due to the accumulation of the CLL cells, patients suffer from disease-specific complications. T cells in CLL patients show a frequent oligoclonality of CD4+ and CD8+ T lymphocytes with an impaired function (Dearden, 2008). Neutrophils and monocytes are also defective in their function (Dearden, 2008). About 70% of the patients develop hypogammaglobulinemia within seven years of diagnosis. Thus, most CLL patients show a reduction in Ig level, correlated to the frequency and severity of bacterial infections (Dearden, 2008). In addition, 10% to 25% of the patients develop autoimmune complications

with autoimmune haemolytic anemia (AHA) as the most common form of autoimmune disorder (Dearden, 2008). CLL patients with early-stage disease are often asymptomatic and therefore monitored without therapy (Hallek et al., 2008). For AHA therapy, prednisolone is used (Dearden, 2008). In case of progression, chlorambucil alone or in combination with steroids was standard therapy (Reynolds et al., 2012). Today it is still administered to elderly patients as front-line therapy (Lu and Wang, 2012) since this regimen is better suited for patients with comorbidities. Although in trials fludarabine yielded a higher response rate compared to chlorambucil as initial treatment (Reynolds et al., 2012) for proper impact of fludarabine an intact p53 gene is necessary (Sturm et al., 2003). Other used chemotherapeutics are cladribine, cyclophosphamide, mitoxantrone, and bendamustine (Lu and Wang, 2012). In addition to chemotherapy some monoclonal antibodies are available, which are often used in combination with chemotherapy (Lu and Wang, 2012). Rituximab was the first approved antibody for treatment of cancer targeting CD20 (Lu and Wang, 2012). Alemtuzumab targets the CD52 cell-surface antigen, and ofatumumab (anti-CD20) has been approved for CLL patients refractory to fludarabine and alemtuzumab (Lu and Wang, 2012). Furthermore, new compounds, such as lenalidomide, high-dose methylprednisolone, cyclin-dependent kinase inhibitors and several kinase inhibitors of B cell receptor signalling pathways are being tested (Lu and Wang, 2012). Standard chemoimmunotherapy is today the combination of fludarabine, cyclophosphamide and rituximab (Foon and Hallek, 2010). However, when patients that do not response to chemotherapy, allogeneic hematopoietic stem cell transplantation is the only therapeutic option left (Hallek et al., 2008).

1.8. Cell-cell interaction and microenvironment in CLL

The microenvironment is a compilation of accessory cells that interacts with malignant cells and promotes tumour growth and drug resistance (Burger et al., 2009). Especially in CLL the microenvironment seems to protect cancer cells from spontaneous and drug-induced apoptosis (Burger et al., 2009). This protection relies on the secretion of chemokines by stromal cells and the expression of adhesion molecules (Burger et al., 2009), but also B cell receptor (BCR) stimulation by stroma-derived antigens contribute to the protective effect (Binder et al., 2010). The stroma derives from so called mesenchymal stromal cells (MSCs) (Seke Etet et al., 2012). These are adult multipotent non-hematopoietic stem cell precursors which support the maintenance and engraftment of hematopoietic stem cells (Seke Etet et al., 2012). Besides MSCs, nurse like cells (NLCs) are found in the spleen and lymphoid tissue of patients (Burger et al., 2009). It was shown that these NLCs differentiate from blood monocytes when cultured together with CLL cells (Burger et al., 2009). *In vitro*, CLL cells undergo spontaneous apoptosis when kept in culture alone, however, coculturing these cells

with feeder cells, stromal cells, NLC or similar, prolongs cell survival (Kurtova et al., 2009). This prosurvival effect includes different factors. Thus, MSC express Notch receptor plus ligands and Notch stimulation had a pro-survival impact on CLL (Seke Etet et al., 2012). In addition the Wnt signalling (Seke Etet et al., 2012) and the stromal produced hedgehog proteins exert a survival effect in CLL (Hegde et al., 2008). Concerning soluble factors, CLL cells can express receptors for pro-angiogenic factors as the vascular endothelial growth factor receptor (VEGFR) 1 and 2, the signalling through these receptors prolongs cell survival (Deaglio and Malavasi, 2009). In receptor ligand pairing, CD40L/CD40 and CD38/CD31 interaction contribute to survival (Deaglio and Malavasi, 2009). Also chemokines like CXCL13, CCL19 and CCL21 exert a survival supporting function by controlling migration (Deaglio and Malavasi, 2009). Furthermore, the interaction between CLL cells and their microenvironment is not only in one direction. CLL cells can secrete angiogenic cytokines and thus modulate the local microenvironment (Lanasa, 2010).

From among all those factors supporting CLL cells, most attention was given to the chemokine CXCL12, also called SDF-1 (stromal derived factor-1) (Burger and Kipps, 2006). CXCL12 is a highly conserved chemokine supporting close cell to cell contact between B cell precursors and the protective stroma (Burger and Kipps, 2006). CXCL12 attracts naive and memory, but not germinal centre B cells (Bleul et al., 1998). CXCL12 is secreted by stromal cells and NLCs (Burger and Kipps, 2006) so that CLL cells are attracted to niches where these feeder cells provide them with pro-survival stimuli (Burger et al., 2009). This chemokine signals through the chemokine receptor CXCR4 (Burger and Kipps, 2006). Like all chemokine receptors, CXCR4 belongs to the 7-transmembrane G protein coupled receptor (GPCR) and is highly expressed in CLL (Burger and Kipps, 2006). It is also the most intensely studied chemokine receptor in CLL. This is due to that antagonizing the CXCR4/CXCL12 axis with a specific CXCR4 specific antagonist, AMD3100 (plerixafor) was shown to sensitize CLL cells to drug-induced apoptosis in coculture (Stamatopoulos et al., 2011). AMD3100 is a bicyclam molecule that selectively and reversibly antagonizes the binding of CXCL12 to its receptor CXCR4 (Pusic and DiPersio, 2010). This compound is already approved for hematopoietic stem and progenitor cell mobilisation in patients with non-Hodgkin's lymphoma (NHL) and there are studies that use AMD3100 as therapy to stimulate re-vascularization after acute tissue injury (Pusic and DiPersio, 2010). Concerning CLL, AMD1300 is tested as sensitizing agent in patients treated with rituximab (Pusic and DiPersio, 2010).

1.9. Cannabinoids and the microenvironment

Concerning the function of cannabinoids in microenvironment it could be shown that they influence chemotaxis in different cells. Thus, BM cells from mice migrate toward 2-AG in a dose dependent manner (Patinkin et al., 2008). Furthermore, with CNR1 stable transfected human embryonic kidney 293 cells migrate toward the cannabinoid agonists HU-210, WIN55212-2 and anandamide (Song and Zhong, 2000) and splenic mouse lymphocytes migrate toward 2-AG, which could be inhibited by SR144528 (Tanikawa et al., 2007). Also B cell trafficking seems to be influenced by cannabinoids. The cannabinoid receptor agonist WIN55-212,2 induces migration of splenic lymphocytes and inhibits CXCL12 induced migration in B lymphocytes (Tanikawa et al., 2011).

Cannabinoids do not only induce migration. In macrophages, treatment with THC results in the inhibition of migration of murine peritoneal macrophages to CCL5 *in vivo* and *in vitro*. This effect was reversed by the CNR2 antagonist SR141716A (Raborn et al., 2008). CXCL12 induced chemotaxis of Jurkat cells (T cell line) and primary CD4⁺ as well as CD8⁺ T lymphocytes was inhibited by incubation with an CNR2 agonist (Ghosh et al., 2006). A similar result was also found by Coopman et al., where JWH133 and 2-AG inhibited CXCL12 induced chemotaxis of activated T lymphocytes (Coopman et al., 2007). However, resting and activated T lymphocytes did not migrate towards both cannabinoids. (Coopman et al., 2007). In the case of breast cancer a CNR2 agonist inhibited the migration of the MCF7 cells and the downstream signalling of the CXCR4 *in vivo* in a breast cancer mouse model (Nasser et al., 2011). The influence of cannabinoids on CLL migration still needs to be investigated.

2. Aim of the study

Cannabinoids were shown to exert pro-apoptotic and anti-proliferative effects in solid tumours and in NHL by binding to the CNR1 and CNR2 receptor. Furthermore, various studies reported that cannabinoids inhibit the migration toward CXCL12 in T lymphocytes and cell lines by interfering with the CXCL12/CXCR4 axis. This axis is described to be a responsible factor in microenvironment induced CLL treatment resistance.

Therefore the aims of the study were:

- To evaluate retrospectively the mRNA expression pattern of CNR1 and CNR2 within CLL patients.
- To investigate the potential of the cannabinoid receptor expression as an additional prognostic maker.
- To test whether protein expression levels of CNR1 and CNR2 follow the mRNA expression.
- To evaluate the cytotoxic effect of CNR1 and CNR2 specific agonists and antagonists on CLL specific and other cancer cell lines and to see whether mRNA receptor expression correlates with sensitivity towards the compounds.
- To test the cytotoxic effect of cannabinoids on primary CLL cells incubated in suspension and in coculture with the mouse fibroblast line M2-10B4 with the compounds and to see whether mRNA receptor expression correlates with sensitivity.
- Compare the sensitivity of primary CLL cells with peripheral blood mononuclear cells (PBMC) from healthy donors (HD) to evaluate the potential of these cannabinoids as therapeutic substances.
- To test the impact of cannabinoids on migration of CLL cells toward CXCL12 and compare their impact with a CXCR4 inhibitor.

2.1. Cannabinoids used in this study

The following compounds were used as agonists, antagonist and inverse agonist in the experiments of this study:

(R)-(+)-Methanandamide is an analogue of the endocannabinoid AEA and a selective agonist for CNR1 (Pertwee, 2006). It was synthesized in 1994, its affinity for CNR1 is lower than that of its natural counterpart AEA but it has a stronger cannabimimetic effect and shows a higher stability to aminopeptidase hydrolysis than AEA (Abadji et al., 1994).

Another anandamide analogue that was used is ACEA. This compound was synthesized 1999 by Hillard et al. and was characterized as a high affinity agonist for CNR1 and only low affinity for CNR2 (Hillard et al., 1999). Its high affinity for CNR1 was shown in a bioassay where rat cerebellar membranes (expressing CNR1) were incubated with radiolabelled [³H]CP55940 that saturably bind to CNR1 and ACEA competed for this [³H]CP55940 binding (Hillard et al., 1999).

As CNR2 agonist, JWH133 was used. This is a Δ^9 -THC analogue, therefore a classical cannabinoid and a selective CNR2 agonist (Pertwee, 2006). Its effect on macrophage IL-12 production can be blocked by a CNR2 but not by a CNR1 antagonist (Correa et al., 2005).

In contrast to most other studies, this study also evaluated the cytotoxic effects of antagonists, for which AM251, AM630, and (-)-Cannabidiol were used.

AM251 is an analogue of SR141716A, which belongs to the diarylpyrazoles group of cannabinoids and is a selective CNR1 antagonist (Howlett et al., 2002; Lan et al., 1996).

As an antagonist of CNR2, AM630 was tested. Its antagonistic effect on cannabinoid receptor agonist was shown in isolated vas deferens of mice (Pertwee et al., 1995). Furthermore AM630 is also described to act as an inverse agonist on CNR2 and as a weak agonist on CNR1 (Ross et al., 1999). In addition it is also presumed to act as a weak inverse agonist on CNR1 (Landsman et al., 1998).

The last compound used in the study was (-)-Cannabidiol. Beside the Δ^9 -THC it is a plant derived compound without psychoactive effects (Guzmán, 2003). It can act as an inverse agonist on the CNR2, it antagonizes CNR1 agonists *in vitro* (Thomas et al., 2007) and it also acts as a weak agonist on the vanilloid receptor (Costa et al., 2004).

3. Methods

A list with all materials, chemicals inclusive manufactures name can be found in the appendix

3.1. Patients and healthy donors

Peripheral blood samples were collected from 108 consecutive patients diagnosed with CLL at the Division of Hematology and Hematology, General Hospital, Vienna. All patients and the four healthy volunteers included in the study signed informed consent according to the Declaration of Helsinki (Ethics Committee Nr: 1011/2012). Of the 108 patients, 102 were used for the investigation of mRNA expression levels. The additional 6 patients were used in FACS analysis, migration assays and in experiments using a microenvironmental model for drug treatment. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll separation; cells were stored viable in liquid nitrogen. For protein analyses, 1×10^6 PBMCs were centrifuged 5 minutes 5000rpm at 4°C, supernatant was discarded and cell pellets were stored at -80°C. For RNA extraction, primary cells were stored at -80°C in TRIzol according to the manual. B lymphocytes from healthy donors were isolated from the PBMCs using the EasySep™ Human B Cell Enrichment Kit without CD43 Depletion following the manufacturer's protocol.

3.2. Cell lines cell culture

The cell lines MEC-1, MEC-2, JURKAT, A-549, and M2-10B4 were cultured at a density of 5×10^5 – 1×10^6 cells/ml under standard conditions (95% humidity, 5% CO₂, 37°C). MEC-1, MEC-2, JURKAT and M2-10B4 were kept in RPMI1640 whereas A-549 was cultured in DMEM, both media containing in addition 10% FCS and 1% Penicillin / Streptomycin. A-549 and M2-10B4 cells were propagated by removing the medium, rinsing with PBS, and incubation with 4x Trypsin-EDTA for three minutes at 37°C prior to dilution with complete medium.

All experiments were performed using the culture medium without phenol red.

3.3. Primary cells cell culture

Cells were thawed 24h before experiment. RPMI1640 + 20% FCS were warmed to 37°C in a 50ml Falcon. An aliquot of the warm medium was carefully added to the cells and cells were resuspended. Thawed cells were transferred to the 50ml Falcon with the rest of warm

medium. This step was repeated until all cells were thawed. Cells were centrifuged for 10min at 1000rpm at room temperature (RT). Supernatant was discarded and pellets resuspended in RPMI1640 + 20% FCS without phenol red, transferred to a small culture flask and incubated over night at 37°C under standard conditions. All experiments were performed using culture medium without phenol red. After 24h cells were transferred to a 50ml Falcon, centrifuged for 10minutes at 1000rpm at RT, pellets were dissolved in RPMI1640 without phenol red and cells counted using a counting chamber before the preparation of experiments.

Cell counting:

The cover glass was placed on the counting chamber. Cell suspension was diluted 1:1 with 0,4% Trypanblue. For primary cells, the cell suspension was additionally diluted 1:10 in RPMI1640 et al. The dilution was introduced into the counting chamber until the mirrored surface was covered. Cells in three of the outer four big squares were counted.

Cell count per ml was calculated by:

$$\text{Mean cell count} \times \text{dilution factor} \times 10.000 = \text{cells/ml}$$

3.4. RNA extraction

Cell lines: Cells from the cell culture were counted and 3×10^6 cells were transferred to 15ml Falcon tubes. After centrifugation for 10minutes at 1000rpm at RT, supernatants were discarded, and pellets were dissolved in 1ml TRIzol and transferred to a 2ml reaction tube.

CLL samples: Cell aliquots stored frozen in TRIzol were used (see chapter 3.1.).

Healthy donors: CD19 sorted cells from two male and two female samples were pooled, respectively, to obtain sufficient material for RNA extraction. Samples had been stored at -80°C in 1ml TRIzol (see chapter 3.1.).

Samples were incubated at RT for 5minutes. Then 200µl of chloroform was added and the tube was vortexed for 15seconds. After 3minutes incubation at RT, samples were centrifuged at 15 000rpm for 15minutes at 4°C. The aqueous phase (RNA Phase) was transferred to a safe lock reaction tube and 500µl of isopropanol was added for RNA precipitation. The samples were incubated at RT for 10minutes, followed by a centrifugation step of 15 000rpm for 10minutes at 4°C. The supernatant was removed, 1ml of 75% ethanol was added for washing, and samples were centrifuged at 12 000rpm 5minutes at 4°C. This washing step

was repeated. Ethanol was discarded and the pellets became air-dried. Finally, the pellet were dissolved in 10µl DEPC water and incubated at 55°C for 10minutes. The amount of isolated RNA and the A260/A280 ratio was measured using NanoDrop 8000 and the software NanoDrop 8000 V. 2.1.0. following the manufacturer's protocol. Until cDNA synthesis samples were stored at -80°C.

3.5. cDNA synthesis

Two µg of RNA was used for cDNA synthesis in maximal 5µl of reaction volume. Two Mastermixes were prepared in separate 2ml reaction tubes. All reagents were stored on ice.

Mastermix 1

For one sample:

0,5µl Oligo (dT) 15 Primer

10,5µl Aqua bidest

Mastermix 1 was vortexed, 11µl were transferred to 0,5ml reaction tubes. Two µg RNA was added and the tubes were incubated at 72°C for 5minutes.

Mastermix 2

For one sample:

5µl 5x Reaction Buffer

4,125µl Aqua bidest

1,25µl dNTPs (10mM)

0,625µl RNAsin Plus

1µl M-MLV Reverse transcriptase

Mastermix 2 was shortly mixed and 12µl were added to the samples. The samples were incubated for 1h at 42°C.

Until Real time PCR, the cDNA was stored at -20°C.

3.6. Real time PCR

Real time PCR was carried out using TaqMan® Gene Expression Assays on demand

CNR1: Hs00275634_m1 FAM reporter labelled

CNR2: Hs00361490_m1 FAM reporter labelled

β-actin Human ACTB (#4326315E) VIC reporter labelled

These assays contain primer and probe.

Real time PCR mix for one cDNA sample

11,4µl Aqua bidest

0,6µl primer assay

12,0µl TaqMan® Universal Master Mix

Reagents were mixed and 24µl were transferred to a semi-skirted 96-Well PCR Plate. 1µl of cDNA was added for a reaction volume of 25µl.

Samples were done in duplicates; two wells without cDNA (only mastermix) were used as negative control. After sealing the plate surface with a cover film, the plate was placed in the ABI Prism 7000 Sequence Detector. The instrument was used following manufacturer's instructions; runs were analyzed using the ABI Prism 7000 SDS Software.

Cycle protocol

1 cycle: 2 minutes 50°C

1 cycle: 10 minutes 95°C

40 cycles: 15 seconds 95°C and 1 minute 60°C

For calculation of mRNA expression, the $\Delta\Delta C_t$ -method was used (Livak and Schmittgen, 2001). β -actin was chosen as housekeeping gene. C_t values of β -actin were subtracted from the C_t values of CNR1 and CNR2 resulting in the ΔC_t value. The $\Delta\Delta C_t$ was obtained by subtracting the mean ΔC_t value of the two CD19 sorted healthy donor pools from the ΔC_t of the patients' samples. By logarithmic transformation using the formula $2^{\Delta\Delta C_t}$, the relative fold increase of CNR1 and CNR2 mRNA expression was calculated.

3.7. Protein isolation

A-549 and JURKAT cells were pelleted directly from cell culture using 5×10^6 and 10×10^6 cells, respectively, and adding 150µl RIPA Buffer complete. Patient samples containing 50×10^6 to 100×10^6 cells were mixed with 500µl to 1000µl RIPA Buffer complete, depending on pellet size.

RIPA Buffer complete

RIPA Buffer

NaCl (in MilliQ)	150mM
Tris-HCl pH7,4 (in MilliQ)	50mM
Na-deoxycholate (in MilliQ)	0,5%
EGTA (1M in NaOH)	2mM
EDTA pH7,4 (in MilliQ)	5mM
NaF (in MilliQ)	30mM
β -Glycerophosphate pH7,2 (in MilliQ)	40mM
Tetrasodium Pyrophosphate (in MilliQ)	10mM
Benzamidine (in MilliQ)	3mM
Nonidet P-40	1%

Adjusted to pH7,4 and filled up to 95ml with MilliQ water

RIPA Buffer complete

RIPA Buffer	1,9ml
200mM Na-Orthovanadate (in MilliQ)	20 μ l
25x Complete (Protease Inhibitor Cocktail Tablets in MilliQ)	80 μ l

After dissolving the pellets in freshly prepared RIPA Buffer complete, samples were vortexed for 10seconds and incubated on ice for 5minutes. These two steps were repeated followed by a centrifugation step at 12 500rpm at 4°C for 30minutes. Finally the supernatant was transferred to a 1,5ml reaction tube.

The protein concentration was determined by Pierce BCA Protein Assay Kit according to the manufacturer's instructions.

3.8. SDS PAGE

The Mini-PROTEAN Tetra Electrophoresis System was assembled according to the manual using 1mm spacer. Then, a 10% separation gel was filled into the gel sandwich and overlaid with Aqua bidest. After polymerization, a 4% stacking gel was prepared. The Aqua bidest was removed from the sandwich and the stacking added on top of the separation gel. Finally, a 10 well comb was added. When the stacking gel was polymerized the gel sandwiches were inserted into the electrophoresis chamber. One x running buffer was added to the chamber. Finally, the combs were carefully removed and the samples were added into the wells.

10% separation gel

MilliQ water	8ml
30% A/B Bis-Acrylamid	6,7ml
1,5M Tris-HCl pH 8,8	5ml
10% SDS	200 μ l
10% APS (in MilliQ)	100 μ l
Temed	10 μ l

3. Methods

4% stacking gel

MilliQ water	7,46ml
30% A/B Bis-Acrylamid	1,67ml
0,5M Tris-HCl pH6,8	3,15ml
10% SDS	125µl
10% APS (in MilliQ)	125µl
Temed	12,5µl

10x running buffer

Tris base	30,3g
Glycine	144,2g
SDS	10,0g
Add MilliQ water up to 1000ml	
Make a 1:10 dilution for 1x running buffer	

For the sample preparation three parts of the sample was mixed with one part of the 4x protein loading buffer.

4x Protein loading buffer

Tris-HCl pH 6,8	125mM
Glycerol	50%
SDS	4%
Orange G (w/v)	0,2%
Add MilliQ water up to 15ml	

Before adding the samples, the loading buffer was diluted 1:10 with β -mercaptoethanol.

Then the samples were incubated at 95°C for 5minutes. As controls, recombinant proteins for CNR1 and 2 were used which were not heat incubated before loading.

CNR1 (Human) Recombinant Protein [# H00001268-G01 ABNOVA]; MW: **52,9kDa**

CNR2 (Human) Recombinant Protein [# H00001269-G01 ABNOVA]; MW: **39,7kDa**

(see Datatsheets)

Sixty µg of the samples, 0,19µg of recombinant CNR1 and 0,1µg of recombinant CNR2 were loaded into the slots. Two µl of pEqGOLD Protein-Marker IV was added to the first slot. Two gels per chamber ran at 60V, 35mA for 20minutes and 50minutes at 130V and 60mA.

3.9. Western blot

For Wet-Western blot the Mini Trans-Blot® Electrophoresis Transfer Cell was used. Before assembly, transfer buffer was freshly prepared and stored at 4°C.

Transfer buffer

Glycine	11,2g
MilliQ water	745ml
1M Tris pH 8,3	50ml
10% SDS	5ml
Methanol	200ml

For each gel, four pieces of Gel Blot Paper and one piece of PVDF Immobilon-FL Transmembran (pore size 0,45µM) was cut to gel size. After SDS PAGE, the gels were put into transfer buffer for 10minutes. The PVDF membrane was activated for 15seconds in methanol and transferred to transfer buffer and incubated for 5minutes. After assembly, samples were blotted for 2hours at 40V and 500mA at 4°C.

Then, membranes were blocked over night in 5% non-fat dry milk in PBS at 4°C. Primary and secondary antibodies were diluted in PBS + 0,1% Tween20 with 10% NaN₃ (1:500).

Primary antibody

Anti-CNR1 polyclonal Antibody [Thermo Scientific #PA1-745] diluted 1:1000

As described in the Datasheet:

Host rabbit; Immunogen: Aminoacids 1-77;

Detects a Band at ~60kDa and lighter Bands at ~23kDa, ~72kDa, ~180kDa

Anti-CNR2 monoclonal Antibody [ABGENT #AF1575a] diluted 1:1500

As described in the Datasheet:

Host mouse Immunogen: Aminoacids 302-360;

Detects a Band at 39,7kDa

Loading control

Anti-GAPDH monoclonal Antibody [CALBIOCHEM #CB1001] diluted 1:8000

As described in the Datasheet:

Host mouse;

Detects Band at ~36kDa for monomeric unit

Membranes were incubated for 16hours at 4°C with the primary antibody. Then they were washed 3x for 5minutes with PBS + 0,1% Tween20. Subsequently, they were incubated 45minutes at RT in the dark with the secondary antibody.

Both secondary antibodies were diluted 1:17 5000

IRDye 680 Conjugated Goat Polyclonal Anti-Mouse IgG (H+L) [LI-COR #926-32220]

Used for anti-CNR2 and GAPDH primary antibody

IRDye 800CW Conjugated Goat Polyclonal Anti-Rabbit IgG (H+L) [LI-COR # 926-32211]

Used for anti-CNR1 antibody

The washing step was repeated and finally the membranes were rinsed with PBS and stored in PBS at 4°C.

3.10. Detection

For Detection of proteins on the membrane, the Odyssey Imager was used. This is a Near-infrared (NIR) fluorescence detection system detecting the fluorescence of the labelled secondary antibodies.

3.11. Viability Assays

Viability assays were performed by CellTiter-Blue® Cell Viability Assay. This is a fluorometric assay, in which the metabolic capacity of cells to reduce resazurin into fluorescent resorufin is measured. At first, cell number and incubation time with the CellTiter Blue reagent were adopted to each cell line and to primary cells following the manual. The viability assay was performed in triplicates in 96 well plates and at a volume of 100µl. Twenty µl of CellTiter Blue reagent was added and the plates were incubated at 37°C under standard conditions. Fluorescence was detected using TriStar at an excitation wave length of 570nm and an emission wave length of 600nm. Results were transferred to Excel 2003 and viability was calculated with medium=blank; and cells plus vehicle set as 100% viability. For creating graphs and calculating IC50 values the software GraphPad Prism5 was used.

3.11.1. Cell lines

Cells were transferred to a 50ml Falcon, centrifuged at 1200rpm for 10minutes at RT. Pellets were resuspended in RPMI1640 without phenol red, DMEM without phenol red was used for A-549. Cells were counted. All cell lines were tested at a density of 25×10^4 cells/ml, except A-549 which were tested at 10×10^4 cells/ml. Adherent cell lines, M2-10B4 and A-549, were seeded directly in 96 flat bottom well plates at a concentration of 25×10^4 cells/ml or 10×10^4 cells/ml and incubated 24h under standard conditions before compound or vehicle was added to the well. For cells in suspension culture (MEC-1, MEC-2, JURKAT), aliquots of

400µl were prepared in reaction tubes. Then, compounds or vehicle (1%) were added to the reaction tubes, using concentrations of 5 – 100µM. Tubes were shortly vortexed and 100µl in triplicates were transferred into 96 well plates.

