

Expression of cannabinoid receptors type 1 and type 2 in non-Hodgkin lymphoma: Growth inhibition by receptor activation

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Endogenous and synthetic cannabinoids exert antiproliferative and proapoptotic effects in various types of cancer and in mantle cell lymphoma (MCL). In this study, we evaluated the expression of cannabinoid receptors type 1 and type 2 (CB1 and CB2) in non-Hodgkin lymphomas of B cell type ($n = 62$). A majority of the lymphomas expressed higher mRNA levels of CB1 and/or CB2 as compared to reactive lymphoid tissue. With the exception of MCL, which uniformly overexpresses both CB1 and CB2, the levels of cannabinoid receptors within other lymphoma entities were highly variable, ranging from 0.1 to 224 times the expression in reactive lymph nodes. Low levels of the splice variant CB1a, previously shown to have a different affinity for cannabinoids than CB1, were detected in 44% of the lymphomas, while CB1b expression was not detected. In functional studies using MCL, Burkitt lymphoma (BL), chronic lymphatic leukemia (CLL) and plasma cell leukemia cell lines, the stable anandamide analog R(+)-methanandamide (R(+)-MA) induced cell death only in MCL and CLL cells, which overexpressed both cannabinoid receptors, but not in BL. *In vivo* treatment with R(+)-MA caused a significant reduction of tumor size and mitotic index in mice xenografted with human MCL. Together, our results suggest that therapies using cannabinoid receptor ligands will have efficiency in reducing tumor burden in malignant lymphoma overexpressing CB1 and CB2.

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Key words: cannabinoid; lymphoma; cancer; apoptosis; xenograft

Non-Hodgkin lymphomas represent clonal expansions of malignant B cells at various stages of maturation. Our understanding of the molecular biology and pathogenesis of malignant lymphoma has improved substantially during the last few years. As a result, targeted therapies and interference with critical signaling pathways are evolving as new treatment modalities.¹ The malignant B cells might also be dependent on interactions with the tumor microenvironment for their survival.^{2,3} This is supported by the fact that it is very difficult to grow lymphoma cells *in vitro* in the absence of stroma or CD40 stimulation. In follicular lymphoma (FL), interactions with T cells, macrophages and dendritic cells have been suggested to influence the clinical behavior of the malignancy.^{2,4,5} The expression pattern of cytokines, chemokines, prostaglandins, leukotrienes and their receptors may also promote or inhibit the survival of malignant B cells.

We have previously studied the role of the endocannabinoid system in MCL.^{6–8} The endocannabinoid system encompasses the G-protein coupled receptors, cannabinoid receptor type 1 (CB1) and type 2 (CB2), the endocannabinoids and the enzymes responsible for endocannabinoid synthesis and degradation.⁹ The endocannabinoids are synthesized from arachidonic acid by many cell types including macrophages and dendritic cells. They have properties similar to compounds of *Cannabis sativa* and have recently been shown to have effects on various types of cancer.⁹ Many of the effects of natural and synthetic cannabinoids are mediated through the cannabinoid receptors. CB1 is expressed at high levels in the central nervous system and at lower levels in immune cells. In addition to the full-length CB1 transcript, 2 splice variants denoted CB1a and CB1b have been reported in human brain.¹⁰ Both splice variants have different ligand binding

capacity compared with that of the full length receptor.¹⁰ The CB2 receptor is expressed in lymphocytes and at lower levels in the brain. Accordingly, resting B lymphocytes express the CB2 receptor and low levels of the CB1 receptor.^{11,12} Polyclonal activation has been reported to modulate expression of cannabinoid receptors on lymphocytes.^{13–16} The function of the cannabinoid receptors in the immune system is not fully known, but a role in regulation of cytokine production and cell migration has been suggested.¹⁶

In MCL, CB1 and CB2 were found to be consistently overexpressed compared with reactive lymphoid tissue or normal purified B lymphocytes.^{8,17} Ligation of the receptors with the endocannabinoid arachidonoyl-ethanolamide (anandamide) as well as with the synthetic cannabinoids WIN 55,212-2 and R(+)-methanandamide (R(+)-MA) inhibited cell proliferation and induced cell death in MCL cell lines and patient samples.^{6,7} Normal B cells, which express lower levels of cannabinoid receptors than MCL, were not affected by cannabinoids, suggesting that ligation of cannabinoid receptors could selectively target the malignant cells.⁷ In addition to MCL, high expression of cannabinoid receptors compared to normal epithelium has been reported in a number of different epithelial malignancies (recently reviewed in Ref. 18). Pharmacological doses of cannabinoids are emerging as suppressors of signaling pathways involved in cancer cell growth, suggesting that the endocannabinoid system might be targeted to control cancer (reviewed in Refs. 18–21).