Cells were incubated 24h and 48h with the compounds. Incubation time after 20µl CellTiterBlue reagent was added were 3h for MEC-1, MEC-2, A-549, and M2-10B4, and 4h for JURKAT cells. For each cell line the assay was done twice in triplicates.

3.11.2. CLL and healthy donors' primary cells

CLL cells were tested both in suspension and in coculture. Primary cells from three healthy donors were tested only in suspension culture.

Suspension culture

Experiments were carried out using 3×10^6 cells/ml in 96 well plates. Preparation was done as described for cell lines. Pre-diluted compounds were added, tubes were vortexed and 100µl were pipetted in 96well plates in triplicates. Plates were incubated 48h under standard conditions. CellTiter Blue reagent was added (20µl) and plates were incubated for 3h.

Coculture

For coculture experiments, 2×10^5 M210B4 cells per 630µl medium were seeded in 12 well plates and incubated 24h under standard conditions. On the next day, 3×10^6 CLL primary cells in 370µl medium were transferred to the wells (concentration 3×10^6 cell / ml). Finally the compounds were added and plates were incubated 48h under standard conditions. To determine viability, 100µl of CLL cells were removed from the 12 well plates and pipetted into 96 wells plates in triplicates. Next, 20µl of CellTiterBlue reagent was added and plates were incubated 3h.

3.11.3. Tested Cannabinoids

See 2.1. Cannabinoids used in this study

R-(+)-Methanandamide in EtOH as vehicle

ACEA in EtOH as vehicle

JWH133 in DMSO as vehicle

(-)-Cannabidiol in DMSO as vehicle

AM251 in DMSO as vehicle

AM630 in DMSO as vehicle

Vehicles in wells did not exceed 1%.

3.11.4. Microenvironmental model for drug treatment model

AMD3100 is an established CXCR4 antagonist. Aim was to test whether interfering with the CXCR4/CXCL12 axis makes CLL cells more susceptible to the chemotherapeutic fludarabine.

Furthermore, it was tested whether CNR1 and CNR2 agonists and antagonist exert an additive effect with fludarabine and to compare the impact of cannabinoids with AMD3100.

As described in 3.11.2. coculture, M2-10B4 were seeded 24h before the experiment in 12 well plates. Primary PBMC from a CLL patient were thawed and cultured for 24h at 37°C as previously described. Since the primary cells of a patient are limited, for the agonist and antagonist incubation only samples from two different patients were used.

As a blank two wells were filled up to 1ml volume and 10µl DMSO or Ethanol were added as vehicle. In the other wells 3×10^6 CLL cells/ml were added.

In the first well with M2-10B4/CLL coculture 5µM fludarabine was added. In the second well 0,629mM AMD3100 was pipetted and in a third well, cells were pre-treated 30minutes with 0,629mM AMD3100 and then 5µM fludarabine was added.

To test the effect of cannabinoids alone, three wells were incubated with JWH133 with different concentrations (10µM, 20µM, 40µM). To the next three wells ACEA (10µM, 20µM, 40µM) was added. To evaluate the effect of the antagonist, three wells were incubated with (-)-Cannabidiol (10µM), AM251 (30µM) and AM630 (30µM).

To test the additive effect of cannabinoids with fludarabine additional wells were pre-incubated 30minutes with JWH133 (10µM, 20µM, 40µM), ACEA (10µM, 20µM, 40µM), (-)-Cannabidiol (10µM), AM251 (30µM) and AM630 (30µM). Then 5µM fludarabine was added.

Finally, the plated cells were incubated 48h at 37°C under standard conditions. As control 3×10^6 CLL cells/ml were incubated with the vehicle. After incubation 100µl from the 12 well plate wells were transferred to a 96 well plate. CellTiterBlue viability assay was performed as described before.

3.12. Determining CXCR4 levels in CLL by FACS

Primary cells of a CLL patient were prepared as described in chapter 3.3. One million cells each were aliquoted into three FACS tube. Tubes were centrifuged at 2500rpm for 5minutes at 4°C. After discarding the supernatant, the pellets were vortexed and 200µl FACS buffer was added (FACS buffer = 0,1% BSA in PBS). The centrifugation step was repeated and 50µl FACS buffer were added to the pellets. The first FACS tube was used as a negative control, without staining.

The second FACS tube was used for CXCR4 staining. For this, 2,5µl of Mouse Anti-Human CD184 (CXCR4)-PE [BD Pharmigen #555974] were added and incubated for 20minutes in the dark. The third FACS tube was stained for isotype control using 2,5µl of Mouse IgG2a κ Isotype Ctrl [BioLegend # 401501] and incubated for 20minutes in the dark.

Next, 800µl FACS buffer were added to each tube, cells were centrifuged at 2500rpm 5minutes at 4°C, supernatant was discarded and pellets vortexed. Two hundred µl of FACS buffer were transferred to the negative control and the CXCR4 stained sample. Fifty µl FACS buffer were added to the isotype control for secondary antibody staining. The secondary antibody, goat anti-mouse IgG-Alexa Fluor488 [Invitrogen # A11001], was diluted 1:400 in PBS and 1µl was added to the FACS tube. After 20minutes of incubation in the dark, cells were washed as described previously. Finally 200µl FACS buffer were transferred into the tube and cells were resuspended. The FACS analysis was performed using FACScan [Becton Dickinson] and the software CellQuest Pro.

3.13. Migration assays

Migration assays were performed in 6,5mm diameter, 5,0µm pore size polycarbonate membrane transwell inserts in 24 well plate after pre-incubation with compounds.

Two migration assays were done with pre-incubation settings.

For pre-incubation primary cells of five CLL patients were cultured as described in chapter 3.3. and seeded into 10 wells of a 12 well plate with 5×10^6 cells/ml. Compounds were added into the wells 1-10 followed by incubation at 37°C.

Pre-incubation settings were as follows

1. Control well (no compound added)
2. 0,629mM AMD3100 0,5h incubation
3. 10µM ACEA 1h incubation
4. 0,1µM AM251 0,5h incubation followed by 10µM ACEA for 1h
5. 0,1µM AM251 0,5h incubation
6. 10µM JWH133 1h incubation
7. 0,1µM AM630 0,5h incubation followed by 10µM JWH133 for 1h
8. 0,1µM AM630 0,5h incubation
9. 0,1% DMSO 1h incubation
10. 0,1% EtOH 1h incubation

For migration, 600µl RPMI1640 without phenol red were pipetted into the bottom wells of the 24 well plates. CXCL12 (0,1µg/ml) or vehicle control (0,1% PBS with 0,1% BSA) was added to the medium. Then transwell inserts were added to the wells.

Next, 100µl pre-incubated cells were transferred to the inserts. Migration was allowed to occur for 4h at 37°C. Then, the contents of the transwell inserts and the bottom wells were transferred to separate reaction tubes. Tubes were centrifuged for 5minutes at 2500rpm, the pellets were resuspended in 200µl PBS, and cells were counted. Migration was calculated using the formula:

Cells in transwell + cells in bottom well = 100% of cells

Cells in bottom well = % of cells migrated

3.14. Statistical methods

CNR1/2 expressions are given as median, quartiles and range. The median was set as a cut-off level for *CNR* expression to determine *CNR* high and low expression groups. The impact of *CNR* mRNA expression on survival was illustrated by Kaplan-Meier plots. Progression free survival was calculated from first treatment to progression, overall survival was measured from date of first diagnosis to follow up and treatment free survival was calculated from date of first diagnose till first treatment or follow up. Prognostic markers were compared between groups using χ^2 -tests. As test for the independence of the prognostic marker a coxregression was performed. *P-values* were calculated by Logrank test. *P-values* $\leq 0,05$ were considered statistical significant. All computations were performed using SPSS version 20.

4. Results

4.1. Patient characteristics

Table 1 lists clinical characteristics of the patients included in the study. Of the 108 CLL patients, 102 CLL patients were used for the mRNA expression studies.

The median age at diagnosis was 62 years (range 25-85). There were slightly more male (59,8%) than female patients (40,2%). 83,8% of the patients had Binet stage A with a lymphoid doubling time <1 year in 23,7% of patients. The mutations status of the IGHV genes was unmutated in 45,6% of patients. The median expression for CD38 was 10% (range 0-91). Chromosomal aberrations were found in 74% of the patients.

Table 1: Clinical characteristics at diagnosis for CLL patients included in this study (N=102).
N: Number of patients; Del: Deletion; Tris: Trisomy; Rearr: Rearrangement.

Age at diagnosis (N=100) [years]	62 (25–85)
Median (Range)	
Sex Female : Male (N=102) [%]	40,2 : 59,8
Binet at diagnosis (N=99) [%]	
A	83,8
B	12,1
C	4,0
Mutational status (N=90) [%]	
Unmutated	45,6
Mutated	54,4
Lymphocyte doubling time (N=93) [%]	
High < 1year	23,7
Low > 1year	76,3
CD38 (N=93) [%]	
Low < 30	67,7
High > 30	32,3
Median (Range)	10 (0-91)
Del13q (N= 96) [%]	
Unmutated<5,0	47,9
Mutated>5,0	52,1
Tris12 (N=96) [%]	
Unmutated<3,7	88,5
Mutated>3,7	11,5
Del11q (N=96) [%]	
Unmutated<8,6	76,0
Mutated>8,6	24,0
p53 (N=96) [%]	
Unmutated<10,2	91,7
Mutated>10,2	8,3
Rearr14q (N=96) [%]	
Unmutated<3,0	88,5
Mutated>3,0	11,5

4.2. CNR1 mRNA expression

Median mRNA expression for CNR1 was 1,34 (range 0,00-140,39) in our cohort (Fig. 1A). Expression was normalized to four CD19 sorted healthy donor PBMCs. Using the median as a cut-off, patients were categorized into high and low expression groups (Fig. 1B). Median for low expression group was 0,20 (range 0,00-1,12) and for high expression group 5,94 (range 1,34-140,39) (Fig. 1B).

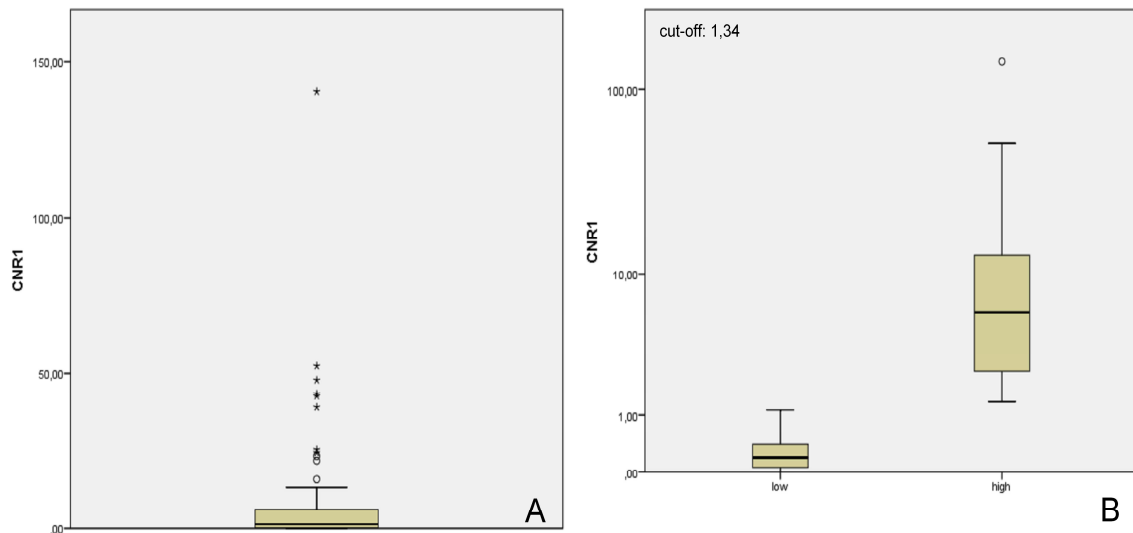


Figure 1: CNR1 mRNA expression in a cohort of 102 CLL patients.

A: Box plot CNR1 expression.

B: Box plot of CNR1 low/high expression groups (N=50 vs. 52).

Note logarithmic scale on y-axis.

After the CLL patients were categorized into high and low CNR1 expressing subgroups, prognostic markers were compared between the two groups. As Table 2 shows, almost equal numbers of patients fell into the CNR1 high and the CNR1 low expression group, respectively.

To test for correlation between expression and established prognostic markers a Chi²-test was done.

4. Results

Table 2: Patient characteristics for CNR1 high (N=52) and CNR1 low (N=50) expressing subgroups.

N: Number of patients; Del: Deletion; Tris: Trisomie; Rearr: Rearrangement.
P-values ≤ 0,05 significant.

CNR1 low	CNR1 low	CNR1 high	CNR1 high	p-value
Age at diagnosis (N=48) [years] Median (Range)	59 (25-85)	Age at diagnosis (N=52) [years] Median (Range)	63 (39-82)	
Sex Female : Male (N=50) [%]	44 : 56	Sex Female : Male (N=52) [%]	36,5 : 63,5	
Binet at diagnosis (N=47) [%] A B/C	91,5 8,5	Binet at diagnosis (N=52) [%] A B/C	76,9 23,1	0,049
Mutational status (N=45) [%] Unmutated Mutated	31,1 68,9	Mutational status (N=45) [%] Unmutated Mutated	60,0 40,0	0,006
Lymphocyte doubling time (N=46) [%] High < 1year Low > 1year	15,2 84,8	Lymphocyte doubling time (N=47) High < 1year Low > 1year	31,9 68,1	0,058
CD38 (N=46) [%] Low < 30 High > 30 Median (range)	78,3 21,7 4 (0-91)	CD38 (N=47) [%] Low < 30 High > 30 Median (range)	57,4 42,6 24 (0-89)	0,032
Del13q (N= 45) [%] Unmutated<5,0 Mutated>5,0	44,4 55,6	Del13q (N=51) [%] Unmutated<5,0 Mutated>5,0	51,0 49,0	0,522
Tris12 (N=45) [%] Unmutated<3,7 Mutated>3,7	88,9 11,1	Tris12 (N=51) [%] Unmutated<3,7 Mutated>3,7	88,2 11,8	0,920
Del11q (N=45) [%] Unmutated<8,6 Mutated>8,6	82,2 17,8	Del11q (N=51) [%] Unmutated<8,6 Mutated>8,6	70,6 29,4	0,183
p53 (N=45) [%] Unmutated<10,2 Mutated>10,2	88,9 11,1	p53 (N=51) [%] Unmutated<10,2 Mutated>10,2	94,1 5,9	0,355
Rearr14q (N=45) [%] Unmutated<3,0 Mutated>3,0	93,3 6,7	Rearr14q (N=51) [%] Unmutated<3,0 Mutated>3,0	84,3 15,7	0,166

A clear association between CNR1 expression and advanced clinical stage could be observed, CNR1 high expressing patients more often were diagnosed with Binet stages B and C compared to low expressing patients (p=0,049). Similarly, CNR1 high expressing patients were more likely to have unmutated IGVH genes (60% vs. 31,1%, p=0,006) and high CD38 expression (42,6% vs 21,7%, p=0,032).

4. Results

Lymphocyte doubling time (LDT) was high in 15,2% of patients within the CNR1 low expressing and in 31,9% within the high expressing group. With a p -value of 0,058 this could indicate a trend for an association between LDT and CNR1 mRNA expression.

The prevalence of cytogenetic aberrations (Del13q, Tris12, Del11q, p53, Rearr14q) did not differ significantly between the groups.

4.3. Survival analysis for CNR1 mRNA expression

In order to determine whether CNR1 expression levels correlate with survival, a Kaplan-meier survival analysis with N=100 patients was calculated (Fig. 2).

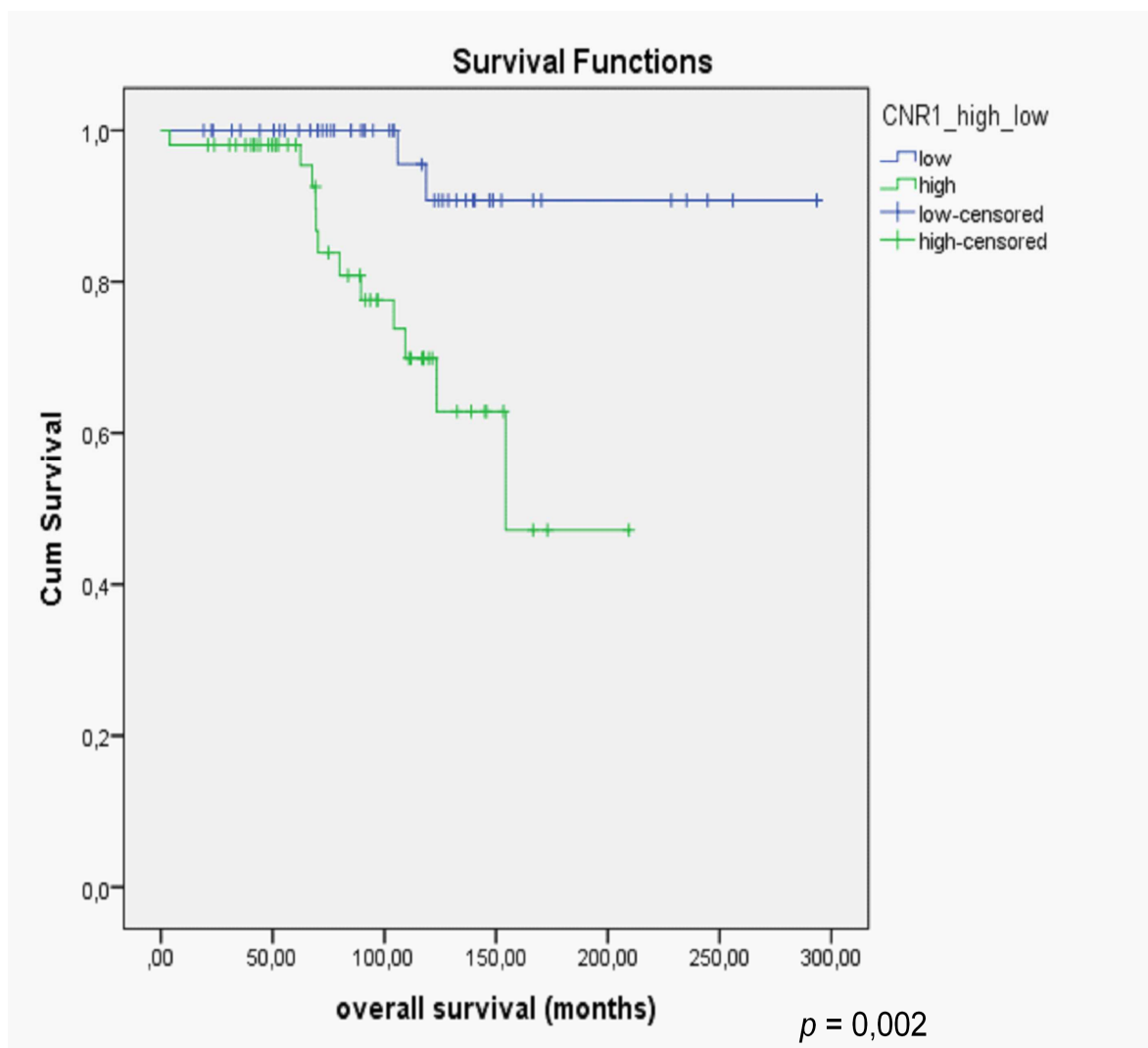


Figure 2: Overall survival for CNR1 high (N=52) and low expressing CLL patients (N=48).

Low CNR1 expression was indicative for longer overall survival (mean 277 months vs. 155 months, $p=0,002$). The low expression group showed 90% survival after 200 months whereas

the high expression cohort had a survival of 50%. The logrank test revealed a significant difference between the two cohorts (p -value=0,002).

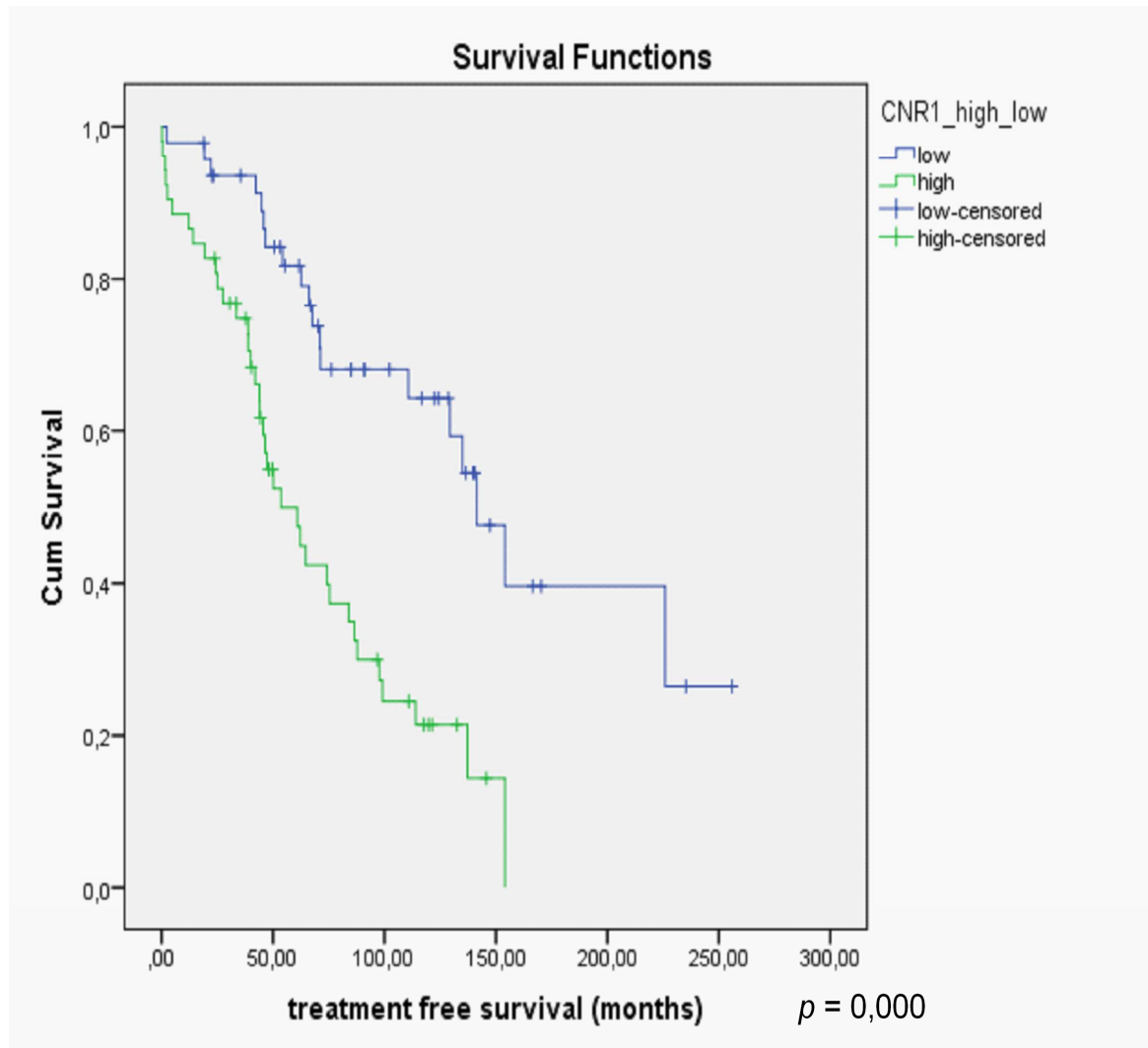


Figure 3: Treatment free survival for CNR1 high (N=52) and low (N=47) expressing CLL patients.

In addition to overall survival (OS), a significant difference were also observed for treatment free survival (TFS) in N=99 patients ($p=0,000$) (Fig. 3). Mean TFS was 152 months in the low expression group, compared to 70 months in the high expression group. A multivariat analysis revealed that the influence on OS and TFS is independent from CD38 expression, Binet stage and LDT, but depends on IGHV mutation status.

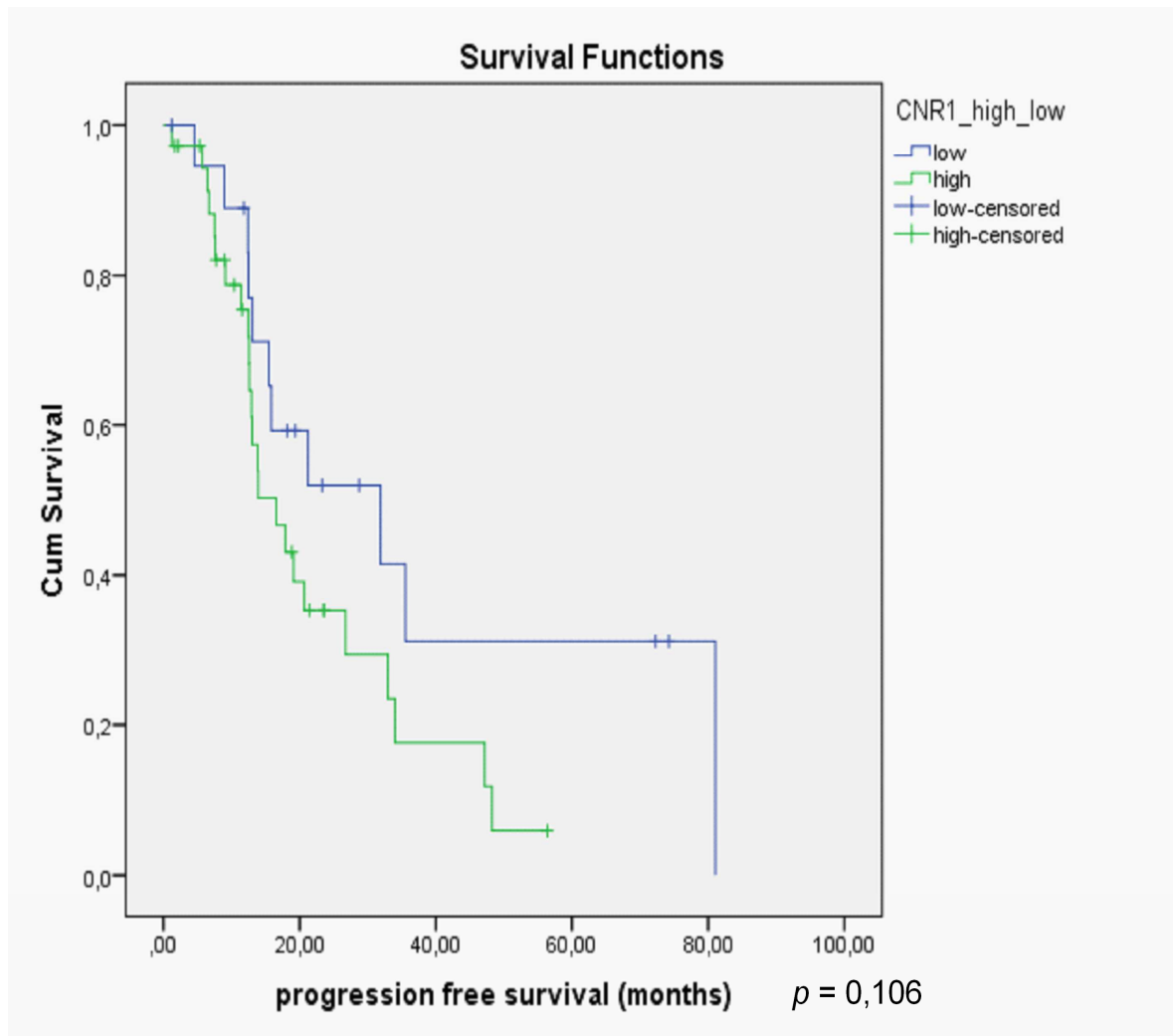


Figure 4: Progression free survival for CNR1 high (N=36) and low (N=19) expressing CLL patients.

In contrast to OS and TFS, progression free survival between the CNR1 high and low expressing groups was not significantly different (N=55) (Mean 34 months low expression group vs. 32 month high expression group; $p=0,106$) (Fig. 4).

4. Results

4.4. Protein expression of CNR1

To investigate whether protein levels follow the mRNA expression levels, Westernblots with high CNR1 mRNA expression CLL patients (N=10) (Fig. 5A+B) and with low CNR1 mRNA expression patients (N=10) were done (Fig. 6A+B). The relative fold increase in mRNA expression for CNR1 is listed in the table below the picture. To account for the case that the CNR1 antibody might bind unspecific to CNR2, CNR2 mRNA expression also was included.

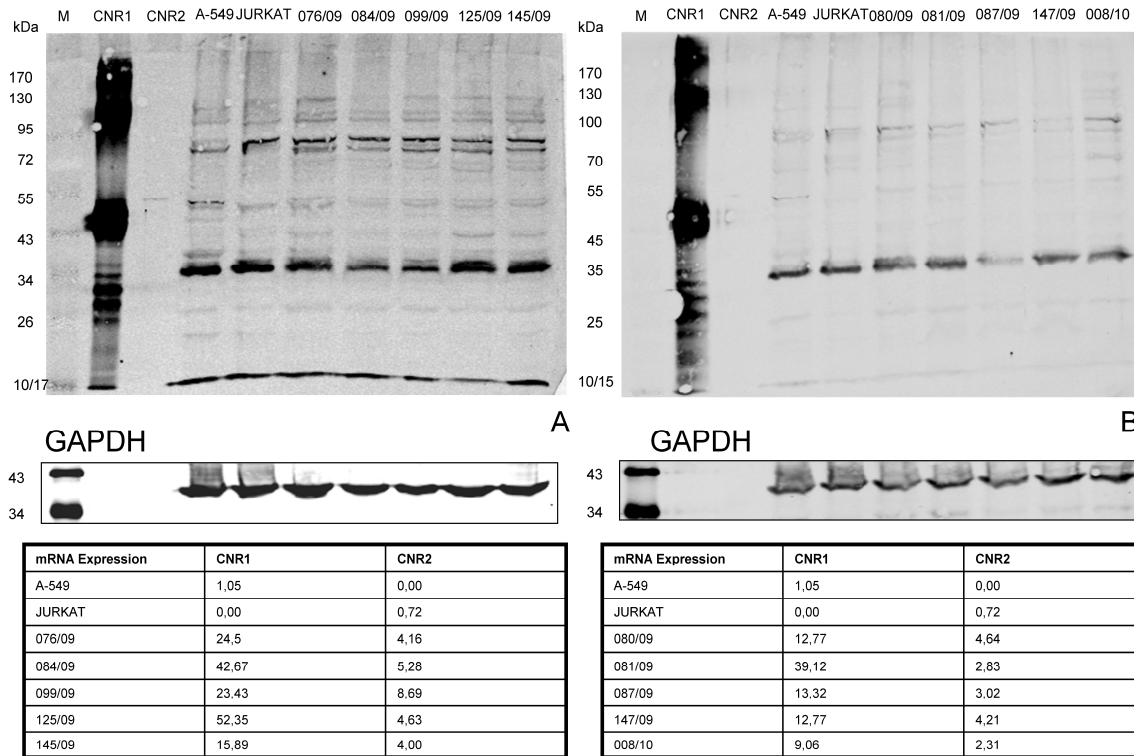


Figure 5: CNR1 protein expression in CNR1 mRNA high expressing CLL patients (N=10).

As positive controls, the cell lines A-549 and JURKAT and recombinant CNR1 and CNR2 proteins were used. Sixty µg of the samples, 0,19 µg of recombinant CNR1 and 0,1µg of recombinant CNR2 were loaded into the slots. As protein marker 2µl of pEqGOLD Protein-Marker IV was used. The IRDye 800CW [LI-COR] as secondary antibody for CNR1 was detected in the 800nm channel of the Odyssey, the IRDye 680 labelled secondary antibody for GAPDH was detected in the 700nm channel. A + B: Westernblot with 5 different CLL patients each.

The calculated size for CNR1 is around 53kDa and the CNR1 antibody is described to show bands at ~60kDa and lighter bands at ~23kDa, ~72kDa, ~180kDa. In the recombinant protein control, the antibody detected a band at the described 53kDa for the CNR1 protein plus bands under 34kDa and bands at 130kDa and 170kDa (Fig. 5A+B). Since there were no bands detected for the CNR2 recombinant protein, the antibody seemed to be specific for CNR1. However, the CNR1 antibody detected bands in JURKAT cells, which do not express CNR1 mRNA. All CLL patients and the cell lines showed bands at the same size (34kDa, 72kDa) and additional at 52kDa for the A-549.

4. Results

The same results were obtained, when patients with a low mRNA expression were screened for protein expression (Fig. 6A+B).

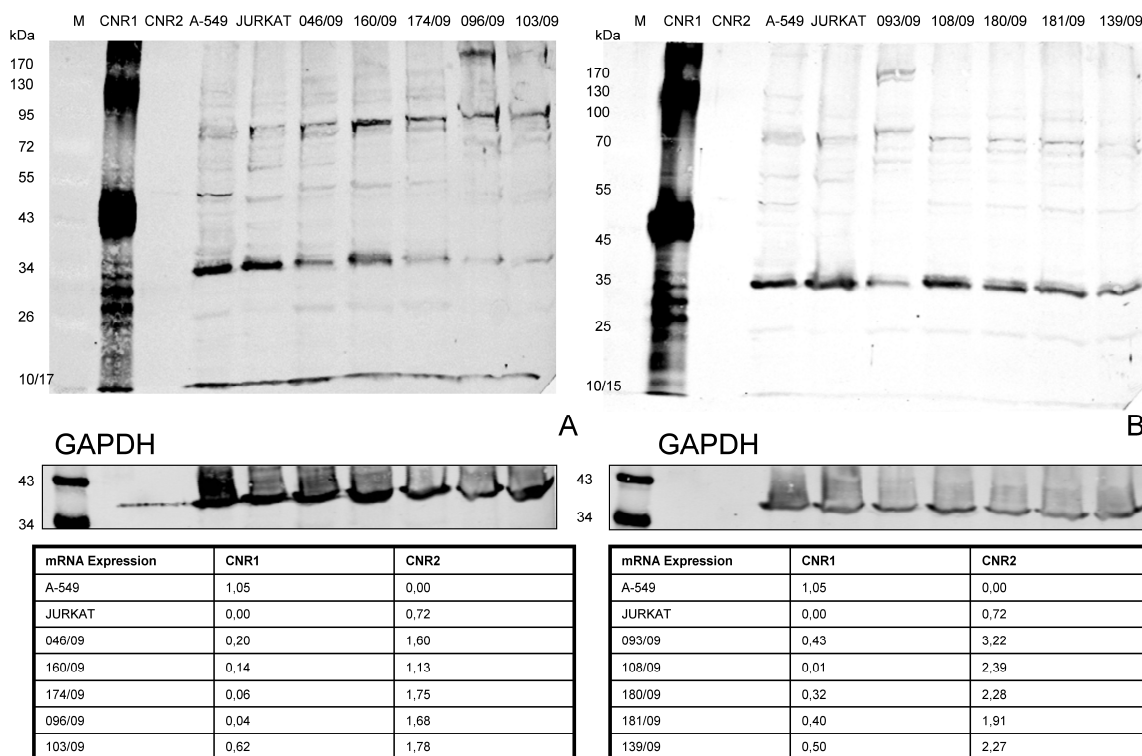


Figure 6: CNR1 protein expression in CNR1 mRNA low expressing CLL patients (N=10).

As positive controls, the cell lines A-549 and JURKAT and recombinant CNR1 and CNR2 proteins were used. Sixty µg of the samples, and 0,19 µg of recombinant CNR1 and 0,1µg of recombinant CNR2 were loaded into the slots. As protein marker 2µl of peqGOLD Protein-Marker IV was used. The IRDye 800CW [LI-COR] as secondary antibody for CNR1 was detected in the 800nm channel of Odyssey, the IRDye 680 as secondary antibody for GAPDH was detected in the 700nm channel.

A + B: Westernblot with 5 different CLL patients each.

Comparing the high and low CNR1 expressing patients (Fig. 5+6), no association between mRNA expression level and protein expression could be observed. In addition, no association between CNR1 protein expression with CNR2 mRNA high or low expression levels could be detected in the westernblots. As a control, the GAPDH loading control revealed that the amounts of protein loaded within one westernblot were similar.

4.5. CNR2 mRNA expression

As for CNR1, CNR2 mRNA expression levels were determined for the 102 CLL patients (Fig. 7A). Median CNR2 mRNA expression was 3,60 ranging from 0,06 to 14,37. The median of 3,60 was used as a cut-off and the cohort was split into CNR2 high and low expression groups (Fig. 7B). The median for the low expression group was 2,28 (range 0,06-3,59) compared to 5,12 (range 3,61-14,37) of the high expressing groups (Fig. 7B).

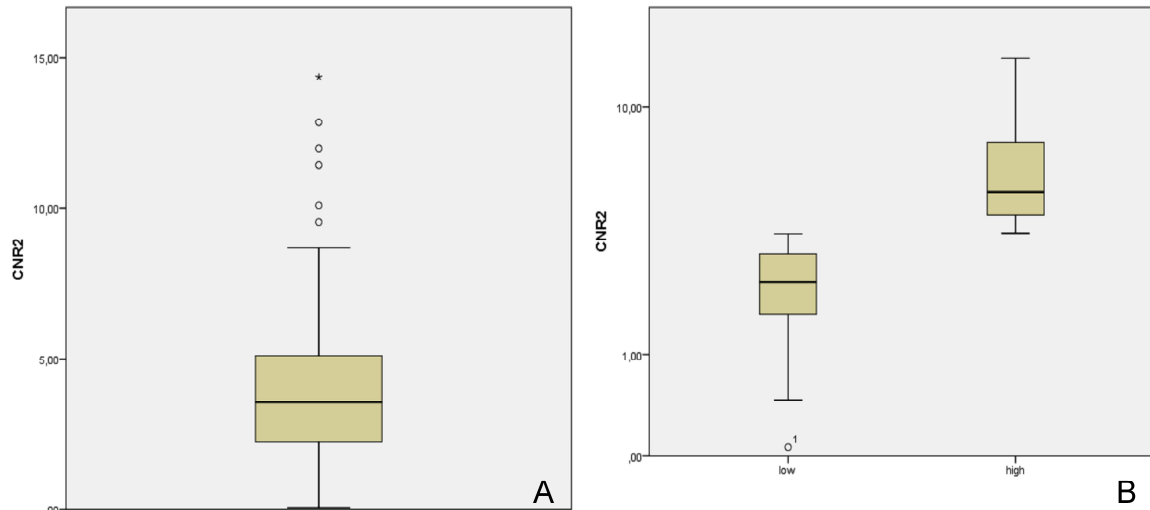


Figure 7: CNR2 mRNA expression in a cohort of 102 CLL patients.

A: Box plot CNR2 expression.

B: Box plot of CNR2 low/high expression groups (N=51 vs. 51).

Note logarithmic scale on y-axis.

Also for CNR2, patient characteristics were analyzed for high (N=51) and low (N=51) expression groups separately and then compared.

4. Results

Table 3: Patient characteristics for CNR2 high (N=51) and low (N=51) expressing subgroups.

N=number of patients, Del: Deletion; Tris: Trisomie; Rearr: Rearrangement.

P-values ≤ 0,05 significant.

CNR2 low	CNR2 low	CNR2 high	CNR2 high	p-value
Age at diagnosis (N=50) [years] Median (Range)	65 (39-85)	Age at diagnosis (N=50) Median (Range)	60 (25-80)	
Sex Female :Male (N=51) [%]	45,1 : 54,9	Sex Female :Male (N=51) [%]	35,3 : 64,7	
Binet at diagnosis (N=49) A B/C	85,7 14,3	Binet at diagnosis (N=50) A B/C	82,0 18,0	0,616
Mutational status (N=45) [%] Unmutated Mutated	46,7 53,3	Mutational status (N=45) [%] Unmutated Mutated	44,4 55,6	0,832
Lymphocyte doubling time (N=47) High < 1year Low > 1year	25,5 74,5	Lymphocyte doubling time (N=46) High < 1year Low > 1year	21,7 78,3	0,667
CD38 (N=48) [%] Low < 30 High > 30 Median (range)	68,8 31,3 7,5 (0-85)	CD38 (N=45) [%] Low < 30 High > 30 Median (range)	66,7 33,3 17 (0-91)	0,830
Del13q (N= 46) [%] Unmutated<5,0 Mutated>5,0	54,3 45,7	Del13q (N=50) [%] Unmutated<5,0 Mutated>5,0	42,0 58,0	0,226
Tris12 (N=46) [%] Unmutated<3,7 Mutated>3,7	91,3 8,7	Tris12 (N=50) [%] Unmutated<3,7 Mutated>3,7	86,0 14,0	0,415
Del11q (N=46) [%] Unmutated<8,6 Mutated>8,6	80,4 19,6	Del11q (N=50) [%] Unmutated<8,6 Mutated>8,6	72,0 28,0	0,333
p53 (N=46) [%] Unmutated<10,2 Mutated>10,2	93,5 6,5	p53 (N=50) [%] Unmutated<10,2 Mutated>10,2	90,0 10,0	0,538
Rearr14q (N=46) [%] Unmutated<3,0 Mutated>3,0	84,8 15,2	Rearr14q (N=50) [%] Unmutated<3,0 Mutated>3,0	92,0 8,0	0,267

In contrast to CNR1, no significant association between any of the established prognostic markers, clinical stage, unmutated IGHV genes, LTD, CD38 expression, or cytogenetic aberrations, and CNR2 mRNA expression could be observed (Table 3).

4.6. Survival analysis for CNR2 expression

Again, Kaplan-Meier survival curves were calculated for overall survival for CNR2 high and low expressing groups (N=100) (Fig. 8). Mean OS for high expressing patients was 206 months vs. 234 months for low expressing patients ($p=0,738$). Thus, CNR2 expression is not significantly associated with overall survival in CLL.

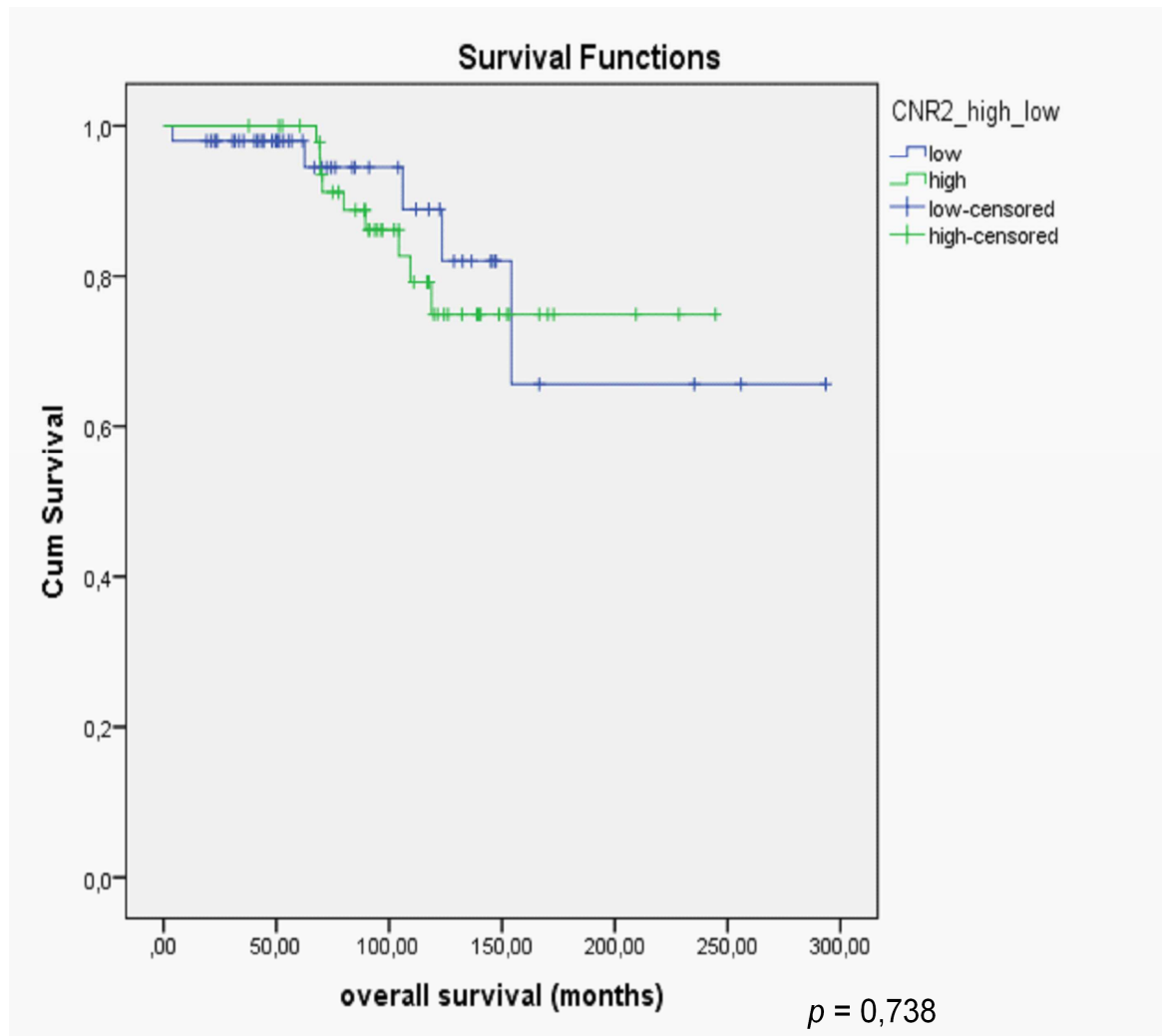


Figure 8: Overall survival for CNR2 high (N=50) and low expressing CLL patients (N=50).

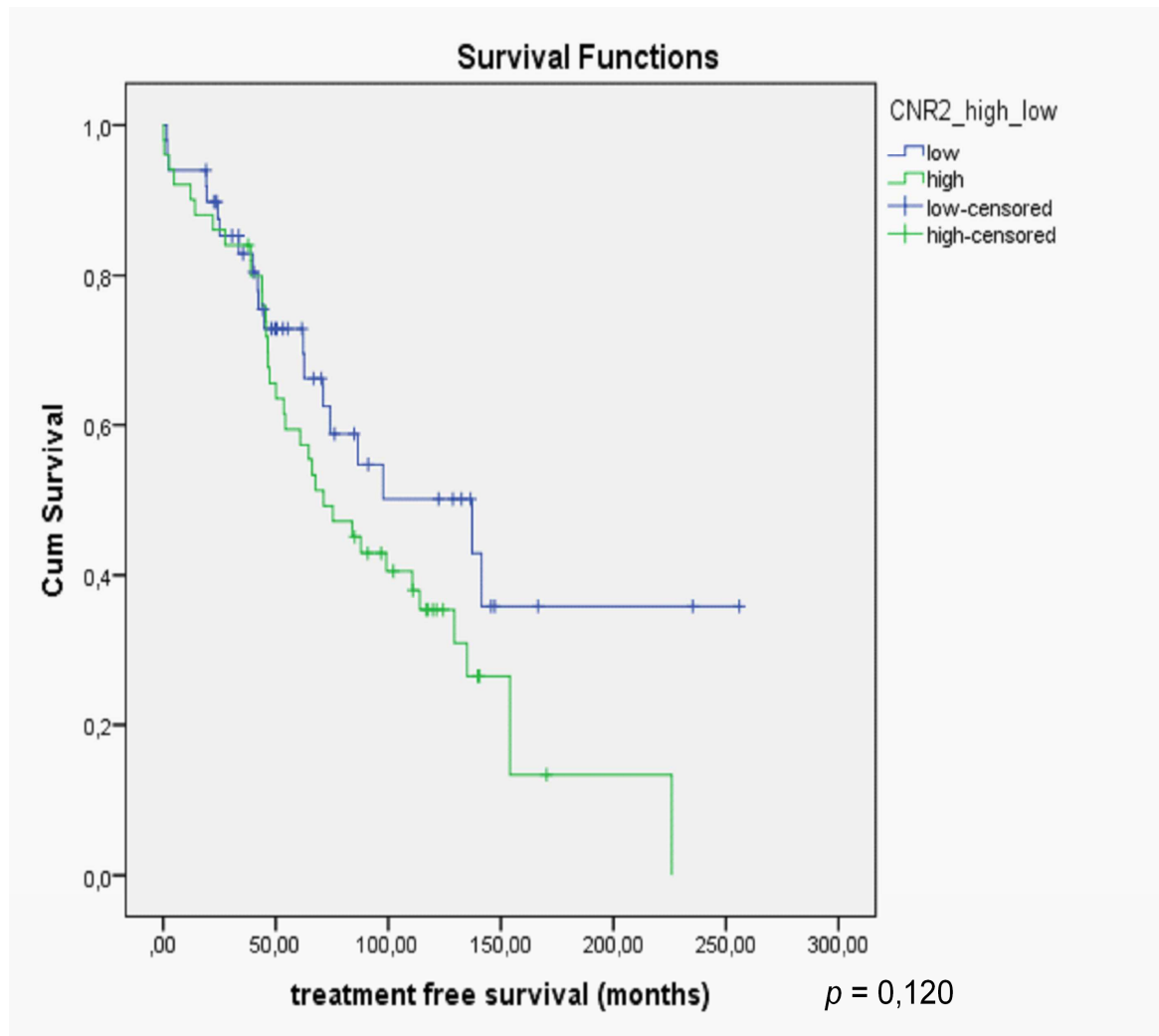


Figure 9: Treatment free survival for CNR2 high (N=50) and low (N=49) expressing CLL patients.

Neither is mRNA expression of CNR2 associated with longer treatment free survival (N=99) ($p=0,120$) (Fig. 9). Mean for low expression 136 months vs. 95 months for high expression.

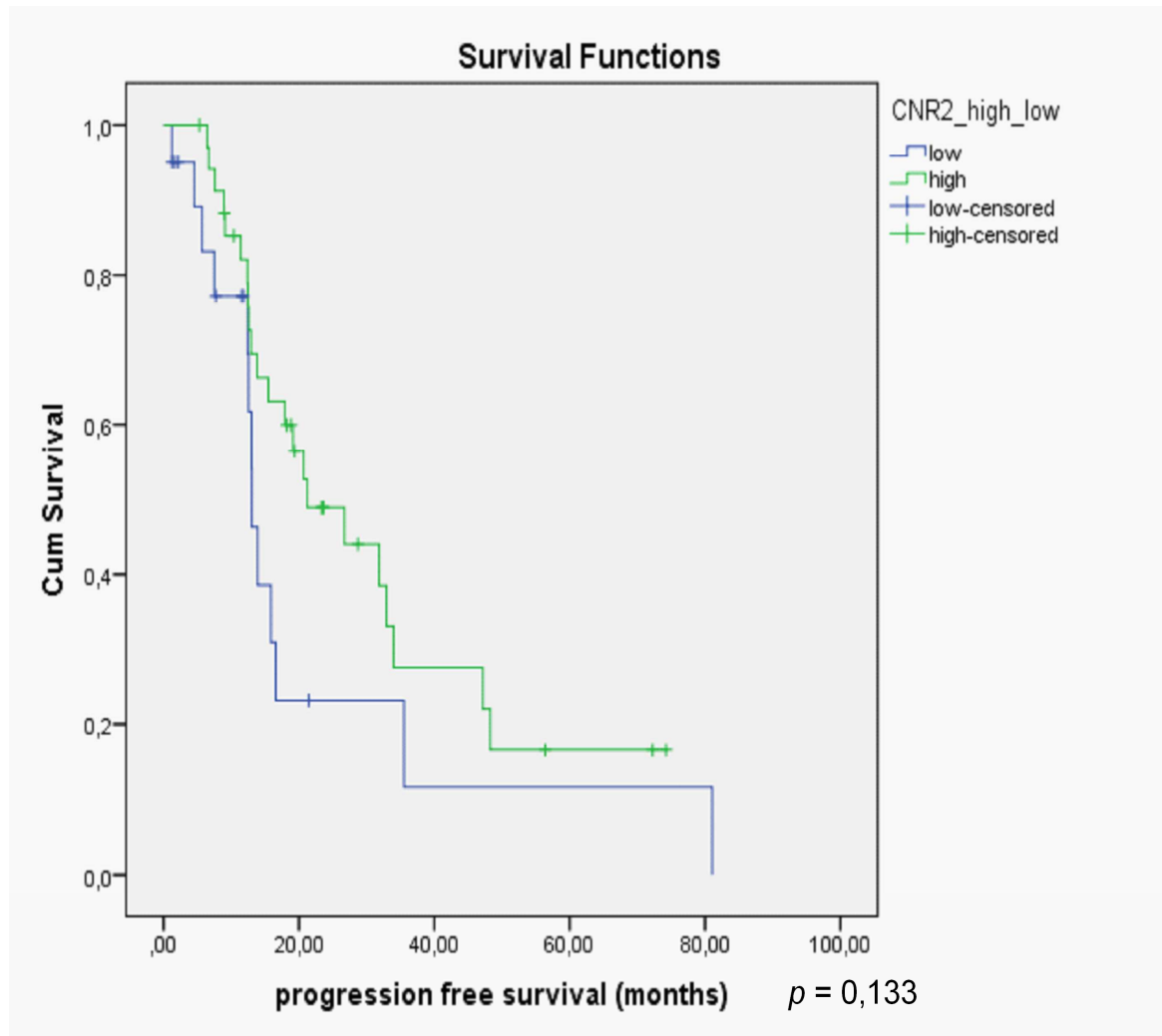


Figure 10: Progression free survival for CNR2 high (N=35) and low expressing CLL patients (N=20).

Likewise, no significant association could be observed for CNR2 mRNA expression levels and progression free survival (N=55) (Fig. 10), underlining that mRNA expression of this gene appears not to have an impact on survival of CLL patients (mean 22 month low expression group vs. 31 month for high expression; p value=0,133).

4.7. Protein expression of CNR2

As for the CNR1, CLL patients with high (N=10) (Fig. 11A+B) and low CNR2 mRNA expression (N=10) (Fig. 12A+B) were tested in Westernblots to see whether protein levels follow the mRNA expression levels. Beside the mRNA expression of CNR2, the CNR1 mRNA levels are also shown in the tables, in case the antibody detects not exclusively the CNR2 protein.

4. Results

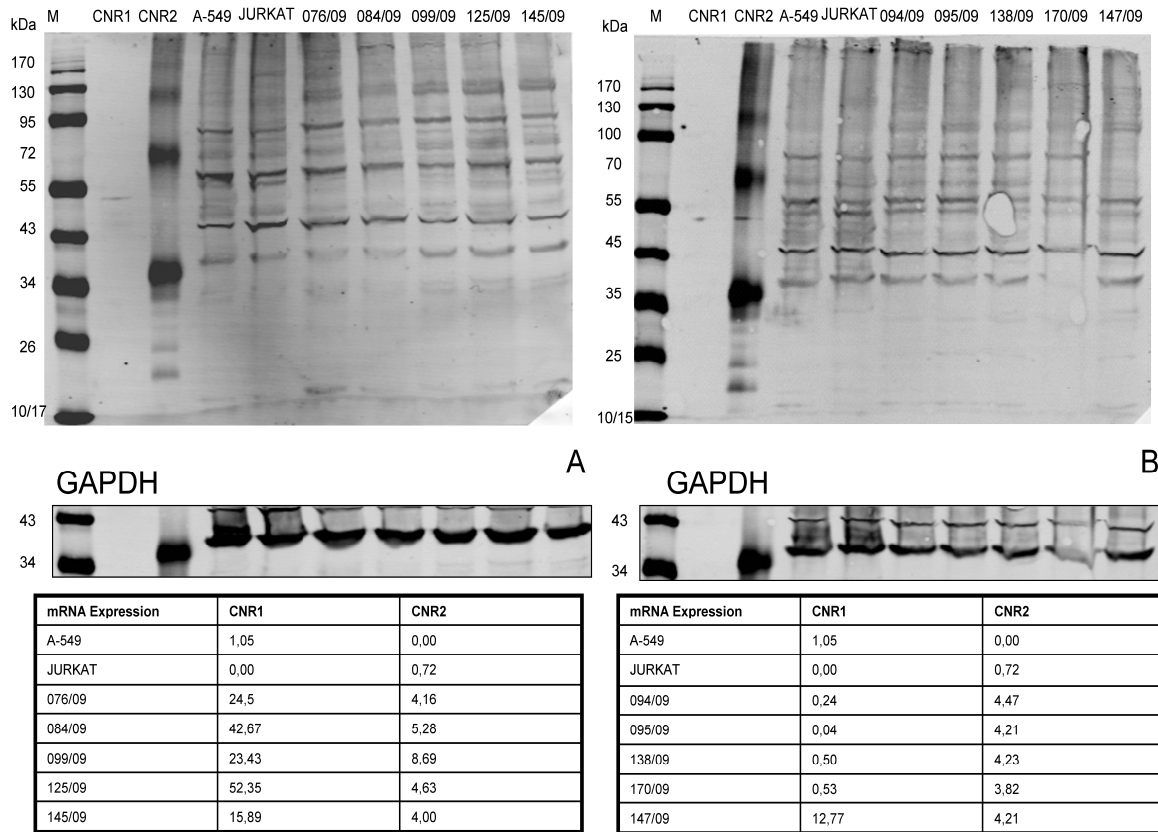


Figure 11: CNR2 protein expression in CNR2 mRNA high expressing CLL patients (N=10).

As positive controls, the cell lines A-549 and JURKAT and recombinant CNR1 and CNR2 proteins were used. Sixty μg of the samples, 0,19 μg of recombinant CNR1 and 0,1 μg of recombinant CNR2 were loaded into the slots. As protein marker 2 μl of pEqGOLD Protein-Marker IV was used. IRDye 680CW [LI-COR] as secondary antibody for CNR2 and GAPDH was detected in the 700nm channel.

A + B: Westernblot with 5 different CLL patients each.

The predicted size for CNR2 is ~40kDa and the antibody is described to detect a band at 39,7kDa. The recombinant protein controls revealed that the antibody detected specific CNR2, since no bands were shown for the recombinant CNR1 (Fig. 11A+B). Beside the specific band at ~40kDa, additional bands at ~72kDa and ~130kDa were seen. Between cell lines and CLL patients, no difference in band size was seen. Even the A-549 cell line, which does not express CNR2 mRNA had the same band pattern as JURKAT cells (Fig. 11A+B). As for recombinant CNR2, the monoclonal antibody did not only detect bands at the predicted size (~40kDa) in CLL cells and cell lines, additional bands at higher size were also detected.

4. Results

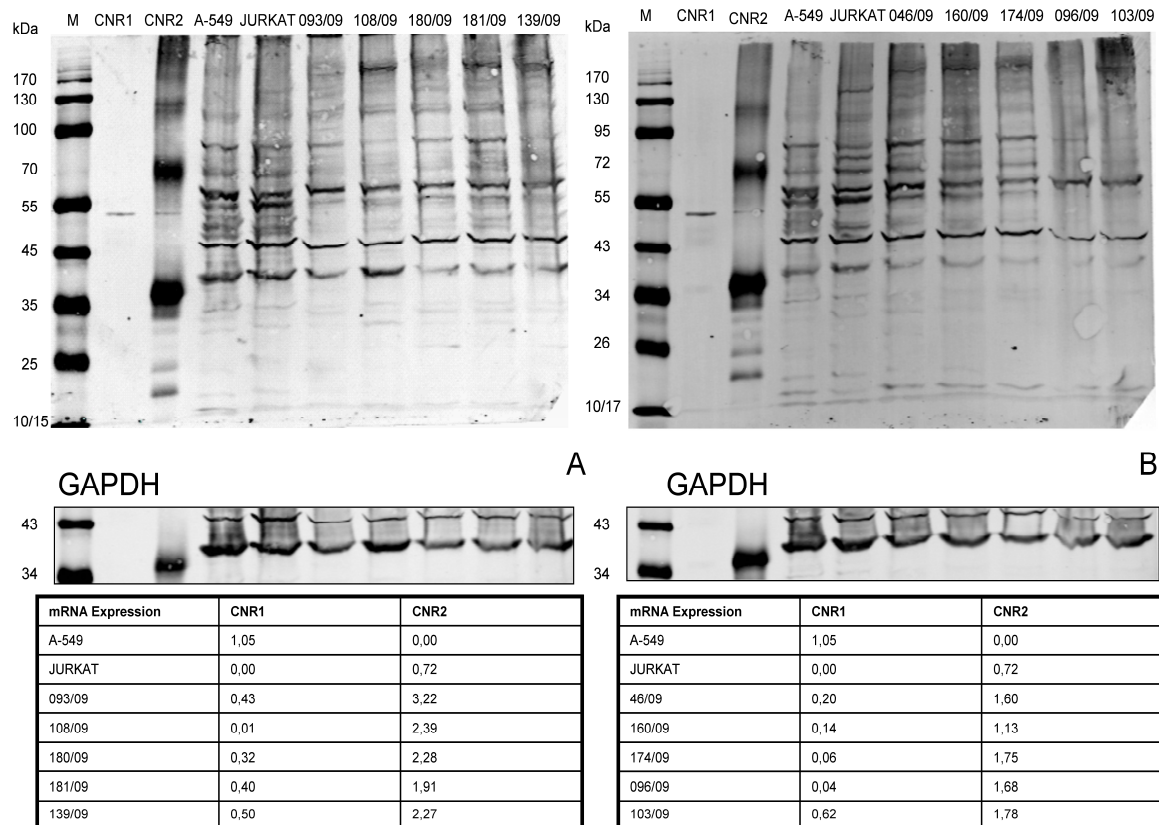


Figure 12: CNR2 protein expression in CNR2 mRNA low expressing CLL patients (N=10).

As positive controls, the cell lines A-549 and JURKAT and recombinant CNR1 and CNR2 proteins were used. Sixty μg of the samples, 0,19 μg of recombinant CNR1 and 0,1 μg of recombinant CNR2 were loaded into the slots. As protein marker 2 μl of pEqGOLD Protein-Marker IV was used. IRDye 680CW [LI-COR] as secondary antibody for CNR2 and GAPDH was detected in the 700nm channel. A + B: Westernblot with 5 different CLL patients each.

Comparing the high expression cohort with the CNR2 low expression cohort, no difference in band size and pattern were seen (Fig. 11A+B + Fig. 12A+B). The GAPDH control revealed for each westernblot an equal protein load (Fig. 11A+B + Fig. 12A+B). It was not possible to evaluate the difference in protein level as seen in the mRNA level.

4.8. Impact of cannabinoids on viability of cell lines

To evaluate the responsiveness of malignant cells to cannabinoids, viability assays were performed. Cell lines were incubated for 24h and 48h with selected compounds, decrease of viability was measured, and IC50 values were determined. In addition, CNR mRNA expression was determined for the four cell lines used (MEC-1, MEC-2, JURKAT and A-549) whether induction of cell death might correlate with gene expression.

4. Results

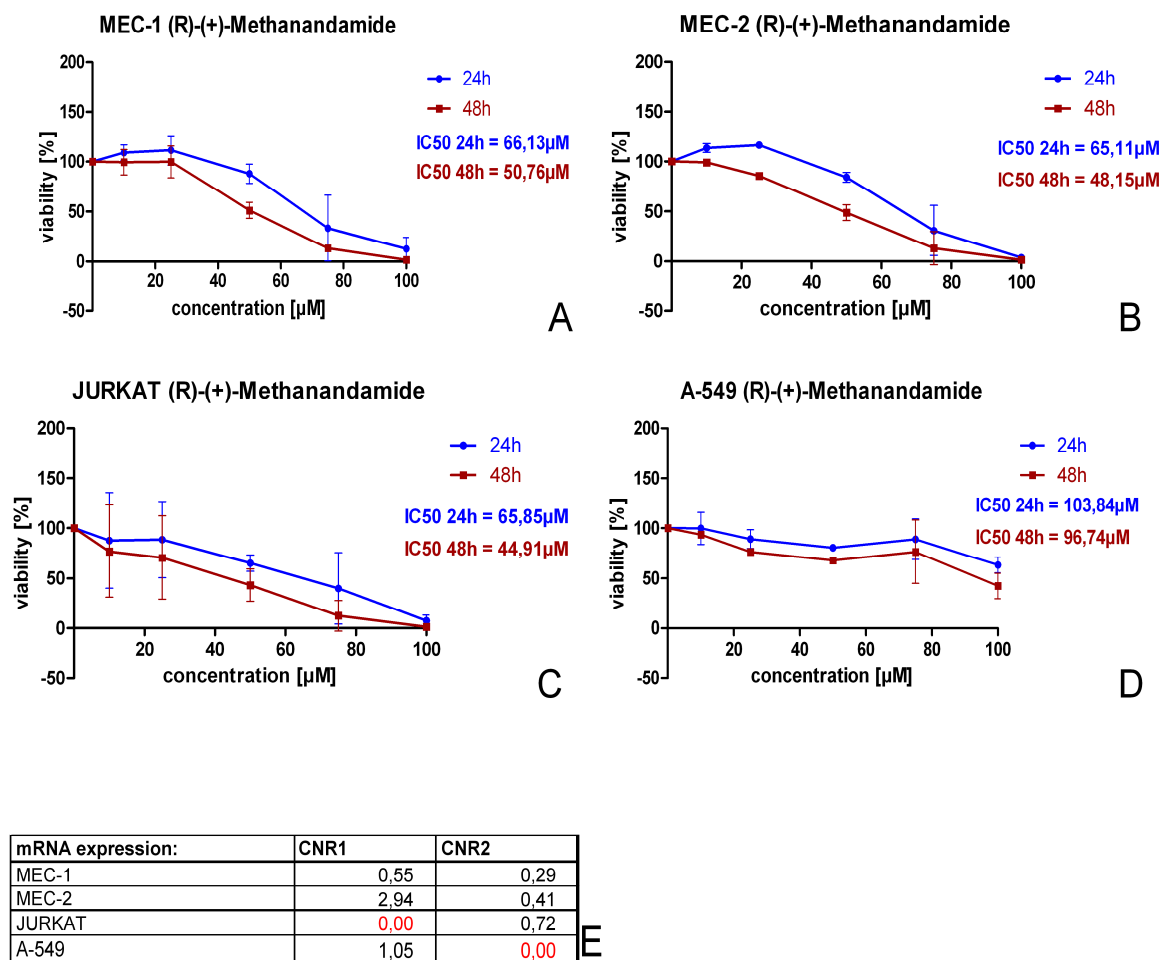


Figure 13: Viability of cell lines incubated with (R)-(+)-Methanandamide for 24 and 48h.

Viability was calculated relative to vehicle control.

A: Viability and IC₅₀ values for MEC-1.

B: Viability and IC₅₀ values for MEC-2.

C: Viability and IC₅₀ values for JURKAT.

D: Viability and IC₅₀ values for A-549.

E: mRNA expression of CNR1 and CNR2 for the cell lines.

Figure 13 shows changes in viability for the four cell lines upon incubation with different concentrations of (R)-(+)-Methanandamide, the stable analog of the endocannabinoid anandamide and agonist of CNR1. For all four cell lines, a time and concentration dependent decrease in viability could be observed (Fig. 13A-D). In particular, A-549 was resistant to treatment with an extrapolated IC₅₀ 103,84 μ M after 24h and 96,74 μ M after 48h of incubation (Fig. 13D).

Levels of mRNA expression of CNR1 and CNR2 are listed in Figure 13E. As reported in the literature, JURKAT cells were positive only for CNR2 mRNA expression and A-549 only for mRNA expression of CNR1. The cell lines MEC-1 and MEC-2 were positive for both receptors (relative to A-549 and JURKAT) although to a varying degree. Despite different CNR1 mRNA expression levels, MEC-1, MEC-2 and JURKAT show similar sensitivities toward the CNR1 agonist (R)-(+)-Methanandamide.

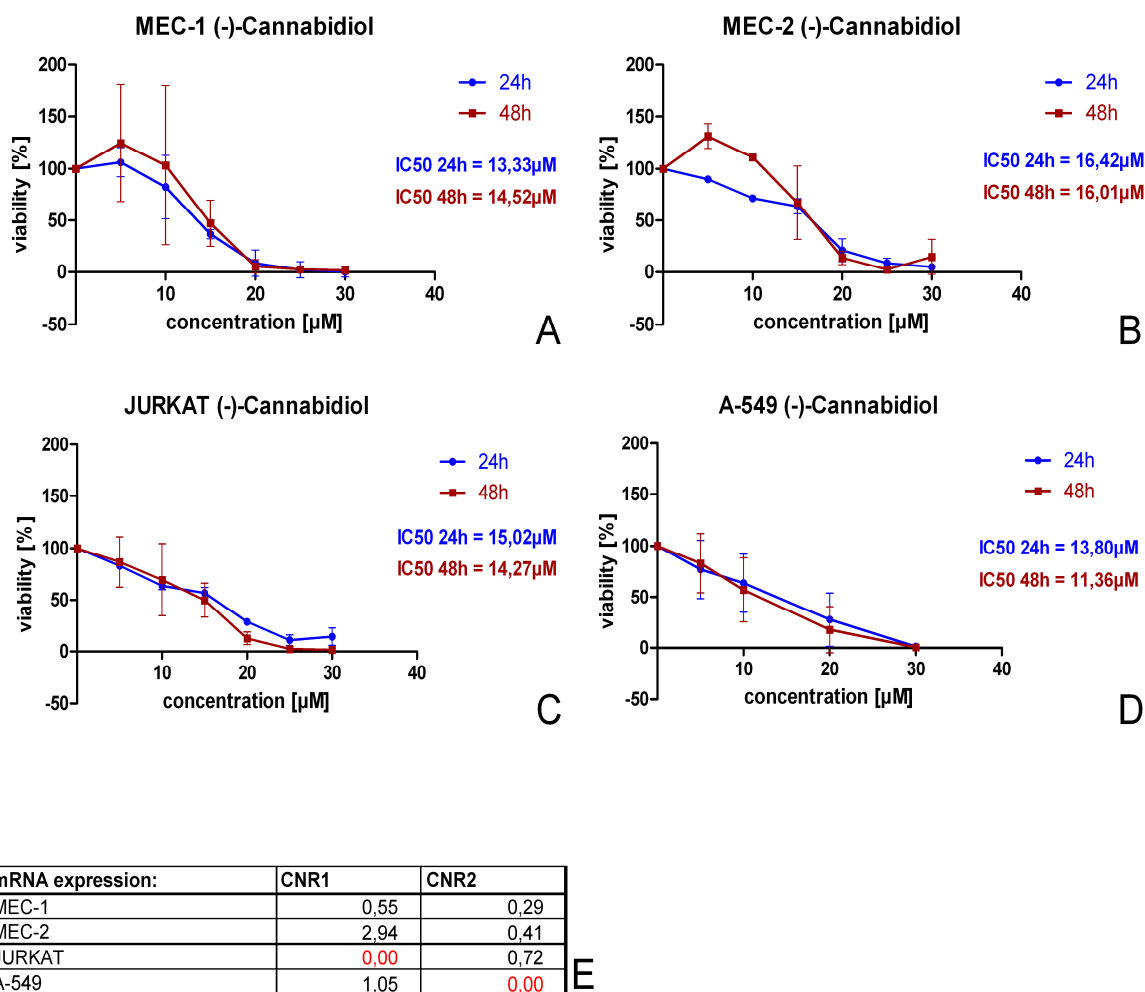


Figure 14: Viability of cell lines incubated with (-)-Cannabidiol for 24 and 48h.

Viability was calculated relative to vehicle control.

A: Viability and IC₅₀ values for MEC-1.

B: Viability and IC₅₀ values for MEC-2.

C: Viability and IC₅₀ values for JURKAT.

D: Viability and IC₅₀ values for A-549.

E: mRNA expression of CNR1 and CNR2 for the cell lines.

Next, the four cell lines were tested for (-)-Cannabidiol, a non-psychotropic constituent of cannabis acting on both cannabinoid receptors. As for (R)-(+)-Methanandamide a dose dependent cytotoxic effect, but not a time dependent one, could be observed. The IC₅₀ values were, generally, 3-4 fold lower than for (R)-(+)-Methanandamide (Fig. 14). All four cell lines showed similar IC₅₀ values, although they express the CNRs to different extents (Fig. 14E).

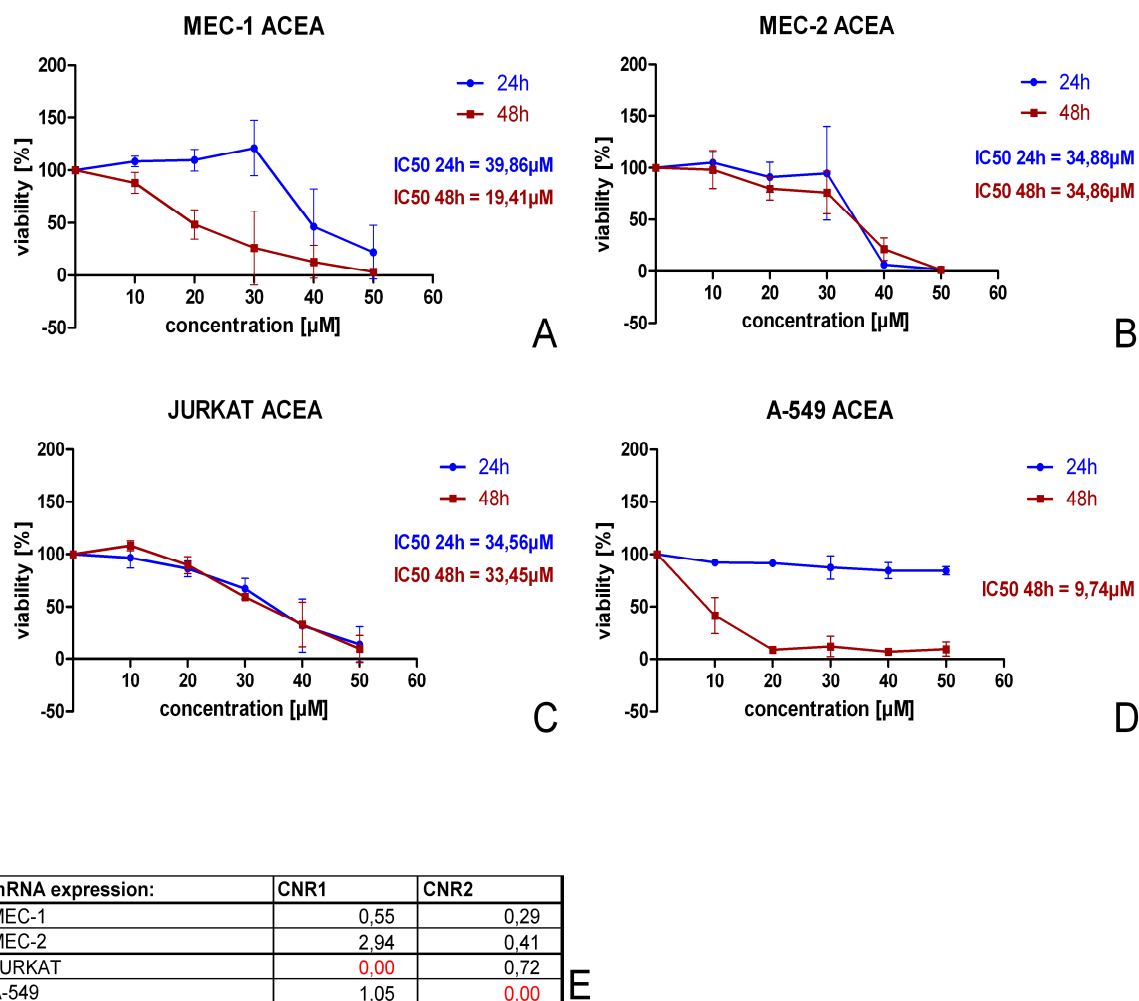


Figure 15: Viability of cell lines incubated with ACEA for 24 and 48h.

Viability was calculated relative to vehicle control.

A: Viability and IC₅₀ values for MEC-1.

B: Viability and IC₅₀ values for MEC-2.

C: Viability and IC₅₀ values for JURKAT.

D: Viability and IC₅₀ values for A-549.

E: mRNA expression of CNR1 and CNR2 for the cell lines.

The following set of experiments was carried out using one selective agonist and antagonist for each receptor, respectively.

In figure 15, the cell lines were incubated with the selective CNR1 agonist ACEA. Whereas MEC-2 and JURKAT showed only a concentration dependent drop in viability, MEC-1 had a concentration and time dependent decrease (Fig. 15A-C). On the A-549 cells, ACEA had the greatest impact on viability after 48h incubation (IC₅₀ = 9,74 μ M), but it had no effect in the 24h incubation assay (Fig. 15D). No association between the sensitivity toward the CNR1 agonist and the CNR1 mRNA expression level could be observed.

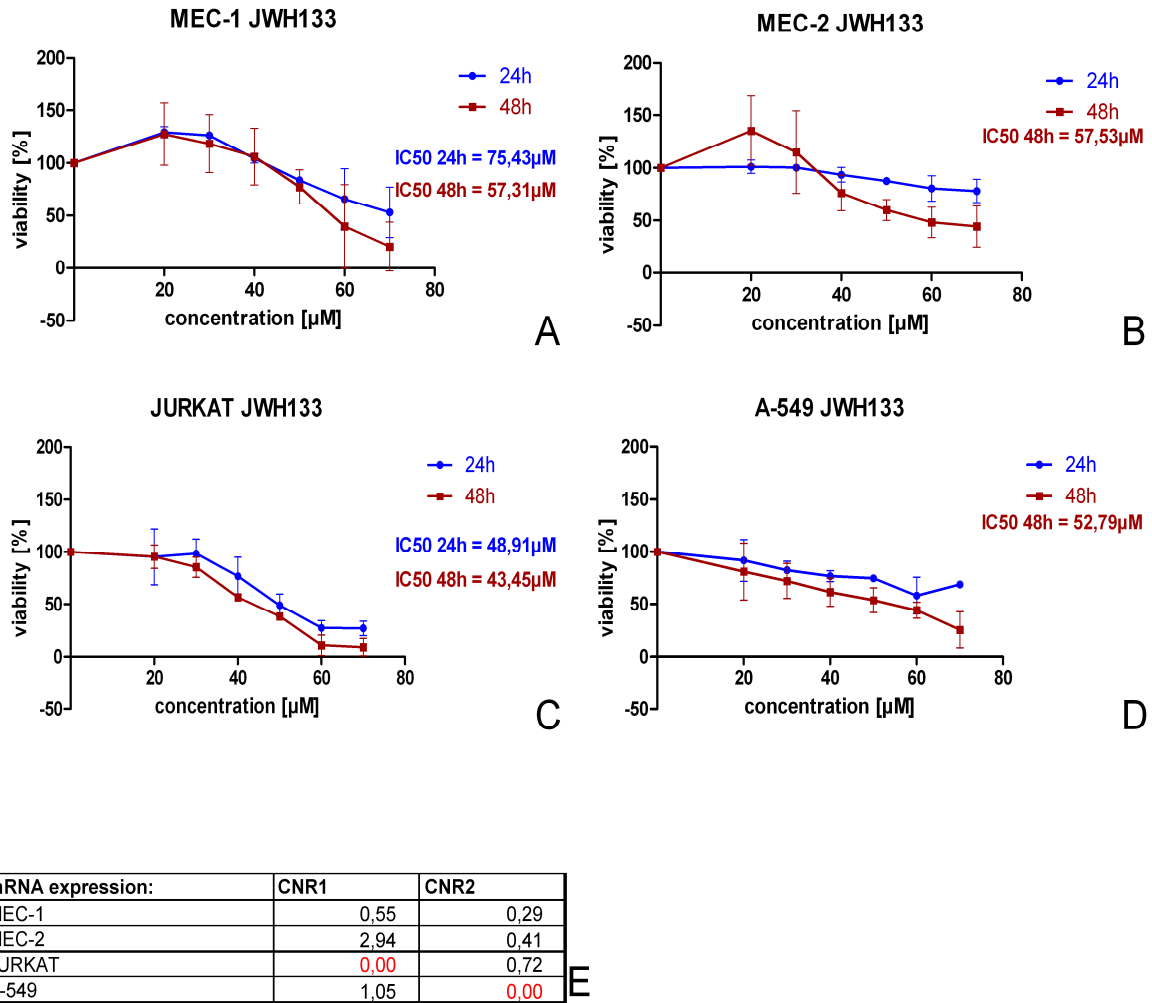


Figure 16: Viability of cell lines incubated with JWH133 for 24 and 48h.

Viability was calculated relative to vehicle control.

A: Viability and IC50 values for MEC-1.

B: Viability and IC50 values for MEC-2.

C: Viability and IC50 values for JURKAT.

D: Viability and IC50 values for A-549.

E: mRNA expression of CNR1 and CNR2 for the cell lines.

In the next experiment, cells were incubated with the CNR2 agonist JWH133 (Fig. 16). All four cell lines showed a time and concentration dependent decrease in viability. In the case of MEC-2 and A-549, the IC50 value was not reached in the 24h incubation setting (Fig. 16B+D). Comparing the different cell lines, JURKAT cells were more sensitive than the other cell lines (Fig. 16C). They also express the highest level of CNR2 mRNA (Fig. 16E). Still, for the other cells there was no association between mRNA expression levels and viability detected.

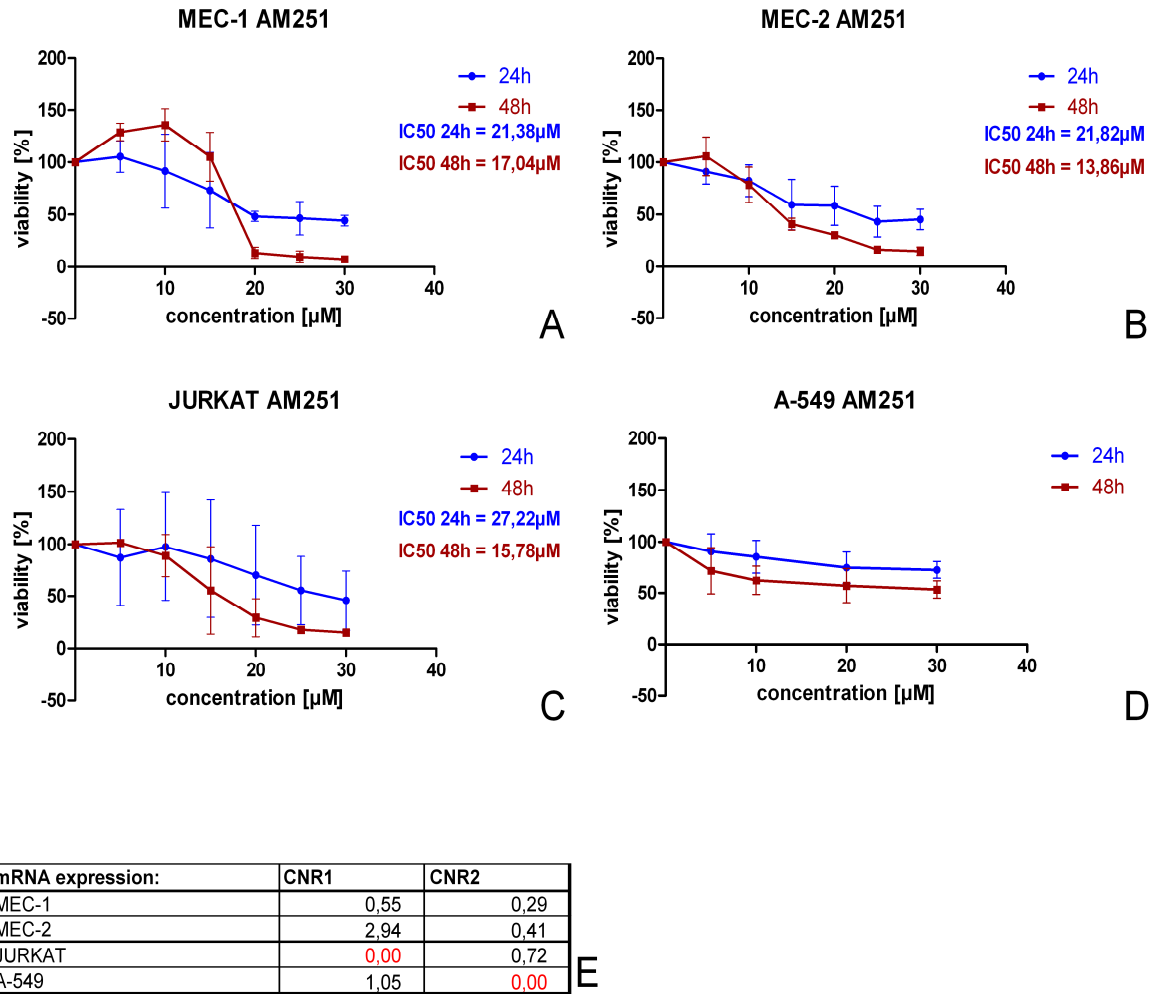


Figure 17: Viability of cell lines incubated with AM251 for 24 and 48h.

Viability was calculated relative to vehicle control.

A: Viability and IC_{50} values for MEC-1.

B: Viability and IC_{50} values for MEC-2.

C: Viability and IC_{50} values for JURKAT.

D: Viability and IC_{50} values for A-549.

E: mRNA expression of CNR1 and CNR2 for the cell lines.

Incubation with the CNR1 antagonist AM251 led to a time and dose dependent decrease in viability for MEC-1, MEC-2 and JURKAT (Fig. 17A-C). The A-549 cells showed some resistance, the IC_{50} value could not be reached in 24h and 48h incubation (Fig. 17D). The mRNA expression was not associated with viability.

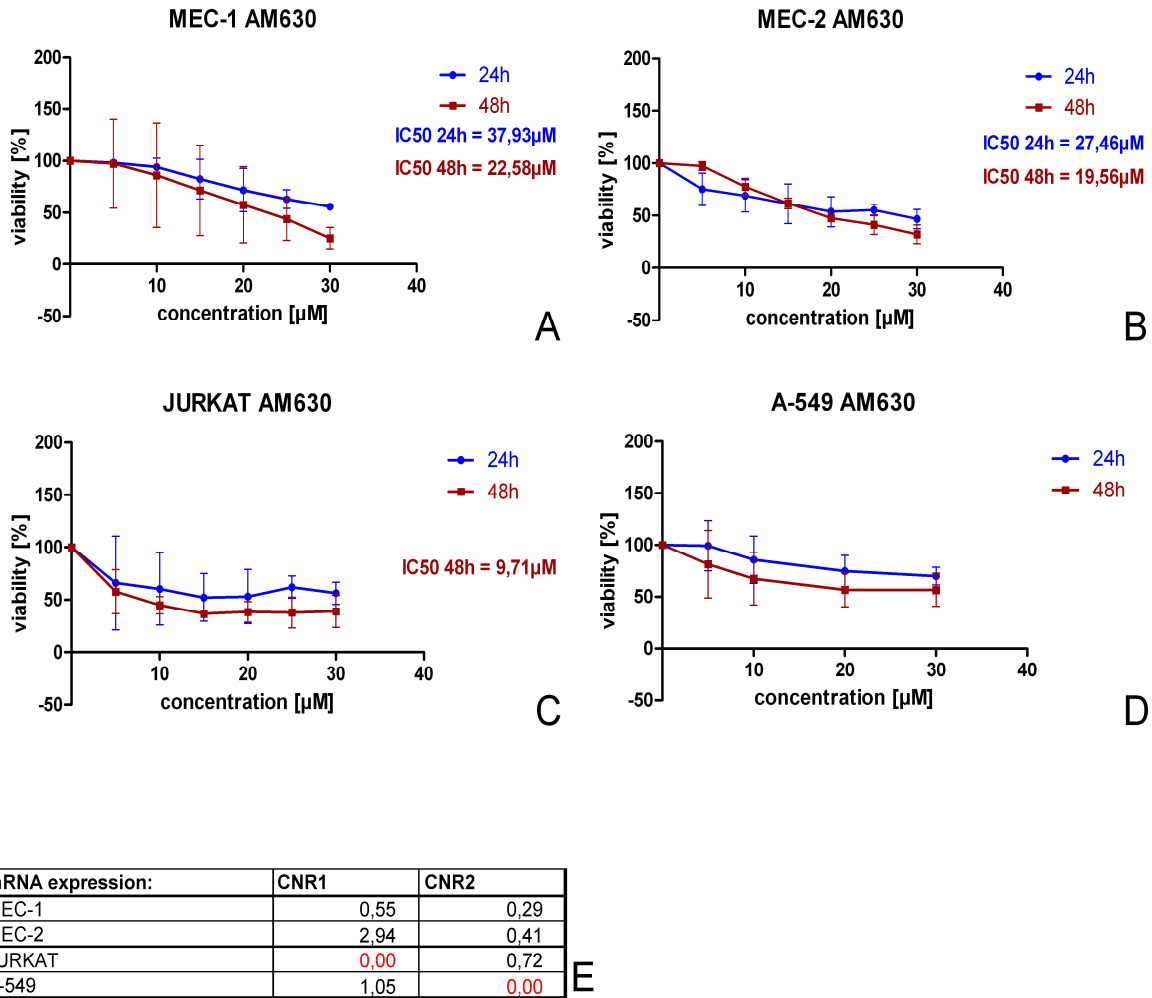


Figure 18: Viability of cell lines incubated with AM630 for 24 and 48h.

Viability was calculated relative to vehicle control.

A: Viability and IC50 values for MEC-1.

B: Viability and IC50 values for MEC-2.

C: Viability and IC50 values for JURKAT.

D: Viability and IC50 values for A-549.

E: mRNA expression of CNR1 and CNR2 for the cell lines.

The cell lines were then incubated with AM630, a CNR2 antagonist (Fig. 18). The IC50 was not reached for the A-549, which do not express CNR2 mRNA, in 24h and 48h incubation (Fig. 18D). The JURKAT cell line, with the highest CNR2 mRNA expression, showed the highest sensitivity toward AM630 in the 48h incubation setting (IC50 = 9,71μM), whereas the IC50 value was not reached in 24h incubation (Fig. 18C). MEC-1 and MEC-2 showed a time and concentration dependent drop in viability, where the MEC-2 were slightly more susceptible. MEC-2 cells have higher CNR2 mRNA expression than MEC-1. Therefore, at least for the AM630 an association between the mRNA level and sensitivity needs to be taken into consideration.

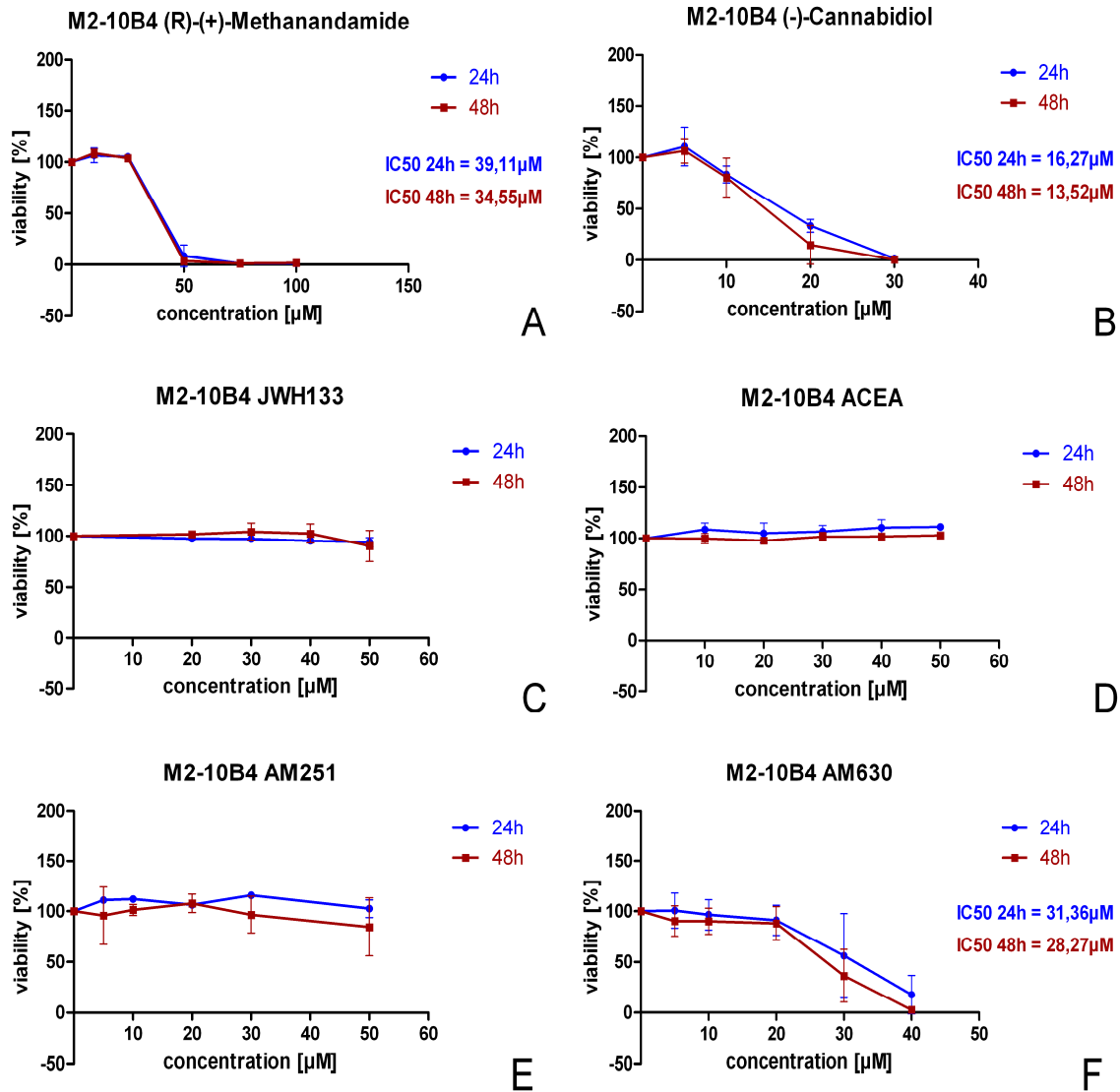


Figure 19: Viability of M2-10B4 incubated with different compounds for 24 and 48h.

Viability was calculated relative to vehicle control.

A: Viability and IC50 values for M2-10B4 incubated with (R)-(+)-Methanandamide.

B: Viability and IC50 values for M2-10B4 incubated with (-)-Cannabidiol.

C: Viability and IC50 values for M2-10B4 incubated with JWH133.

D: Viability and IC50 values for M2-10B4 incubated with ACEA.

E: Viability and IC50 values for M2-10B4 incubated with AM251.

F: Viability and IC50 values for M2-10B4 incubated with AM630.

Note different scales on X-axis.

Finally, viability screens were also carried out with the mouse fibroblast cell line M2-10B4. Since these cells would be used as feeder cells in coculture assays with CLL primary cells, it was important to determine the extent of cannabinoid induced cytotoxicity in the cells. These cells exert resistance toward JWH133, ACEA and AM251 incubation (Fig. 19C-E). For the incubation with AM630, (R)-(+)-Methanandamide and (-)-Cannabidiol they showed a time and concentration dependent drop in viability (Fig. 19A+B+F). (-)-Cannabidiol had the strongest impact on viability (Fig. 19B).

4. Results

4.9. Impact of cannabinoids on viability of primary cells

After testing the CNR1 and CNR2 agonists and antagonists on cell lines, primary cells from CLL patients were screened. In contrast to cell lines, primary cells were incubated only for 48h in suspension culture and with M2-10B4 mouse fibroblasts as feeder layer in coculture (Fig. 20).

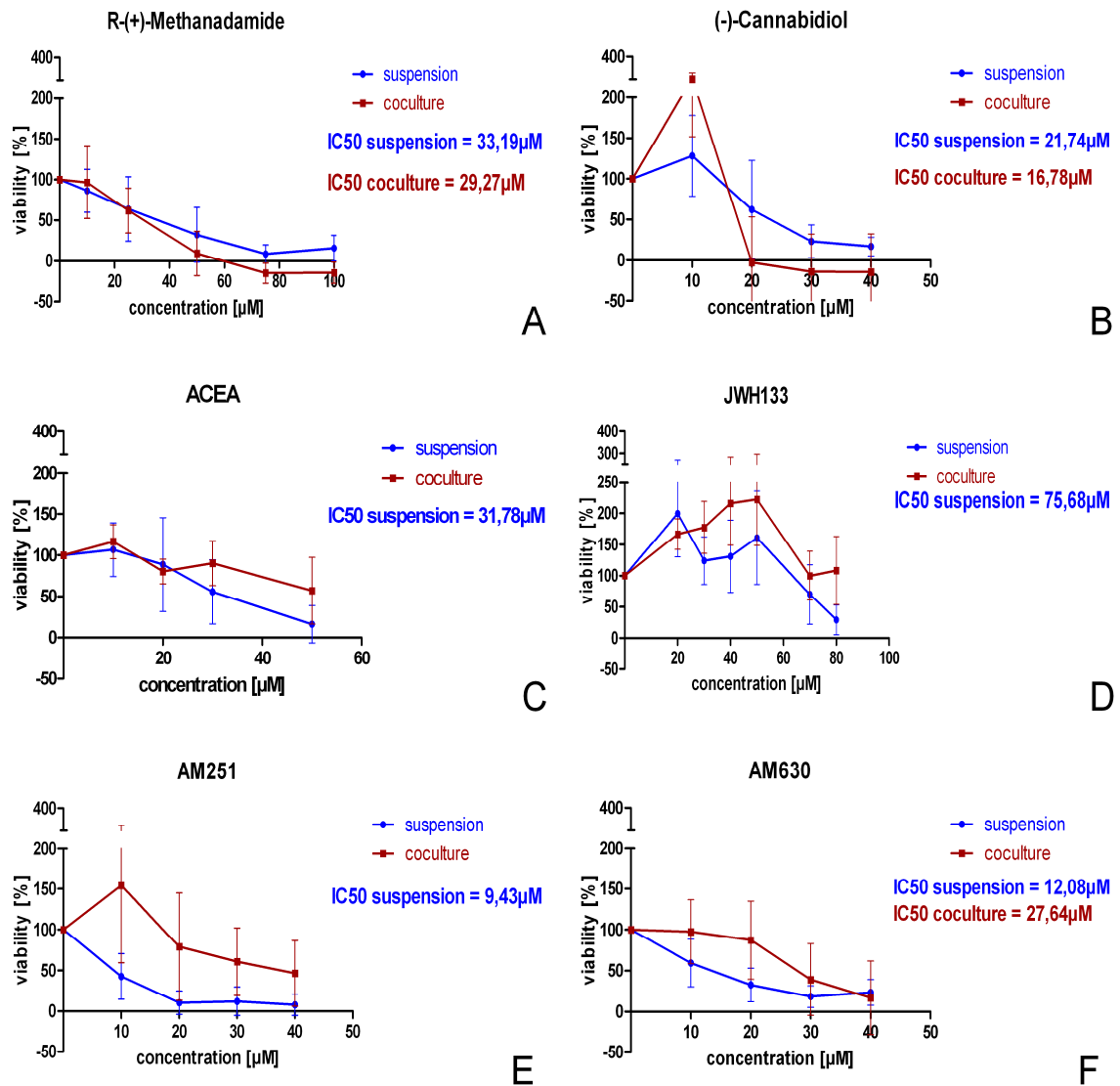


Figure 20: Impact of cannabinoids on primary cells from CLL patients.

Cells were incubated with different concentrations of compounds in suspension culture and coculture for 48h. Viability was calculated relative to vehicle control.

A: CLL primary cells incubated with R-(+)-Methanandamide (N=10).

B: CLL primary cells incubated with (-)-Cannabidiol (N=18).

C: CLL primary cells incubated with ACEA (N=16).

D: CLL primary cells incubated with JWH133 (N=16).

E: CLL primary cells incubated with AM251 (N=16).

F: CLL primary cells incubated with AM630 (N=16).

Note different scales on X-axis.

In the coculture incubation setting, CLL cells were less sensitive toward the cannabinoids and the IC₅₀ value could not be calculated in coculture during incubation with ACEA, JWH133 and AM251. The IC₅₀ value was slightly higher in coculture compared to suspension culture for (R)-(+)-Methandamide incubation (Fig. 20A+C+D+E). Incubations with (-)-Cannabidiol and AM630 were exceptions for the observed resistance in coculture (Fig. 20B+F). Their IC₅₀ values in coculture were similar to the sensitivity of the M2-10B4 after 48h incubation. Comparing the IC₅₀ for suspension culture, JWH133 the CNR2 agonist had the lowest impact on the cells (75,68μM) and the CNR1 antagonist AM251 the strongest (9,43μM) (Fig. 20D+E).

4. Results

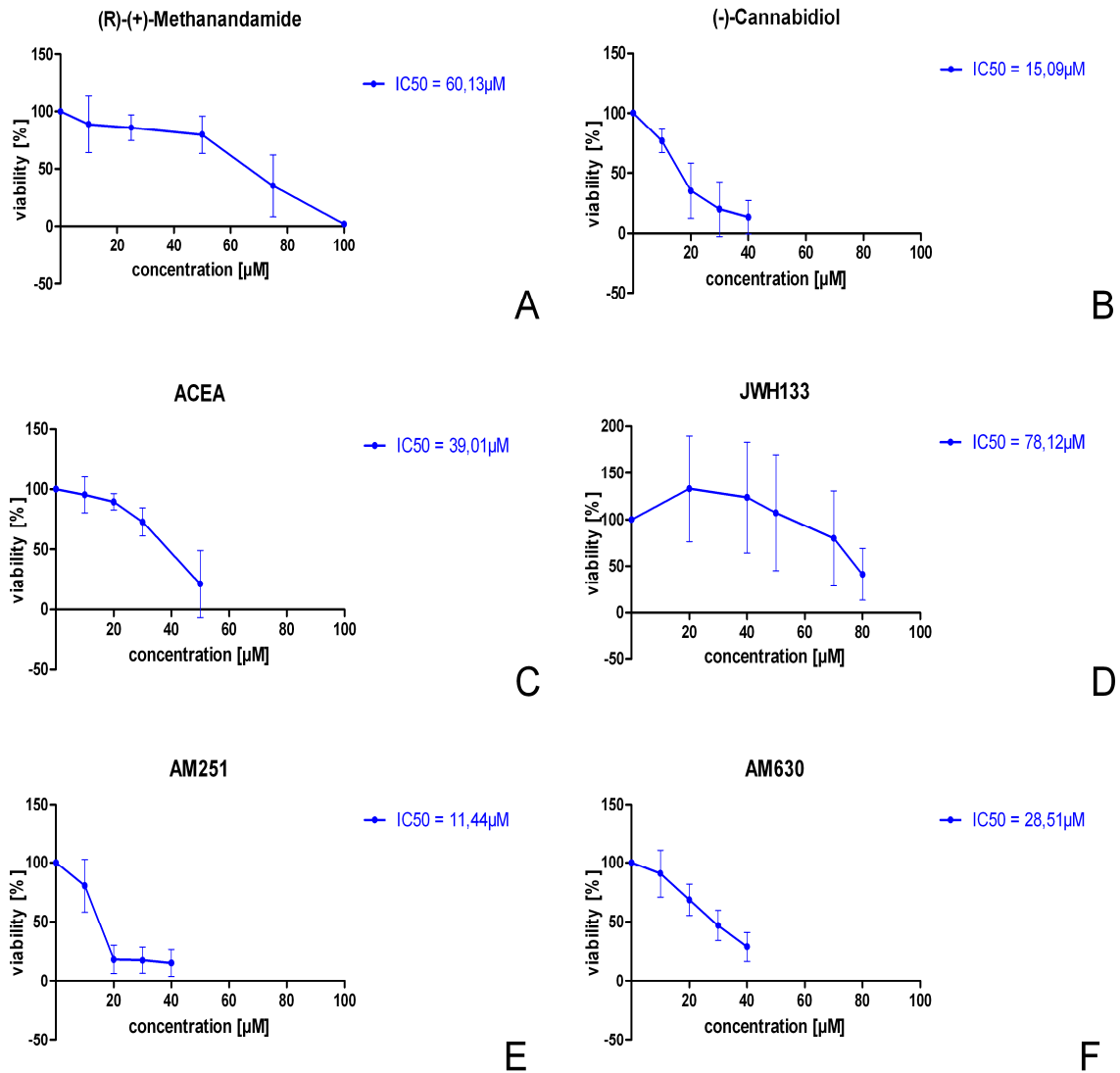


Figure 21: Impact of cannabinoids on primary cells from healthy donors (HD).

Cells were incubated with different concentrations of compounds in suspension culture for 48h. Viability was calculated relative to vehicle control.

A: HD primary cells incubated with R-(+)-Methanandamide (N=2).

B: HD primary cells incubated with (-)-Cannabidiol (N=3).

C: HD primary cells incubated with ACEA (N=3).

D: HD primary cells incubated with JWH133 (N=3).

E: HD primary cells incubated with AM251 (N=3).

F: HD primary cells incubated with AM630 (N=3).

Note the different scale of the y-axis for D (JWH133).

In addition to CLL cells, PBMC from healthy donors (HD) were tested to evaluate cytotoxicity of the compounds in healthy cells. All cannabinoids led to a dose dependent decrease in viability (Fig. 21). The sensitivity and therefore the IC₅₀ values differed between compounds. Thus, incubation with AM251 and (-)-Cannabidiol had the strongest impact on the cells, whereas JWH133 and R-(+)-Methanandamide exerted only moderate cytotoxicity with high IC₅₀ values (Fig. 21). For better comparison of the effects of cannabinoids on the different cells used, the IC₅₀ values after 48h incubation were listed in a table (Table 4).

4. Results

Table 4: Comparison of sensitivity toward cannabinoids. IC50 values in μM after 48h incubation. <i>NR = IC50 not reached.</i>						
	(R)-(+)- Methandamide	(-)- Cannabidiol	ACEA	JWH133	AM251	AM630
MEC-1	50,76	14,52	19,41	57,31	17,04	22,58
MEC-2	48,15	16,01	34,86	57,53	13,86	19,56
JURKAT	44,91	14,27	33,45	43,45	15,78	9,71
A-549	96,74	11,36	9,74	52,79	NR	NR
CLL suspension	33,19	21,74	31,78	75,68	9,43	12,08
CLL coculture	29,27	16,78	NR	NR	NR	27,64
M2-10B4	34,55	13,52	NR	NR	NR	28,27
HD	60,13	15,09	39,01	78,12	11,44	28,51

Considering the overall cytotoxic effects among the different cell types used, (-)-Cannabidiol had the strongest and JWH133 the lowest impact on viability. Among cell lines, A-549 showed some kind of resistance except for (-)-Cannabidiol and ACEA. The other three cell lines MEC-1, MEC-2 and JURKAT were equally affected by the different cannabinoids. Comparing the CLL cell lines MEC-1 and MEC-2 with primary CLL cells in suspension, the primary cells were similarly susceptible to ACEA, less for JWH133 and (-)-Cannabidiol and more affected by AM251, AM630 and (R)-(+)-Methandamide. In coculture, the CLL cells were more resistant to the cannabinoids except for (-)-Cannabidiol and AM630. For those two compounds, however, as can be seen in table 4, the feeder cells, M2-10B4, were affected to an equal extent. Compounds that have a higher influence on the cocultured CLL cells than on the primary cells of healthy donors are of interest as potential therapeutic drug. But only (R)-(+)-Methandamide was more cytotoxic for CLL cells than for the healthy cells (Table 4).

4. Results

To evaluate a potential association between sensitivity to cannabinoids and gene expression, IC50 values were plotted versus mRNA expression levels. In some cases, IC50 values could not be calculated due to that 50% reduction in viability was not reached, the compound was not tested on the CLL sample, or additional data points would have been needed for calculation.

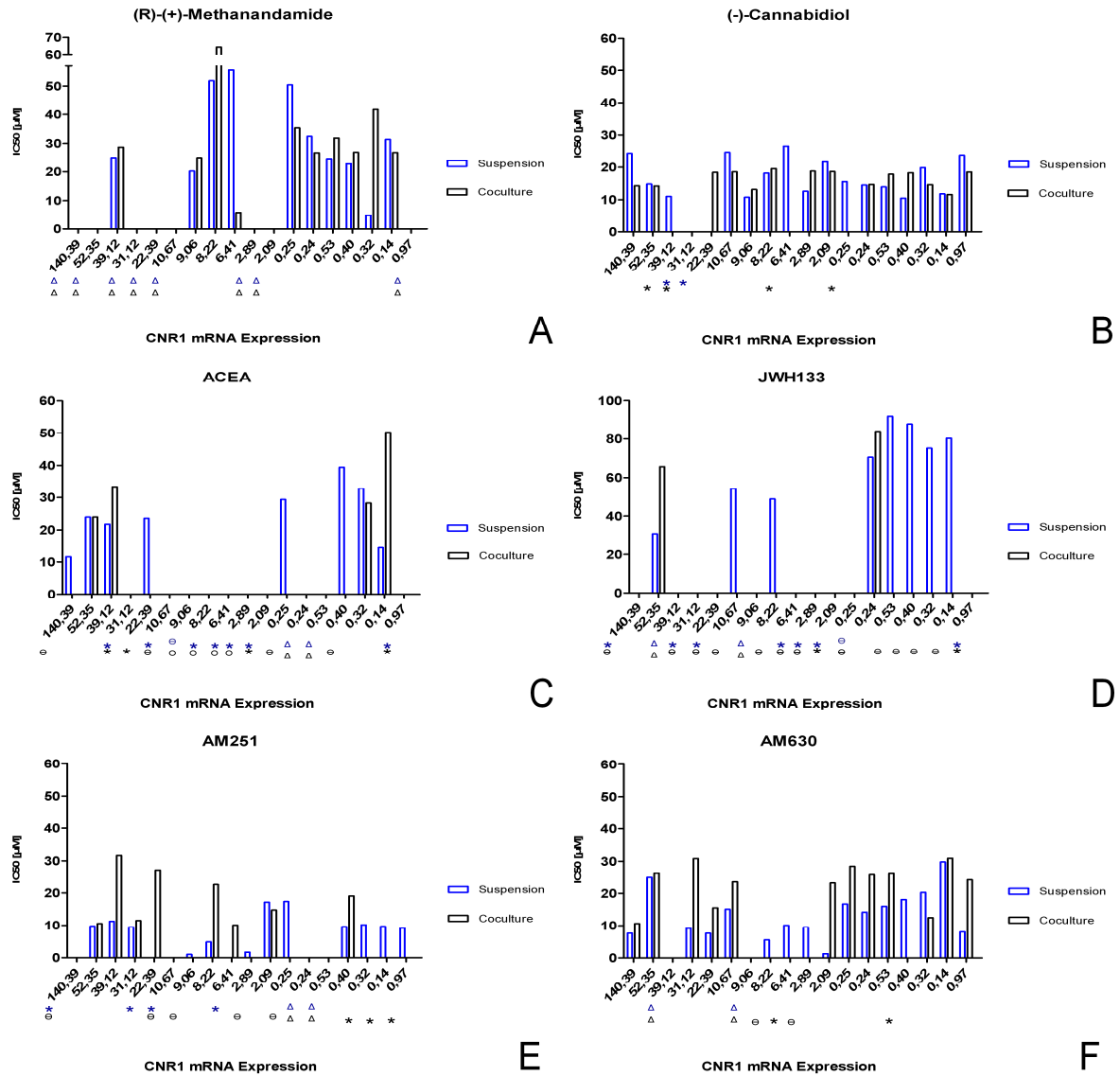


Figure 22: Decrease in viability in relation to CNR1 mRNA expression levels.

CLL primary cells were incubated in suspension culture and in coculture with M2-10B4.

A: CLL primary cells incubates with R-(+)-Methanandamide (N=10).

B: CLL primary cells incubates with (-)-Cannabidiol (N=18).

C: CLL primary cells incubates with ACEA (N=16).

D: CLL primary cells incubates with JWH133 (N=16).

E: CLL primary cells incubates with AM251 (N=16).

F: CLL primary cells incubates with AM630 (N=16).

Note different scales on y-axis. IC50 values could not be calculated due to:

Θ: 50% viability was not reached; Δ: Not tested for the compound; *: Too few data points.

Figure 22 shows relative CNR1 mRNA expression levels vs. IC50 values for the compounds tested. For incubation with (R)-(+)-Methandamide, it can be seen that patients with a high CNR1 mRNA expression showed similar IC50 values as patients with low CNR1 mRNA expression (Fig. 22A). The same results was shown for the incubation with (-)-Cannabidiol (Fig. 22B). Incubation of cells in coculture with ACEA and JWH133 did not induce a reduction of viability of > 50% so that IC50 values could not be calculated in most cases. However, comparing IC50 values in suspension culture with CNR1 expression levels, no association could be observed (Fig. 22C+D). Also for the incubation with the antagonists AM251 and AM630 an association between CNR1 expression and sensitivity toward the compounds could not be shown (Fig. 22E+F).

4. Results

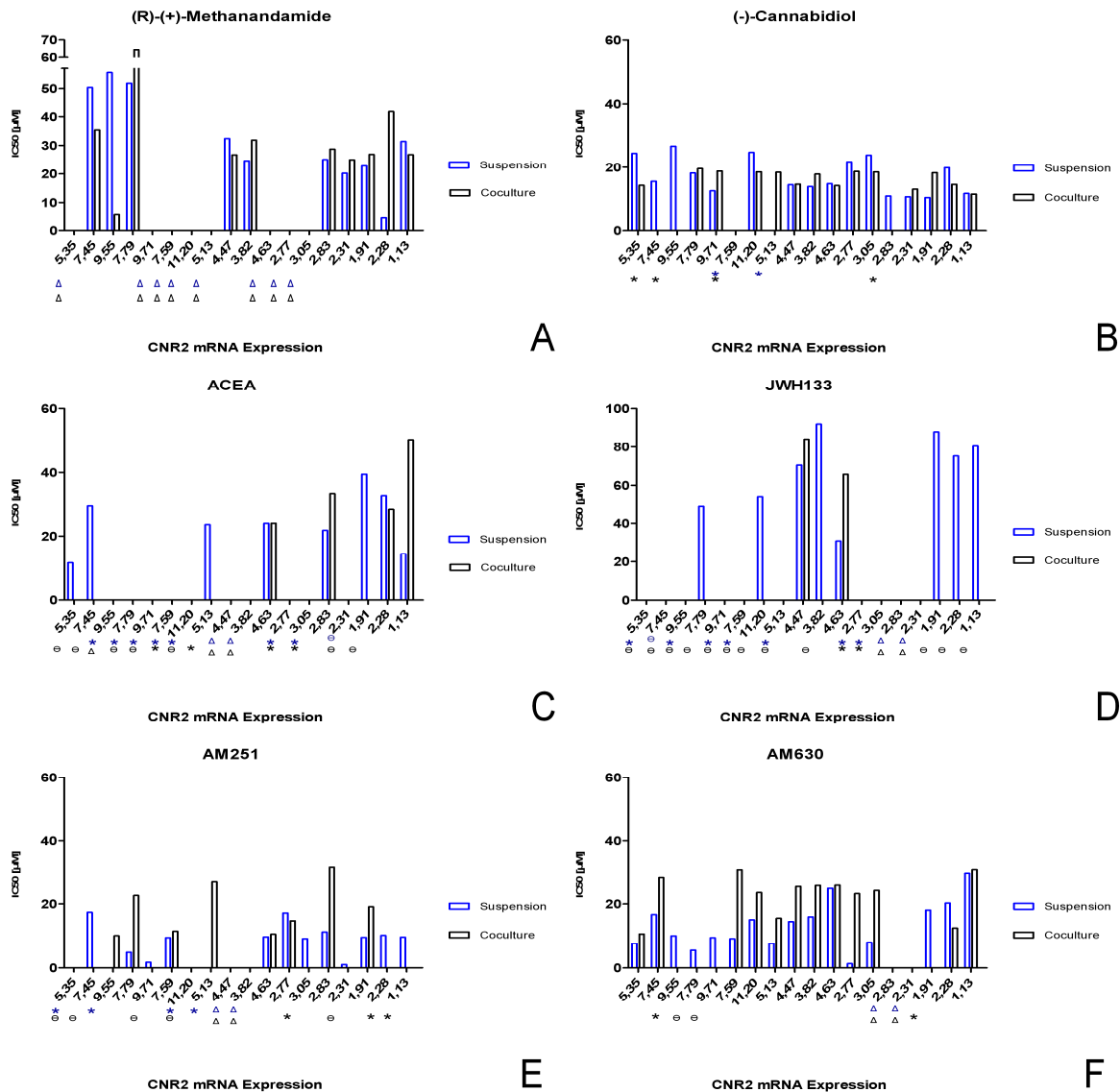


Figure 23: Decrease in viability in relation to CNR2 mRNA expression levels.

CLL primary cells were incubated in suspension culture and in coculture with M2-10B4.

A: CLL primary cells incubates with R-(+)-Methanandamide (N=10).

B: CLL primary cells incubates with (-)-Cannabidiol (N=18).

C: CLL primary cells incubates with ACEA (N=16).

D: CLL primary cells incubates with JWH133 (N=16).

E: CLL primary cells incubates with AM251 (N=16).

F: CLL primary cells incubates with AM630 (N=16).

Note different scales on y-axis. IC₅₀ values could not calculated due to:

Θ: 50% viability was not reached; Δ: Not tested for the compound; *: Too few data points.

Since there was no clear association between CNR1 mRNA expression and the IC₅₀ values, the association between the CNR2 mRNA expression and sensitivity to the compounds was also tested (Fig. 23). For (R)-(+)-Methanandamide and (-)-Cannabidiol there was no association between the high and low CNR2 expressing samples and the corresponding IC₅₀ values (Fig. 23A+B). ACEA and JWH133 showed similar results as for the CNR1 expression meaning that in coculture IC₅₀ values were mostly not available, since 50% reduction of viability was not reached. For incubation in suspension culture, no difference in

sensitivity in relation to CNR2 expression levels was found (Fig. 23C+D). Finally there was no association between the CNR2 expression and viability reduction for incubation with AM251 and AM630 (Fig. 23E+F).

4.10. Determining CXCR4 levels in CLL by FACS

The microenvironment plays a critical role in CLL providing survival signals and thus contributing to resistance to treatment. Particularly the CXCR4/CXCL12 axis has been shown to contribute to survival of leukemic cells. In the literature, CLL cells are described to express CXCR4. Therefore, a representative FACS Screen for CXCR4 expression on CLL cells was done.

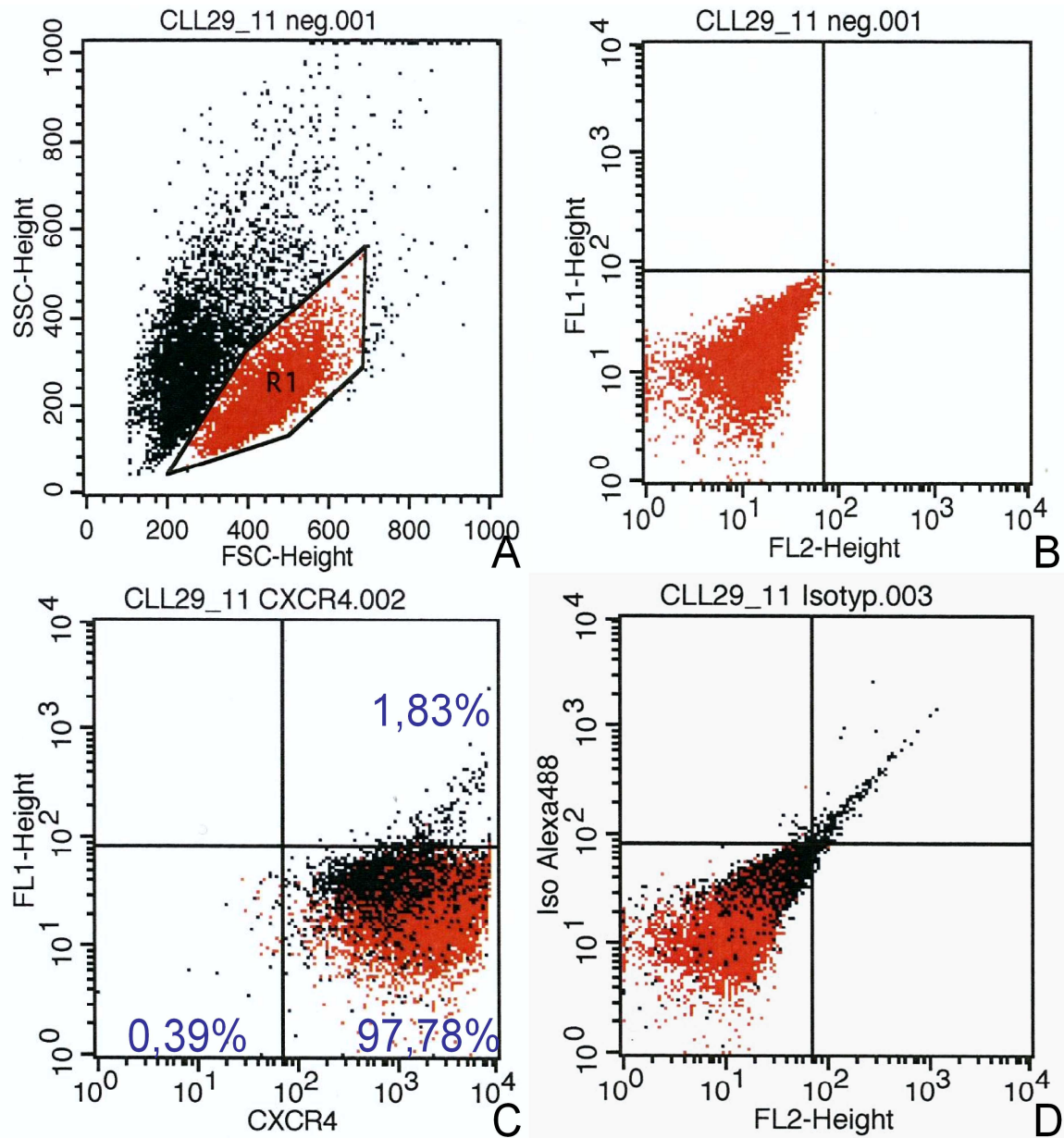


Figure 24: Representative FACS analysis for CXCR4.

Cells were stained for CXCR4 with Mouse Anti-Human CD184 PE, mouse IgG2a κ was used as isotype control with Alexa Fluor488-labeled goat anti-mouse as secondary antibody.

A: Control in FSC and SSC.

B: Control auto fluorescence.

C: CXCR4 stained.

D: Isotype control.

Figure 24 shows one CLL sample analysed with a flow cytometer FACSscan. In forward scatter (FSC) and sideward scatter (SSC) each dot represents one CLL cell which is located on the plot due to its size and granularity. Size was depicted by FSC and granularity by SSC. Cells were gated (R1) due to their size and granularity (Fig. 24A). In a next step the threshold for auto fluorescence, which was detected by detector FL1 and FL2, was set (Fig. 24B). The CXCR4 antibody was PE labelled which was detected in detector FL2. Figure 24C shows that 97,78% of the gated cells (red coloured) are located in the lower right and therefore are

CXCR4 positive. Therefore the CLL cells tested in the migration assay express the CXCR4. An additional isotype control was performed to ensure that the used mouse IgG2a antibody used for CXCR4 staining did not bind unspecific to the CLL cells. All cells in the isotype control were located in the lower left and are therefore negative (Fig. 24D). They showed no signal for Alexa Fluore488 in the FL1 detector. Therefore the CXCR4 detection was specific.

4.11. Microenvironmental model for drug treatment model

Cannabinoids have been implicated in interfering with the CXCL12/CXCR4 axis in T-cells. A set of experiments was conducted to explore additive effects of cannabinoids with fludarabine, a standard drug in CLL treatment, as has been described for AMD3100, a CXCR4 targeting antibody.

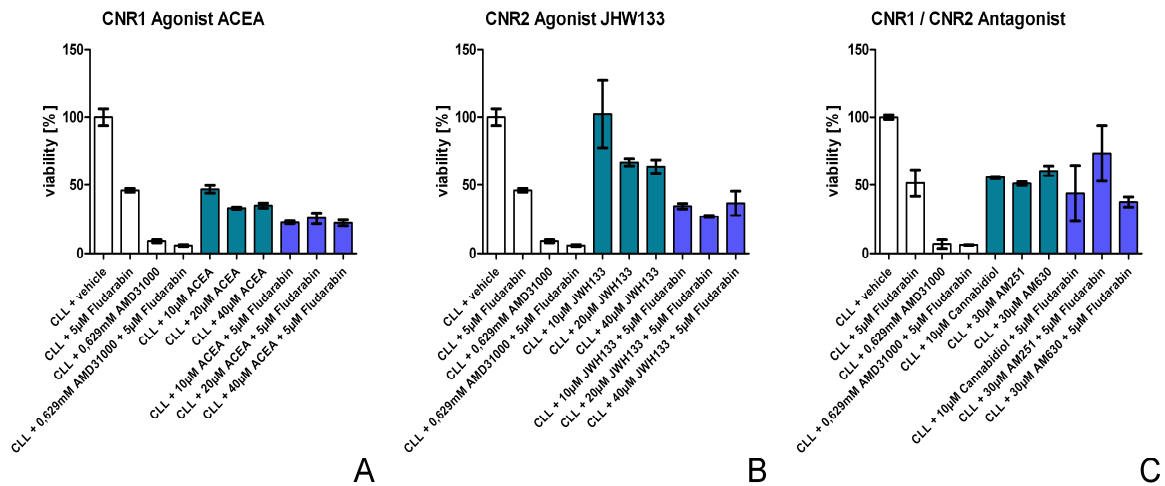


Figure 25: Interference of cannabinoids with CXCR4/CXCL12 interaction.

Decrease in viability in CLL primary cells incubated 48h with 5µM fludarabine after 30 minutes pre-incubation with AMD3100 or CNR1 agonist (ACEA) or CNR2 agonist (JWH133). Mean values and SD were calculated from triplicates in 96well plates.

A: Pre-incubation with CNR1 agonist (ACEA) (N=1).

B: Pre-incubation with CNR2 agonist (JWH133) (N=1).

C: Pre-incubation with CNR1 antagonist (AM251) or CNR2 antagonist (AM630), CNR1 antagonist & CNR2 inverse agonist ((-)-Cannabidiol) (N=1).

Figure 25 depicts the viability of PBMCs of two CLL patients after 48h incubation in different settings. CLL sample incubated with 0,5% DMSO were set as 100% viability. In all settings fludarabine reduced viability to 50% (Fig. 25A-C). The strongest impact on viability, even more than fludarabine, had the CXCR4 inhibitor, viability was reduced to 9%. This effect was not much enhanced when 5µM fludarabine were added for 48h. Incubation with the CNR1 agonist ACEA at all three concentrations (10µM, 20µM and 40µM) had a similar impact as fludarabine alone (33-47% viability) (Fig. 25A). ACEA showed only a minimal additive effect to fludarabine (22-25%) when fludarabine was added after ACEA pre-treatment (Fig. 25A).

A similar result could be seen for the incubation with the CNR2 agonist JWH133. Pre-incubation with JWH133 resulted in a reduction of viability to ~66%. Viability for the three tested concentrations was similar, concerning the high SD (standard deviation) within the 10µM setting. By adding fludarabine the viability was reduced to 27-37% (Fig. 25B). Thus fludarabine and JWH133 had a small additive effect. Finally the same tests were done for the CNR1 and CNR2 antagonists. Pre-incubation with 10µM (-)-Cannabidiol, 30µM AM251 or 30µM AM630 reduced the viability of the CLL cells to 51-61%. The additional incubation with fludarabine led only to a slight reduction in viability for the (-)-Cannabidiol and AM630 pre-incubated cells. There was no additional reduction in viability by adding fludarabine, when cells were previously incubated with the CNR1 antagonist AM251 (73% viability) (Fig. 25C).

4.12. Impact of cannabinoids on migration of CLL cells towards CXCL12

To further investigate a potential role of cannabinoids in interference with the microenvironment, migration assays were performed. Aim was to test whether CLL cells expressing the CXCR4 migrate toward the ligand CXCL12 and how incubation with cannabinoids alters the migration behaviour.

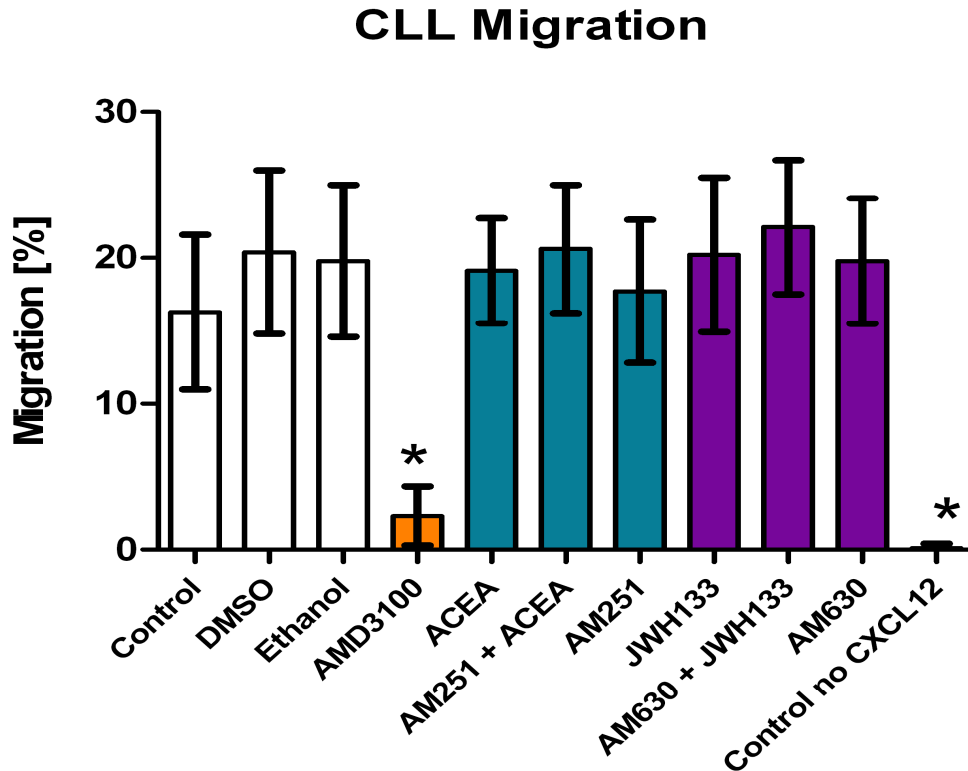


Figure 26: Impact of cannabinoids on migration of CLL primary cells (N=5) towards CXCL12.

Total cell number of cells in bottom well plus cells in transwell inserts were set as 100%. Percent of migrated cells was calculated by dividing the number of cells in the bottom well by the number of total cells. The *p*-values were calculated by Mann-Whitney U test.

P-value $\leq 0,05$ significant.

Figure 26 shows that 16% of untreated primary CLL cells migrated towards CXCL12 compared to up to 20% in the vehicle controls (DMSO or Ethanol). In the negative control, CXCL12 was not added to the bottom well, only 0,1% of cells migrated into the bottom well compared to control ($p=0,007$). When cells were incubated with the CXCR4 inhibitor AMD3100, migration was reduced significantly (2,3% migration, $p=0,014$) compared to control. Incubation with the CNR1 agonist ACEA as well as incubation with the CNR2 agonist JWH133 led to migration similar to the vehicle treated cells. Incubation with the cannabinoid receptor antagonist, AM251 for CNR1 and AM630 for CNR2, respectively, resulted in only slightly decreased migration compared to vehicle control. When cells were pre-treated with the antagonist before incubation with the corresponding agonist, migration was slightly increased, however, not statistically significant to incubation either with agonist or with antagonist alone. Thus, and in contrast to AMD3100, the cannabinoids did not exert a significant effect on the migration of the primary CLL cells towards CXCL12.

5. Discussion

Cannabinoids are the unique active compound of *Cannabis sativa* L. (Velasco, Sánchez, & Guzmán, 2012): Preparations of this plant have been used in medicine for centuries (Velasco et al., 2012). Today it is known that cannabinoids exert an anti-proliferative and pro apoptotic effect on cancer cells (Guzmán, 2003). In this study, emphasize was put on Chronic lymphocytic leukemia (CLL). CLL is the most prevalent leukemia of adults in Western countries (Müller-Hermelink et al., 2008). The clinical course is heterogeneous (Malavasi et al., 2011), and for the patients, who do not response to treatment and progress rapidly, novel therapeutic options are needed. Due to the cytotoxic effects reported for cannabinoids and the need for therapeutic drugs in CLL, we wanted to study the cannabinoid receptors and their ligands in this malignancy in more detail.

As first aim of this study, we screened a cohort of CLL patients for the expression of cannabinoid receptor 1 (CNR1) and cannabinoid receptor 2 (CNR2).

As typical for CLL, the percentage of female was slightly lower than that of male (1:1,5), the median age was 62 years. In comparison, Müller-Hermelink et al. observed the median age for CLL at 65 years with a female:male ratio of 1:1,5-2 (Müller-Hermelink et al., 2008). Delago et al. showed in a study dealing with deletion of chromosome 17p a median age of 68 years in 294 patients (Delgado et al., 2012).

Literature about CLL reports that about 80% of the CLL cases show cytogenetic abnormalities, 50% of the cases have a deletion of chromosome 13q, 20% trisomy of chromosome 12 and deletions of chromosome 17, 11 and 6 are found (Müller-Hermelink et al., 2008). Similar abundancies of these cytogenetic abnormalities were described by Döhner et al. with worse prognosis for deletion of 17 and 11 (Döhner et al., 1999).

In comparison, in this cohort 74% of the patients showed chromosomal aberrations and display similar percentage of deletions and rearrangements as described in the literature.

Also, 40-50% of patients have unmutated immunoglobulin heavy chain variable region genes (IGHV) (Müller-Hermelink et al., 2008). In this cohort 45,6% of the patients showed unmutated IGHV genes.

Thus, our cohort of 102 patients can be considered to be a well representative group of CLL patients.

After normalization of mRNA expression to CD19+ sorted healthy donors, an elevated CNR1 expression with a high variability within the cohort could be observed. Also CNR2 expression was increased compared to healthy donors. In CLL, Gustafson et al. described already an enhanced expression of the two receptors compared to control tissue, with only a moderate correlation between the expression levels of the two receptors (Gustafsson et al., 2008).

Furthermore, comparing the expression medians, a three times higher expression of CNR2 was observed among CLL patients compared to CNR1.

Such a 3:1 ratio (CNR2 :CNR1) was also found in PBMCs from healthy groups of different age and sex (Nong et al., 2002), and in mantle cell lymphoma (MCL) cells, CNR1 was higher expressed than CNR2 (Richardson et al., 2007).

Since we found an overexpression of the two cannabinoid receptors in CLL, the second aim of the study was to evaluate the potential of the cannabinoid receptor mRNA expression levels as prognostic makers. Using median expression as cut-off, CLL patients were split into *CNR* high and *CNR* low expressing cohorts and the data were analyzed accordingly. The results revealed that high CNR1 expression was significantly associated with Binet stages B + C. These later stages B and C reflect advanced and/or progressive disease, with more involved lymph nodes or organomegaly and anemia/thrombocytopenia (Hallek et al., 2008). Furthermore the results showed an association of high CNR1 expression with the IGHV mutational status, the CNR1 high expression group showed a higher percentage of unmutated IGHV genes. Patients with an unmutated sequence have a worse outcome (Müller-Hermelink et al., 2008). The CNR1 high expression group showed also a shorter lymphocyte doubling time (LDT), with LDT under one year being associated with adverse prognosis (Müller-Hermelink et al., 2008). Finally, high CNR1 expression was associated with high CD38 expression. High CD38 expression is associated with worse clinical outcome (Malavasi et al., 2011). There was no association between CNR1 expression and any of the cytogenetic markers.

Kaplan-Meier survival analysis revealed a significantly shorter overall and treatment free survival in the CNR1 high expression group. For progression free survival no significant correlation was found. All together, the data support that CNR1 high expression is a novel prognostic maker. Multivariate analysis demonstrated that CNR1 as prognostic marker was independent of CD38 expression, Binet stage and LDT, but associated with mutation status.

On the other hand, while CNR2 is higher expressed in CLL patients, high mRNA expression was not associated with any of the tested prognostic marker. Also overall, treatment free and progression free survival was not significantly different when comparing high and low expressing groups. A higher expression of CNR2 in CLL compared to CNR1 was also found by Piszcz et al. on the surface of neoplastic lymphocytes from CLL patients detected by flow cytometry (Piszcz et al., 2004). An explanation for the higher CNR2 expression might be that CLL is a disease of neoplastic B cells with an increase in the clonal B cells in the peripheral blood (Hallek et al., 2008). B lymphocytes show the highest CNR2 expression within normal PBMC (Mackie and Stella, 2006). Hence, the number of cells with high CNR2 expression is increased in CLL patients. The prognostic value of CNR2 was also tested in diffuse large B-cell lymphoma (DLBCL). The analysis of 104 DLBCL samples in a tissue microarray found

no correlation between CNR2 expression and clinical outcome (Rayman et al., 2011). In contrast, for human hepatocellular carcinoma (HCC) a significant disease-free survival for patients with high CNR1 and CNR2 expression was demonstrated and both cannabinoid receptors correlated with two clinicopathological markers (Xu et al., 2006). The authors recommended both receptors as potentially prognostic factors.

Since the present study revealed no association between CNR2 expression and survival, for CLL only the CNR1 expression seems to be of importance.

The next aim was to evaluate whether the protein expression levels follow CNR1 and CNR2 mRNA expression levels. In this study westernblots were used to determine protein expression in CLL patients with CNR1 high/low and CNR2 high/low mRNA expression levels. As positive control, recombinant CNR1 and recombinant CNR2 protein was used. Additional positive controls were the cell lines A-549 and JURKAT. A-549 cells, which only express CNR1, were used for the datasheet of the CNR1 antibody from GENTEX and the JURKAT cells were already used by others as a positive control for CNR2 expression (Small-Howard et al., 2005).

For CNR1 protein, it was difficult to detect bands of the correct size. The amino acid (AA) length of the CNR1 is 472AA (Howlett et al., 2002), therefore the predicted size is 53kDa (Daaka et al., 1996). Preliminary data led to the decision to use the polyclonal CNR1 antibody from Thermo Scientific, since antibodies from two other manufacturers resulted in less intense bands at the predicted size (data not shown). In the datasheet of the CNR1 antibody used, the detection of one band at ~60kDa and lighter bands at ~23kDa, ~72kDa, ~180kDa is reported. In fact, the antibody detected a big band at the calculated ~53kDa for the recombinant protein and a lighter band of the same size in A-549 cells. In the patients' samples and other cell lines a band at the in the datasheet described 72kDa were also detected. In all samples, additional bands were detected at under 34kDa, 130kDa and 170kDa, which were not reported in the datasheet. Other groups described similar problems for the detection of CNR1 at the calculated size (~53kDa) with the use of different polyclonal antibodies. Grimsey et al. tested a number of N-terminal CNR1 polyclonal antibodies from different manufactures and none detected bands between 53kDa and 60kDa (Grimsey et al., 2008). Take together; this suggests an inherent problem regarding the detection of CNR1 protein with the commercially available CNR1 antibodies. There are several possible explanations for the different band sizes. A slightly higher molecular weight than 53kDa could be explained by glycosylation. Three glycosylation sites at the N-terminus were reported for CNR1 (Shire et al., 1995). Larger band sizes than the detected 130 – 170kDa were also described by others. Wager-Miller et al. developed an antibody against CNR1 401-473AA, which detected bands at 53kDa, 60kDa as well as at 160kDa and 200kDa (Wager-Miller et al., 2002). They suggested that the high molecular weight forms were trimeric and tetrameric

forms of the CNR1 receptor. Another explanation could be that the CNR1 receptor builds dimers with other receptors. A study revealed that CNR1 exists as receptor complex with the Dopamin D2 receptor (Kearn et al., 2005). At a first thought, the bands, smaller than 53kDa might be due to splice variants of the CNR1, in addition to the full length CNR1, two splice variants were reported, CNR1a and CNR1b (Ryberg et al., 2005). Where CNR1a is described to be by 61AA shorter (Shire et al., 1995) and CNR1b shows an in-frame deletion of 33AA (Ryberg et al., 2005). Concerning the molecular weight, CNR1a has a calculated size of 45,9kDa and CNR1b of 49,1kDa

(<http://www.proteinatlas.org/ENSG00000118432/gene> visited on 13.11.12). Thus, the splice variants cannot explain the detected bands at under 34kDa, which remain questionable.

In order to compare protein and mRNA expression, two controls were used. As reported previously and as detected by us (Small-Howard et al., 2005; Datasheet CNR1 GENTEX), A-549 cells do not express CNR2 mRNA and JURKAT cells lack CNR1 expression. Also other groups found that naive JURKAT cells express only low levels of CNR1 transcripts (Börner et al., 2007; Daaka et al., 1996). This might be changed by changing the activation status of the cell. Thus, CNR1 mRNA expression was enhanced in JURKAT cells after IL-4 (Interleukin-4) (Börner et al., 2007) or THC treatment (Börner et al., 2007). Bands of similar size were detected for the proteins of the two cell lines using the CNR1 antibody. Since only the CNR1 and not the CNR2 recombinant protein showed an intense band at 53kDa, the used antibody seemed to be specific for CNR1, which would suggest that the CLL cells and the two cell lines do not express sufficient amounts of the protein to be detectable. In this study, although patients with both high and low CNR1 mRNA expression were tested, no association between mRNA expression and protein expression levels could be observed. Due to the problems in detection of bands at the correct size and the independence of band patterns from mRNA expression levels, the protein levels of CNR1 could not be evaluated.

As for the CNR1, CNR2 high/low mRNA expressing patients were screened to determine the CNR2 protein expression in CLL. Similarly, the cell lines A-549 and JURKAT together with the recombinant CNR1 and recombinant CNR2 proteins were used as controls.

Again, the westernblots revealed problems with regard to the detection of CNR2 protein. The amino acid length for the CNR2 is 360AA (Howlett et al., 2002), which predicts a size of 38kDa for the protein (Small-Howard et al., 2005). In the datasheet a band at 39,7kDa is described for CNR2 (ABGENT) antibody, which corresponds with the ~40kDa band observed for the recombinant CNR2 protein. Although this antibody is monoclonal, additional bands at 72kDa and 130kDa were detected with recombinant CNR2 protein. Since the antibody did not show any bands for the CNR1 recombinant protein, the detection of CNR2 protein seemed to be specific. In the cell lines JURKAT and A-549 and in the tested CLL samples similar bands were detected at 45kDa, 55kDa, 72kDa together with a light band at 40kDa.

Considering that A-549 cells do not express CNR2 mRNA, it is rather unlikely that the CNR2 antibody detected CNR2 protein only. Also for the CLL samples, no correlation could be observed between protein band intensity and mRNA expression level. The 40kDa band was more intense for the recombinant protein and less intense for all samples tested (cell lines and CLL samples), independent of mRNA expression level. One explanation could be that the cells do not express sufficient CNR2 protein for detection, and that protein expression might be enhanced in activated cells. For macrophages it could be shown that expression of CNR2 protein was undetectable in resident peritoneal macrophages, but that it was expressed to a high extent in thioglycolate-elicited macrophages (Carlisle et al., 2002). Since in macrophages protein expression appeared to follow mRNA expression (Carlisle et al., 2002), and in this study protein expression was not associated with mRNA expression, the problem might not be due to low protein expression but to the antibody used. This leads to the question what the monoclonal antibody detects at 45kDa, 55kDa, 72kDa and 130kDa. A second isoform of CNR2 has been reported which is expressed to another extent in different tissues (Liu et al., 2009). Still, CB2 antibodies that are commercial available, could not distinguish the two isoforms (Liu et al., 2009) and the predicted size, referring to the human protein atlas is for both splice variants 39,7kDa

(<http://www.proteinatlas.org/ENSG00000188822/gene> visited 13.11.12). Another explanation might be the sequence homology between the two receptors. Both receptors show 68% amino sequence homology within the transmembrane domain and 44% throughout the whole protein (Jean-Gilles et al., 2010). The data suggest that the CNR2 antibody also detects the CNR1 protein, due to the bands at 45kDa, 55kDa, 72kDa and 130kDa, since for CNR1 recombinant protein bands were also detected at 52kDa, 72kDa and 130kDa.

In conclusion, using westernblot for direct protein detection of CNR1 and CNR2 it was not possible to detect an association between mRNA level and protein level. While in prostate cancer, the two cannabinoid receptors are expressed to a higher extent than in normal prostate epithelial cells (Olea-Herrero et al., 2009), in CLL an enhanced expression of the *CNRs* on protein level could not be shown. In future experiments, the specificity of the antibodies could be tested by using blocking peptides as described by Blázquez (Blázquez et al., 2006). Further solutions to overcome the detection problems with the available antibodies might be to evaluate downstream activation in the signal cascade of the cannabinoid receptors. Thus, to test whether an mRNA increase of CNR1 after receptor activation is followed by an increase in CNR1 protein, CNR1 mediated MAPK phosphorylation was detected by westernblot (Börner et al., 2007).

The third aim of the study was to evaluate the cytotoxic effect of CNR1 and CNR2 specific agonists and antagonists on cell lines per se and with regard to CNR1 and CNR2 expression levels. MEC-1 and MEC-2 were used, since they are CLL cell lines (Stacchini et al., 1999).

A-549 and JURKAT were used as positive controls for CNR1 and CNR2, respectively. For all tested cell lines there was a time and concentration depended reduction in viability, with a difference in sensitivity between the cell lines. The exception for a time dependent effect was (-)-Cannabidiol, the compound most effective overall in the study, for which similar IC50 values were determined for the two incubation times. A time dependent drop was also shown for ACEA, AM251 and AM630 in human pancreatic cancer cells (Fogli et al., 2006). Differences in sensitivity were reported by two groups. A much lower IC50 was found for (R)-(+)-Methandamide after 48h incubation in a human prostate cell line, with ~50µM for the cell lines in this study and 10µM for the prostate cell line (Olea-Herrero et al., 2009). JWH133, which showed high IC50 values in this study (~50µM), inhibited already significantly at a concentration of 100nm the secretion of IL12-p40 by LPS/INFγ stimulated macrophages (Correa et al., 2005).

In the cell lines studied here, no clear association between mRNA expression levels and sensitivity could be observed. JURKAT cells, which do not express CNR1, had a comparable IC50 for the CNR1 agonist ACEA compared to MEC-1 and MEC-2 that express both cannabinoid receptors. A-549 cells which only express CNR1 displayed the highest reduction in viability, but only after 48h. Overall, these cells were the least affected cell line cells in this study. (R)-(+)-Methanadamide, the analogue of the endocannabinoid anandamide and a CNR1 agonist (Pertwee, 2006), and JWH-133, CNR2 agonist (Pertwee, 2006), showed the highest IC50 values in all four cell lines. These data would suggest that cannabinoids that act only on one of the two cannabinoid receptors are less cytotoxic. However, this is in contrast to another study, where MEC-1 and MEC-2, which express both receptors to some extent, were more affected by (R)-(+)-Methandamide than cell lines with either low CNR1 or CNR2 expression (SK-MM-2; Raji, Namalwa) (Gustafsson et al., 2008). In addition, ACEA, a selective CNR1 agonist (Hillard et al., 1999) and a compound that acts only on one receptor, such as JWH133 and (R)-(+)-Methandamide, showed even lower IC50 values than JWH133 and (R)-(+)-Methandamide. (-)-Cannabidiol was the most effective compound in this study, and, considering that this compound acts as inverse CNR2 agonist and CNR1 antagonist (Thomas et al., 2007), one would assume that this could be due to the fact that it acts on both receptors. On the other hand, AM251 was more effective than AM630, at least in MEC-1 and MEC-2 cells. Considering that AM630 acts as CNR2 antagonist (Pertwee et al., 1995), as inverse CNR2 agonist and weak CNR1 agonist (Ross et al., 1999), and as inverse CNR1 agonist (Landsman et al., 1998), thus influencing both receptors, while AM251 only antagonizes CNR1, the hypothesis that compounds acting on both receptors have higher impact on cells definitely is not straight forward.

All together, the data suggest both a cell type specific and a compound specific cytotoxic activity regardless of mRNA expression. A reason for the independence of viability reduction

from mRNA level could be differential regulation of protein expression after translation in different cell types, which would not be detected on the mRNA level and which would have escaped detection in this study due to unspecificity of the antibodies available. Another reason for the independence of sensitivity from mRNA expression levels could be that cannabinoids do not exert their function restricted to the cannabinoid receptors. For AM251 and AM630 it was shown that they also act via TRP channels (Patil et al., 2011). Also, (-)-Cannabidiol was shown to act on the vanilloid receptor TRPV1 by generating a TRPV-1 receptor mediated intracellular calcium elevation in TRPV1 transfected HEK293 cells (Ligresti et al., 2006).

The concentrations needed for an effective inhibition of viability for all compounds were highly variable resulting in IC50 values mostly larger than ~15µM. In this respect, it has been reported that cannabinoids are less effective in serum containing medium than in serum free medium which could be the result of an interaction between the drugs and albumin. Thus, over 99% of the plasma endocannabinoid anandamide is bound to albumin. Also, BSA showed a high affinity binding site for anandamide (Bojesen and Hansen, 2003). In addition, serum has been described to protect against apoptosis based on its nutrition composition (Zanghi et al., 1999), consequently cells are higher susceptible to cytotoxic agents in serum-free medium. In mantle cell lymphoma (MCL), anandamide and the synthetic cannabinoid WIN-55,212-2 were more effective in lower serum concentrations (Flygare et al., 2005). The same was observed by Richardson et al, MCL cells were less sensitive to THC under natural conditions with 10% serum than under serum-free conditions (Richardson et al., 2007).

Which precise pathway was involved in the reduction in viability in this study was not tested. In the literature, different pathways have been reported to be involved in cannabinoid signalling depending on the cells. In melanoma cells the incubation with cannabinoids inhibited AKT, a key element in prosurvival pathway that is deregulated in many tumours, whereas ERK, JNK, p38 MAPK were not affected (Blázquez et al., 2006). Analysing the gene expression profile after AM251 treatment in MIA PaCa-2 cells revealed that this antagonist affects JAK/STAT, MAPK and cell cycle related pathways (Fogli et al., 2006). THC in leukemic cell lines act on the MAPK pathway by deactivation of ERK2 (Powles et al., 2005) and the treatment of MCL with (R)-(+)-Methanandamide induced cell death via an increase in ceramide by upregulation in the *de novo* ceramide synthesis pathway (Gustafsson et al., 2009).

In addition to cell lines, primary cells from CLL patients were incubated in suspension and in coculture with the cannabinoids. For the evaluation of the cannabinoids as therapeutic substances also PBMC from healthy donors (HD) were screened and therefore incubated in suspension for 48h. Comparing 48h incubations in suspension culture, CLL primary cells

were more sensitive towards (R)-(+)-Methandamide and the antagonists AM251 and AM630 compared to the CLL cell lines MEC-1 and MEC-2, whereas they showed higher IC₅₀ for ACEA and JWH133. These different results show that for evaluating the potentials of cannabinoids in therapy, the testing of cell lines instead of primary cells is but just a first step. In addition, the primary cells showed higher standard deviations than the cell lines due to biological variation between single patients. In healthy donors, all compounds except (R)-(+)-Methandamide displayed IC₅₀ values similar to CLL patients. Comparable data were observed for THC, cell death occurred at similar concentrations in leukemia cell lines and non-malignant cells (Powles et al., 2005). This stands in contrast to other studies where (-)-Cannabidiol at concentrations, which corresponded to the IC₅₀ values of epithelial cells derived from various tumours, had no cytotoxic effect on non-tumour cell lines (human keratinocytes, rat preadipocytes, mouse monocyte-macrophages) (Ligresti et al., 2006). Furthermore, Shrivastava et al showed that (-)-Cannabidiol induced cell death in breast cancer cells but was significantly less effective on non cancer cell lines (MCF10-A) (Shrivastava et al., 2011).

To take into account the protective effect of the microenvironment for CLL cells, CLL samples were also tested in coculture with the mouse fibroblast cell line M2-10B4. Overall, CLL cells in suspension were more susceptible towards most cannabinoids compared to experiments in coculture. Only two of the tested compounds, (-)-Cannabidiol and (R)-(+)-Methanandamide, had similar influence on the cells in suspension and in coculture. CLL primary cells undergo spontaneous apoptosis when cultured in absence of microenvironment (Hegde et al., 2008). Also, M2-10B4 and human marrow stromal cells protect CLL from spontaneous and drug induced apoptosis equally effective (Kurtova et al., 2009). In other studies it was demonstrated that after 48h under standard culture conditions only 50% of CLL cells remained viable whereas in coculture with M2-10B4 viability was enhanced up to 81% (Buchner et al., 2010). This protective effect of M2-10B4 is due to the expression of pro survival signals for the CLL cells. Thus, vimetin provides anti-apoptotic signals to CLL cells through the BCR (Binder et al., 2010). Other protective effects of the microenvironment are Notch ligands which are expressed by MSC (Seke Etet et al., 2012) and hedgehog proteins produced by stromal cells that promote survival in CLL cells *in vitro* (Hegde et al., 2008). To determine to which degree the particular resistance of CLL samples in coculture was associated with the sensitivity of M2-10B4 toward cannabinoids, the mouse fibroblasts were tested alone. For ACEA, JWH133 and AM251 IC₅₀ values were not reached similar to incubations of CLL cells in coculture with M2-10B4. For AM630 the IC₅₀ value was higher, but for (-)-Cannabidiol and (R)-(+)-Methanandamide the IC₅₀ values were similar to primary CLL cells. This would explain, why no difference between IC₅₀ values of primary CLL cells between suspension and coculture assays for (-)-Cannabidiol and (R)-(+)-Methanandamide

could be observed. As mentioned earlier, (R)-(+)-Methandamide was the only tested compound with a lower impact on HD cells than on CLL cells. Since M2-10B4 cell incubation revealed a strong effect of (R)-(+)-Methandamide on these feeder cells, it still needs to be tested how strong this compound affects human stromal cells compared to mouse fibroblasts for better evaluation of the therapeutic potential of this substance.

Whether the reduction in viability was due to a direct induction of apoptosis or other physiological changes was not studied here. Other groups showed that (R)-(+)-Methanandamide directly induced apoptosis and cell death in cell lines representing MCL and CLL (MEC-1 and MEC-2) (Gustafsson et al., 2008).

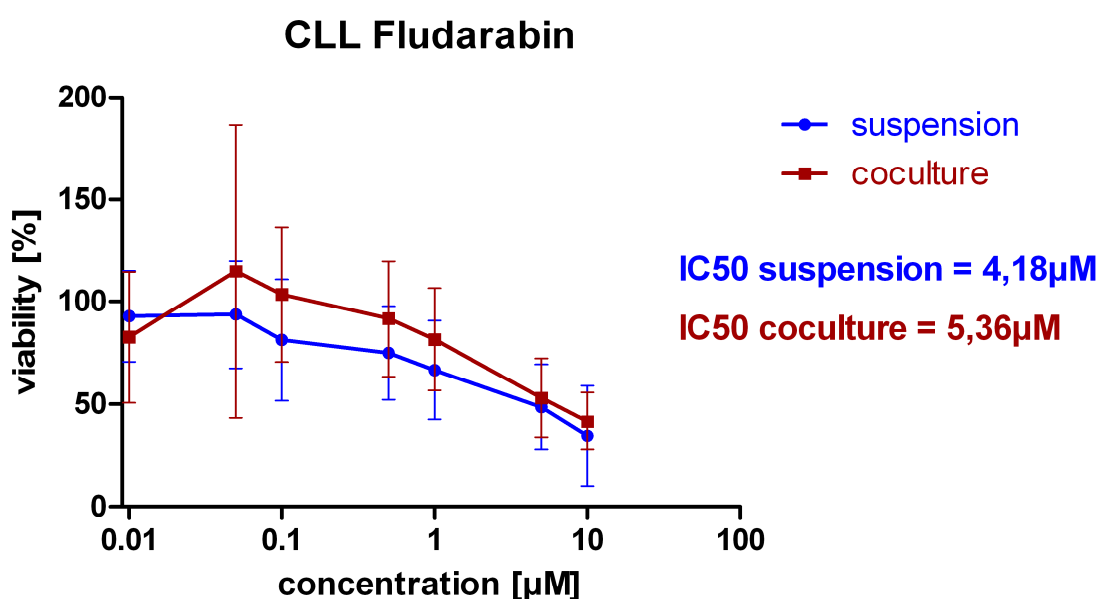


Figure 27: CLL primary cells incubated with fludarabine.

Primary CLL PBMCs were incubated with different concentrations of fludarabine in suspension and coculture (M2-10B4) for 48h. Data were provided by Clemens Pausz.

Compared to other compounds, relatively high cannabinoid doses are needed for comparable effects on viability. For comparison figure 27 depicts primary CLL cells incubated with fludarabine, a standard chemotherapeutic drug in CLL. For this compound IC50 values of 4,18μM in suspension and 5,36μM in coculture could be reached, whereas for (R)-(+)-Methanandamide 29,27- 33,19μM were determined. Thus, cannabinoids have only limited use as novel chemotherapeutics, in particular since the administration of cannabinoids into the body is difficult. Oromucosal uptake of cannabinoids is lower than by inhaling, in addition drug absorption depends on the inhaling technique. For THC the total amount of absorption is 10-30% (Karst et al., 2010), however, inhalation might lead to lung impairment (Karst et al., 2010). High doses would be needed which might lead to side effects. In human studies using cannabinoids for chronic pain, adverse effects were sedation, dizziness, cognitive

impairment, anxiety and dry mouth, these effects increased with higher dosages (Karst et al., 2010). Thus, rimonabant, a CNR1 antagonist/inverse agonist, used in treating obese people was withdrawn from the market, due to its side effects (Ward and Raffa, 2011). Furthermore, the cytotoxic effect of THC in MCL cells *in vitro* was low when using concentrations close to the real achievable plasma level (Richardson et al., 2007). On the other hand, dronabinol (Marinol) and nabilone are administered as capsules (Velasco et al., 2012) and are approved and already used successfully for stimulation of appetite in AIDS patients (dronabinol) and for the suppression of nausea and vomiting during chemotherapy (nabilone) (Pertwee, 2008). Still, the use of cannabinoids in anti-cancer therapy thus is questionable.

Also in primary cells it was tested whether high CNR1 or CNR2 mRNA expressing patients were more sensitive to cannabinoids than CNR1/CNR2 low expressing patients. Similar to the cell lines there was no correlation between mRNA expression levels of the two cannabinoid receptors and susceptibility to the compounds of the primary cells. As mentioned earlier, others showed that high cannabinoid receptor expressing cells are more affected than low expressing cells. The viability of Rec-1 cells with high CNR1 expression was significantly reduced after 48h 10 μ M anandamide treatment whereas SKMM-2 cells, which do not express CNR1, remained unaffected (Flygare et al., 2005).

However, also the opposite - drug activity independent of CNR expression - has been reported in the literature. Thus, (-)-Cannabidiol induced apoptosis in activated hepatic stellate cells which could not be blocked by either AM251 or AM630 (Lim et al., 2011). The same independence of (-)-Cannabidiol from cannabinoid receptor expression was seen for the human glioma cell lines U87 and U373 (Massi et al., 2004) and for breast cancer cells (Shrivastava et al., 2011).

Although binding also on CNR1 and CNR2, it was reported that (-)-Cannabidiol mainly acts as antagonist to GPR55 (Ryberg et al., 2007) and as weak agonist of VR1 vanilloid receptors (Costa et al., 2004). Finally, also Powles et al found that cannabinoid receptor expression levels did not correlate with cytotoxic response to THC in CEM, HEL-92 and HL60 cell lines (Powles et al., 2005). In CLL the microenvironment protects cancer cells from spontaneous and drug-induced apoptosis (Burger et al., 2009). Especially the interaction of CXCL12 and CXCR4 contributes to the drug resistance (Burger and Kipps, 2006). Stromal cells and NLCs secrete CXCL12, which signals through the chemokine receptor CXCR4 (Burger and Kipps, 2006), so that CLL cells are attracted to niches where these feeder cells provide them with pro-survival signals (Burger et al., 2009). CLL cells express a high amount of the CXCR4 (O'Callaghan et al., 2012; Burger and Kipps, 2006), which was also revealed in the FACS analysis in this study. Considering that cannabinoids were reported to interfere with CXCL12/CXCR4 interaction (Ghosh et al., 2006), it was tested in coculture whether cannabinoids exert an additive effect with fludarabine, the CXCR4 specific inhibitor

AMD3100 (O'Callaghan et al., 2012) was used as positive control. Highest impact on viability had AMD3100 alone which was even stronger than fludarabine itself. Compared to fludarabine, cannabinoids exerted only a small impact on the viability of primary CLL cells. In addition, all tested cannabinoid agonists and antagonist showed only a small or even non additive effect with fludarabine. This indicates that a) cannabinoids are not suited for combination therapy with fludarabine in CLL, and b) in contrast to observations in JURKAT cells, cannabinoids do not interfere with the CXCL12-CXCR4 axis in chronic lymphocytic leukemia. In contrast, AMD3100, also known as plerixafor, was shown to decrease the expression of CXCR4 (Stamatopoulos et al., 2011). The observed viability reducing effect of AMD3100 was also described by others. Buchner et al. described the used M2-10B4 as CXCL12 secreting cells. They also found that AMD3100 reversed the protective effect of M2-10B4 which led to a reduction in viability. They also showed that CLL cell survival was not affected by AMD3100 in the absence of M2-10B4 (Buchner et al., 2010). A similar result was observed by Stamatopoulos et al. They showed that when mononuclear cells (MNC) from CLL patients were cultured alone, AMD3100 did not have a significant effect on apoptosis or viability but significantly decreased the viability when cells were cultured with AMD3100 under microenvironmental conditions (Stamatopoulos et al., 2011).

The final aim of this study was to investigate the effect of cannabinoids in interference with CLL cell migration. It was tested whether CLL cells expressing the CXCR4 migrate toward the ligand CXCL12 and how incubation with cannabinoids alters the migration behaviour. The results revealed that CLL primary cells migrate towards CXCL12, with cell migration significantly reduced when no CXCL12 was available. The migration of CLL cells toward CXCL12 was also described by O'Hayre et al. Furthermore, they reported that B cells from healthy donors show an even higher migration than cells from CLL patients (O'Hayre et al., 2010). Migration was reduced strongly after AMD3100 incubation, an effect also described by Stamatopolous et al. Pre-incubation of MNC of CLL patients resulted in a marked decrease of migration in response to CXCL12 (Stamatopoulos et al., 2011). This interruption of the CXCR4/CXCL12 axis is of interest in therapy since it renders malignant cells more susceptible to cytotoxic agents. AMD3100 is already clinically used in combination with granulocyte-colony stimulation factor (G-CSF) to enhance the mobilization of stem cells for transplantation in patients with non-Hodgkin lymphoma (NHL) (O'Callaghan et al., 2012; Pusic and DiPersio, 2010). Furthermore there is an ongoing Phase I /II study assessing the role of AMD3100 as sensitizing agent in patients with CLL treated with rituximab (Pusic and DiPersio, 2010).

In migration assays it was tested whether cannabinoids could be an equally promising substance in this interference with the protective environment. In contrast to AMD3100, however, and as already indicated by the combination experiments with fludarabine, the

incubation with cannabinoids did not significantly reduce migration, although other groups showed that cannabinoids may have a function in migration.

The CNR2 agonist JWH015 inhibited CXCL12 induced chemotaxis, therefore the authors proposed that CNR2 might cross talk with CXCR4 (Nasser et al., 2011). WIN55,212-2 was shown to induce mouse splenic lymphocyte migration toward CXCL12 but to inhibit migration at high doses (Tanikawa et al., 2011). Thus the cannabinoid concentration could play an important role, especially since higher doses exert a cytotoxic effect. Pre-incubation with a cannabinoid antagonist before the agonist incubation, did not enhance and did not reduce migration in our study. While the treatment of non-small lung cancer cells (NSCLC) with WIN55,212-2 and JWH-015 inhibited chemotaxis *in vitro*, this was attenuated by pre-treatment with the antagonists AM251 and AM630 (Preet et al., 2011). Such an effect was also shown for the JURKAT T cell line (Ghosh et al., 2006). Incubation with the CNR2 agonist JWH-015 inhibited the CXCL12 induced chemotaxis; pre-incubation with AM630 partially reversed the inhibitory effect of JWH-015 in migration (Ghosh et al., 2006). Gosh et al. used the same agonist concentration of 10 μ M (Ghosh et al., 2006). Instead of JWH-015 used by Gosh et al., in the recent study JWH133 was used, for which a similar cytotoxicity can be supposed. In addition the inhibition of migration with JURKATS was reached for 10 μ M JWH133 in the recent study (data not shown). Therefore, an inhibition of migration for CLL with 10 μ M JWH133 was expected. However, this could not be shown for CLL cells. Gosh et al. had the highest reversal of the inhibitory effect of JWH-015 for 0,25 μ M AM630 (Ghosh et al., 2006). In the present study, 0,1 μ M of the antagonist AM630 was used. Since a higher or lower concentrations of the antagonist had no effect in the Gosh study (Ghosh et al., 2006), the concentration of 0,1 μ M might have been too low for an effect. However, neither the incubation with the agonist (JWH133) nor the incubation with the antagonist (AM630) influenced the CLL migration toward CXCL12. Therefore, it could not be supposed that the combination of both compounds might exert an effect, as shown for the JURKATS (Ghosh et al., 2006). Summarizing, although cannabinoids have been reported to play a role in the interaction with cell-cell cross-talk, they did not interrupt the protective effect of the microenvironment in CLL cells, neither did they show an appreciable additive effect with the therapeutic drug fludarabine. The concentrations needed for a cytotoxic effect on CLL cell viability were much higher than for fludarabine. Importantly, they exert a similar toxic impact on healthy cells. In contrast to other studies, it has to be concluded that the usefulness of the tested synthetic cannabinoids as therapeutic drugs in CLL is questionable. The mRNA expression of cannabinoid receptor 1, however, could be determined as a novel and reliable prognostic marker.

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Appendix

I. List of abbreviations

APS	Ammonium Persulfate
BSA	Bovine Serum Albumine
<u>Cannabinoids:</u>	
(R)-(+)-Methanandamide	(R)-N-(2-Hydroxy-1-methylethyl)-5Z,8Z,11Z,14Z eicosatetraenamide
AMD3100 octahydrochloride	1,1'-[1,4-Phenylenebis-(methylene)]-bis-(1,4,8,11 tetraazacyclotetradecane) octahydrochloride
ACEA	N-(2-Chlorethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide
JWH133	(6aR,10aR)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran
(-)-Cannabidiol	2-[(1R,6R)-3-Methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol
AM251	N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl) 4-methyl-1H-pyrazole-3-carboxamide
AM630	6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxid
NaN ₃	Sodium azide
Na-deoxycholate	Sodium-deoxycholate
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
FCS	Fetal Calf Serum
HCl	Hydrochlorid acid
NaCl	Sodium chloride
NaF	Sodium fluoride
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline

APPENDIX

RPMI medium	Roswell Park Memorial Institute medium
RT	Room temperature
SDS	Sodium dodecyl sulfate
Tris	Tris(hydroxymethyl)aminomethane

II. Material

ABI Prism 7000 SDS Software	[Applied Biosystems]
ABI Prism 7000 Sequence Detector	[Applied Biosystems]
CellQuest Pro Software	[Becton Dickinson]
CELLSTAR Cell Culture Flasks	[Greiner Bio-One]
Centrifuge Allegra X-12R	[BECKMAN COULTER]
Centrifuge 5417c	[Eppendorf]
Centrifuge Universal 32R	[Hettich]
Combitip	[Eppendorf]
Counting chamber	[BRAND]
CRYO STORAGE VESSEL <i>BSS-4800</i>	[VWR]
EasySep® kit	[StemCell Technologies]
Electrophoresis power supply E455	[CONSORT]
Excel 2003	[Microsoft]
FACScan	[Becton Dickinson]
FACS tubes	[Becton Dickinson]
Freezer -80°C	[Thermo Scientific]
Gel Blot Paper	[WHATMAN]
GraphPad Prism5	[GraphPad Software, Inc.]
Immobilon-FL Transfermembran	[MILLIPORE]
Incubator Heraeus	[Thermo Scientific]
Kimwipes	[Kimberly-Clark]
Medline freezer -20°C	[LIEBHERR]
Medline fridge 4°C	[LIEBHERR]
Microprocessor pH537	[WTW]
Microscope Axiovert 40C	[ZEISS]
MicroAmp Optical Adhesive Film	[Applied Biosystems]
MikroWin 2000	[Berthold Technologies]
Mini-PROTEAN Tetra Electrophoresis System	[BIO-RAD]
Mini Trans-Blot® Electrophoretic Transfer Cell	[BIO-RAD]
Multipette® Plus	[Eppendorf]

APPENDIX

NanoDrop 8000	[Thermo Scientific]
NanoDrop 8000 V.2.1.0.	[Thermo Scientific]
Odyssey Imager	[LI-COR]
Odyssey software V3.0	[LI-COR]
Pierce BCA Protein Assay Kit	[Thermo Scientific]
Pipettor Easypet	[Eppendorf]
Reaction tubes	[Eppendorf]
Polycarbonate Membrane Transwell Inserts	[CORNING]
Research® Pipette	[Eppendorf]
Safety cabinet HERAsafe	[Thermo Scientific]
Safe-lock tubes	[Eppendorf]
SafeSeal Tips	[Biozym]
Semi-Skirted 96-Well PCR Plate	[STARLAB]
SPSS Statistics Version 20	[IBM]
Stripette® pipets	[CORNING]
Thermomixer	[Eppendorf]
TriStar LB941	[Berthold Technologies]
Vortex mixer L24	[Labinco]
96 Well Polystyrene Microplates	[Greiner Bio-One]
12 Well tissue culture treated	[Greiner Bio-One]
24 Well cell culture cluster	[CORNING]

III. Chemicals

Antibodies:

Alexa Fluor488 goat anti-mouse IgG (H+L)	[Invitrogen]
Anti-CNR1 polyclonal Antibody	[Thermo Scientific #PA1-745]
Anti-CNR2 monoclonal Antibody	[ABGENT #AF1575a]
Anti-GAPDH monoclonal Antibody	[CALBIOCHEM #CB1001]
IRDye 680 Conjugated Goat Polyclonal Anti- Mouse IgG (H+L)	[LI-COR #926-32220]
IRDye 800CW Conjugated Goat Polyclonal Anti-Rabbit IgG (H+L)	[LI-COR #926-32211]
PE Mouse Anti-Human CD184	[BD Pharmigen #555974]
Purified Mouse IgG2a, κ Isotype Ctrl	[BioLegend #401501]
A/B Bis-Acrylamid	[NATIONAL DIAGNOSTICS]
APS	[SERVA]
Aqua bidest	[Braun]

APPENDIX

Benzamidine	[SIGMA]
<u>Cannabinoids:</u>	
ACEA	[TOCRIS]
AM251	[TOCRIS]
AM630	[TOCRIS]
(-)-Cannabidiol	[TOCRIS]
JWH133	[TOCRIS]
(R)-(+)-Methanandamide	[TOCRIS]
AMD3100 octahydrochloride	[TOCRIS]
Albumin from bovine serum	[SIGMA]
CellTiter-Blue®	[PROMEGA]
Chloroform	[MERCK]
CNR1 (Human) Recombinant Protein	[# H00001268-G01 ABNOVA]
CNR2 (Human) Recombinant Protein	[# H00001269-G01 ABNOVA]
25x Complete	[ROCHE]
DEPC	[SIGMA]
DMSO	[SIGMA]
dNTP Mix U1515	[Promega]
EDTA	[CALBIOCHEM]
EGTA	[SIGMA]
Ethanol	[VWR]
Ficoll (Biocoll)	[BIOCHROM]
Fludarabine	[SIGMA]
Isopropanol	[FLUKA]
GIBCO DMEM	[Life Technologies]
GIBCO DMEM (free of phenolred)	[Life Technologies]
GIBCO FCS Gold	[Life Technologies]
GIBCO PBS	[Life Technologies]
GIBCO RPMI1640	[Life Technologies]
GIBCO RPMI1640 (free of phenored)	[Life Technologies]
Glycerol	[CALBIOCHEM]
β-Glycerophosphate	[FLUKA]
Glycine	[MERCK]
HCl	[SIGMA]
β-Mercaptoethanol	[SIGMA]

APPENDIX

Methanol	[SIGMA]
Mikrozid	[Schuelke]
MilliQ water	[MILLIPORE]
M-MLV Rev. Transcriptase M1705	[Promega]
NaCl	[SIGMA]
Na-deoxycholate	[SIGMA]
NaF	[SIGMA]
NaN ₃	[SIGMA]
Na-Orthovanadate	[SIGMA]
Non-fat dry milk	
Nonidet P-40	[USB]
Oligo (dT) 15 Primer C1101	[Promega]
Orange G	[SIGMA]
Penicillin / Streptomycin	[PAA]
peqGOLD Protein-Marker IV	[PEQLAB]
Recombinant Human CXCL12	[R&D Sytems #350-NS]
5x Reaction Buffer	[Promega]
RNAasin® Plus N2611	[Promega]
SDS	[BIO-RAD]
TaqMan® Gene Expression Assays	[Applied Biosystems]
TaqMan® Universal Master Mix	[Applied Biosystems]
Temed	[BIO-RAD]
Tetrasodium Pyrophosphate	[SIGMA]
Tris	[SIGMA]
Tris-HCl	[BIO-RAD]
TRIzol	[Life Technologies]
Trypanblue	[SIGMA]
Trypsin-EDTA	[PAA]
Tween20	[SIGMA]

Abstract

Cannabinoids are the active compounds of the marijuana plant *Cannabis sativa* L.

For hundreds of years, these plant derived phytocannabinoids have been used for medical indications, in particular for their psychoactive properties, and a number of synthetic cannabinoids with similar activity have been developed in recent years. They exert their function by binding the two cannabinoid receptors, CNR1 and CNR2, as agonist, antagonist or inverse agonist. Of the two receptors, CNR1 is mostly expressed in the nervous while CNR2 mainly is found in the immune system. Clinical applications for cannabinoids are, for instance, the suppression of nausea and vomiting in patients during chemotherapy, or the stimulation of appetite in AIDS patients. In addition, various studies reported that cannabinoids induce apoptosis and inhibit cell proliferation, invasion, and metastasis and block angiogenesis in solid tumors. Similar antiproliferative effects were observed in non-Hodgkin lymphomas.

Chronic lymphocytic leukemia (CLL) is a non-Hodgkin lymphoma and the most prevalent leukemia of adults in Western countries. The clinical course is heterogeneous, and standard therapeutic regimens consist of chemo- or immuno-chemotherapy. But one third of patients never responds to treatment, progress rapidly and succumbs to the disease. Great progress has been made in the development of therapeutics in recent years. However, in particular for these high risk patients novel therapeutic options are urgently needed.

Considering the cytotoxic effects reported for cannabinoids and the need for therapeutic drugs in CLL, we wanted to study the cannabinoid receptors and the effect of their ligands in this malignancy in more detail. A cohort of 102 well characterized patients was screened by real time PCR for CNR1 and CNR2 mRNA expression. Expression was calculated relative to the mean of CD19 sorted healthy cells. Protein expression was analyzed in selected samples. Cell lines and peripheral blood mononuclear cells (PBMC) from CLL patients and healthy donors were incubated with cannabinoids in different concentrations and in comparison to fludarabine to establish cytotoxic efficacy. IC50 values were calculated based on standard viability assays. Both cannabinoid receptors were over-expressed in the study cohort. Whereas CNR2 mRNA expression did not correlate with any bad prognostic marker, high CNR1 mRNA expression was associated with advanced Binet stage (p-value = 0,049), unmutated IGHV (p-value = 0,006) and high CD38 expression (p-value = 0,032).

Furthermore, CNR1 high expressing patients had a shorter overall survival (p-value = 0,002) and treatment free survival (p-value = 0,000). Protein expression levels could not be analyzed, due to the lack of specificity of the commercially available CNR1 and CNR2 antibodies. In a first step, the tumor cell lines MEC-1, MEC-2 (both CLL), JURKAT (T-cell acute leukemia), and A-549 (lung carcinoma) were incubated with R-(+)-Methanandamide,

ACEA, (-)-Cannabidiol, JWH133, AM251, and AM630 at different concentrations. In these cell lines, a time and dose dependent decrease in cell viability could be determined. Differences in sensitivity between cell lines was observed, which were, however, not associated with CNR1 and CNR2 mRNA expression levels. Next, PBMC from CLL patients were incubated with the compounds both in suspension and in a pre-clinical, coculture model using the mouse fibroblast cell line M2-10B4. In suspension, comparing the CLL with the cell lines MEC-1 and MEC-2, the primary cells were similar susceptible to ACEA, less for JWH133 and (-)-Cannabidiol and more affected by the other three compounds. The coculture with the feeder cells exerted a protective effect, except for the incubation with (R)-(+)-Methanandamide and (-)-Cannabidiol. These two compounds had also a toxic effect on the M2-10B4 cells. Similar cytotoxic effects of the tested cannabinoids were observed in healthy donor PBMC except for R-(+)-Methanandamide.

Finally, emphasize was put on the microenvironment, particular the CXCR4/CXCL12 axis, which has been shown to contribute to survival of leukemic cells. Cannabinoids have been implicated in interfering with this chemokine axis in T cells. Therefore the additive effect of cannabinoids with fludarabine was explored and compared to the impact of AMD3100, a CXCR4 targeting compound with experimental usage in therapy. In inhibiting cell viability, cannabinoids were less effective compared to AMD3100 and exerted only a small additive effect when tested in combination with fludarabine. Finally, the effect of cannabinoids on the migration of CLL cells, expressing the CXCR4, toward the CXCL12 ligand was evaluated. In contrast to AMD3100, the cannabinoids did not exert a significant inhibiting effect on the migration of the primary CLL cells towards CXCL12.

Referring to the low impact of cannabinoids on microenvironment in survival and migration and the similar cytotoxic effect on CLL cells and healthy cells, cannabinoids seem to be a poor therapeutic substance in CLL. Although the mRNA expression of CNR2 is not of prognostic value, the CNR1 mRNA expression levels could be established as new prognostic marker.

Zusammenfassung

Cannabinoide sind die aktiven Inhaltsstoffe der Cannabispflanze *Cannabis sativa L.*. Diese aus der Pflanze stammenden Phytocannabinoide wurden, besonders aufgrund ihrer psychoaktiven Eigenschaften, schon vor hunderten von Jahren in der Medizin angewandt. In den vergangenen Jahren wurden diverse synthetischen Cannabinoide mit ähnlicher Wirkung entwickelt. Sie üben ihre Funktion aus indem sie als Agonist, Antagonist oder inverser Agonist an die zwei Cannabinoidrezeptoren CNR1 und CNR2 binden. Dabei wird CNR1 hauptsächlich im Nervensystem exprimiert, während man CNR2 meist im Immunsystem findet. In der Klinik werden Cannabinoide bereits gegen Übelkeit und Erbrechen bei Chemotherapiepatienten und zur Appetitstimulation von AIDS Patienten eingesetzt. Zudem wurde in verschiedenen Studien die Wirkung von Cannabinoiden bei soliden Tumoren beschrieben. Sie inhibieren Apoptose, Zellproliferation, Invasion, Metastase und blockieren Angiogenese. Ähnliche anti-proliferative Wirkungen wurden auch bei non-Hodgkin Lymphomen beobachtet.

Chronische lymphatische Leukämie (CLL) ist ein solches non-Hodgkin Lymphom und die häufigste Leukämie bei Erwachsenen in westlichen Ländern. Der klinische Verlauf ist sehr heterogen. Die Standardtherapie besteht aus Chemo- oder Immuno-chemotherapie. Ein Drittel der Patienten reagiert jedoch nicht auf die Behandlung, und erliegt nach schneller Progression der Erkrankung. Obwohl in den vergangenen Jahren große Fortschritte in der Entwicklung von Therapien gemacht wurden, werden für die Hoch-Risiko-Patienten neue Therapiemöglichkeiten dringend benötigt. Daher und wegen der berichteten cytotoxischen Effekte der Cannabinoide, wollten wir die Cannabinoidrezeptoren und ihre Liganden in der CLL genauer studieren. Eine Kohorte von 102 gut charakterisierten Patienten wurde in einer real time PCR auf CNR1 und CNR2 mRNA Expression untersucht. Die Expression wurde relative zum Mittelwert von CD19 sortierten gesunden Zellen berechnet. Zudem wurde in ausgewählten Proben die Proteinexpression analysiert. Um den cytotoxischen Effekt nachzuweisen, wurden Zelllinien und mononukleare Zellen des peripheren Bluts (PBMC) von gesunden Probanden und CLL Patienten mit Cannabinoiden in verschiedenen Konzentrationen inkubiert und mit Fludarabin verglichen. Die IC50 Werte wurden basierend auf Standardviabilitätstests kalkuliert.

Beide Cannabinoidrezeptoren waren in der Studienkohorte überexprimiert. Während die CNR2 mRNA Expression mit keinem prognostischen Marker korrelierte, war eine hohe CNR1 Expression mit einem fortgeschrittenen Binet Stadium (p-Wert=0,049), unmutiertem IGHV (p-Wert=0,006) und erhöhter CD38 Expression (p-Wert=0,032), assoziiert. Zudem zeigten CNR1 hoch exprimierende Patienten ein kürzeres Gesamtüberleben (p-Wert=0,002) und behandlungsfreies Überleben (p-Wert=0,000). Die Proteinexpression konnte nicht

analysiert werden, da die kommerziell erhältlichen Antikörper sich als nicht spezifisch für CNR1 und CNR2 herausstellten. Bei der Inkubation der Tumorzelllinien MEC-1, MEC2 (beide CLL), JURKAT (T-Zell akut Leukämie) und A-549 (Lungenkarzinom) mit (R)-(+)-Methandamide, ACEA, (-)-Cannabidiol, JWH133, AM251 und AM630 mit verschiedenen Konzentrationen, konnte eine zeit- und konzentrationsabhängige Abnahme der Zellviabilität gezeigt werden. Zudem reagierten die Zelllinien, unterschiedlich sensibel, was nicht mit der mRNA Expression des CNR1 und CNR2 assoziiert war. Danach wurden die PBMC von CLL Patienten in Suspension- und in einem Cokulturmodell mit der Maus Fibroblasten Zelllinie M2-10B4 mit den Substanzen inkubiert. In dem Suspensionsmodell, zeigten die primären CLL Zellen eine ähnliche Anfälligkeit gegen ACEA, eine geringere gegen JWH133 und (-)-Cannabidiol und eine höhere Sensitivität gegen die anderen drei Cannabinoide.

Das Cokulturmodell zeigte einen protektiven Effekt für die Cannabinoidinkubation, außer bei (R)-(+)-Methandamide und (-)-Cannabidiol. Diese hatten ebenfalls einen toxischen Effekt auf die M2-10B4. Außer (R)-(+)-Methandamide, hatten die Cannabinoide einen ähnlichen toxischen Effekt auf die gesunden Zellen.

Im letzten Teil der Studie wurde das Mikroumfeld, besonders die CXCR4/CXCL12 Interaktion untersucht. Diese trägt zum Überleben von Leukämie Zellen bei und Cannabinoide wurden beschrieben, diese Interaktion in T-Zellen zu stören. Daher wurde der additive Effekt von Cannabinoiden mit Fludarabin untersucht und mit der Wirkung von AMD3100, einem CXCR4 Inhibitor, verglichen. Es zeigte sich, dass Cannabinoide, im Vergleich zu AMD3100, einen geringeren Einfluss auf die Zellviabilität und nur einen geringen additiven Effekt mit Fludarabin hatten. Zuletzt wurde die Wirkung von Cannabinoiden auf die Migration von CLL Zellen, die CXCR4 exprimieren, zu CXCL12 getestet. Im Gegensatz zu AMD3100, hatten die Cannabinoide keinen signifikanten inhibierenden Einfluss auf die Migration.

Bezogen auf den geringen Einfluss der Cannabinoide auf das Mikroumfeld in Bezug auf Überleben und Migration, sowie der ähnlichen toxischen Wirkung auf CLL und gesunde Zellen, scheinen Cannabinoide keine vielversprechende therapeutische Substanz in der CLL darzustellen. Obwohl die CNR2 Expression keinen prognostischen Wert ergab, konnten die CNR1 mRNA Expression als neuer prognostischer Marker gezeigt werden.

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PERSONAL DATA

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EDUCATION

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08/1996 – 06/2005	Gymnasium auf der Morgenröthe	Siegen, Germany
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WORK EXPERIENCE

03/2011 – present	Master thesis at the Medical University of Vienna Department of Medicine I, Division of Hematology Title: “The role of the cannabinoid receptors in chronic lymphocytic leukemia”	Vienna, Austria
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04/2003 – 04/2003	District Hospital Siegen Internal Medicine Ward Internship	Siegen, Germany
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