Little is known about the role of the cannabinoid system in malignant non-Hodgkin B cell lymphomas other than MCL. Ligation of CB2 induced programmed cell death in acute T cell lymphoblastic leukemia (T-ALL).^{22,23} Immunoreactivity for CB2 has been reported in both B and T non-Hodgkin lymphomas. However, the 2 different antibodies used gave divergent results, possibly due to different phosphorylation of the receptor, and no functional studies were reported.²⁴

Abbreviations: B-CLL, B cell chronic lymphocytic leukemia; BL, Burkitt lymphoma; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; pre-B ALL, precursor-B acute lymphoblastic lymphoma; RFI, relative fold increase; R(+)-MA, R(+)-methanandamide; SLL/CLL, small lymphocytic lymphoma/B cell chronic lymphocytic leukemia; SR1, SR141716A; SR2, SR144528; T-ALL, acute T cell lymphoblastic leukemia.

The last two authors contributed equally to this work.

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In the present study, we analysed CB1 and CB2 expression by quantitative real time PCR in a panel of non-Hodgkin lymphomas of B cell type, including small lymphocytic lymphoma/B cell chronic lymphocytic leukemia (SLL/CLL), marginal zone lymphoma (MZL), diffuse large B cell lymphoma (DLBCL), FL, precursor-B acute lymphoblastic lymphoma (pre-B ALL), MCL, immunocytoma (IC) and BL. Our results demonstrate that the majority of malignant B cell lymphomas express CB1 and/or CB2 but at a highly variable level. Treatment with the anandamide analog R(+)-MA induced apoptosis in MCL and in B CLL cell lines, expressing higher levels of CB1 and CB2 than reactive lymphocytes. However, BL cell lines and a plasma cell line were resistant. *In vivo*, treatment with R(+)-MA caused a 40% reduction of tumor weight in mice xenografted with MCL. Reduced proliferation, measured as mitotic index, was observed in the tumor cells from the treated animals. The antiproliferative and proapoptotic effects of cannabinoids make the endocannabinoid system a potential new therapeutic target for individualized therapy in lymphomas which overexpress cannabinoid receptors such as MCL and B CLL.

Material and methods

Reagents

SR141716A (SR1) and SR144528 (SR2) were kind gifts from Sanofi-Reserche (Montpellier, France). R(+)-methanandamide (R(+)-MA) was purchased from Tocris Cookson (Bristol, UK). AIM-V medium was purchased from Invitrogen (Carlsbad, CA).

Tumor and control tissue

Non-Hodgkin lymphomas of B cell type and reactive lymphoid tissues were retrieved from the files of the Department of Pathology. Tissue removed for diagnostic purposes was obtained unfixed at the Department of Pathology and immediately processed. Representative parts of the tissue was fixed in formalin and paraffin embedded. Other parts were snap-frozen and subsequently stored at -70°C . All cases (Table I) were diagnosed and classified using World Health Organization Classification tumors of Haematopoietic and Lymphoid Tissues.²⁵

Cell lines

The MCL cell line Rec-1²⁶ was a kind gift from Dr. Christian Bastard, Ronan, France. The CLL cell lines MEC1 and MEC2, the BL cell lines Raji and Namalwa, the plasma cell line SK-MM-2 and the MCL cell line JEKO-1 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). Cell lines were maintained in RPMI 1640 medium (Invitrogen) supplemented with 2 mM L-glutamine and 10% fetal calf serum (FCS) and 50 μg of gentamicin (Invitrogen) under standard conditions (humidified atmosphere, 95% air, 5% CO_2 , 37°C).

Mouse xenograft model of human MCL

Ten-week-old female SCID/NOD mice (bred at Karolinska Institutet, Southern Campus, Stockholm, Sweden) were injected subcutaneously on the back with a suspension of 10×10^6 JEKO-1 cells in 0.4 ml PBS. A stock solution of R(+)-MA in EtOH was diluted to 500 $\mu\text{g}/\text{ml}$ in PBS. Three days after injecting the JEKO-1 cells, the mice within the group of treated animals ($n = 11$) were injected subcutaneously with R(+)-MA, 5 mg/kg, at the previous injection site. The mice included in the group of mock-treated animals ($n = 12$) were injected subcutaneously with PBS containing the same amount of EtOH as the diluted R(+)-MA. Tumor diameters were measured daily. Animals were sacrificed at day 33 when the diameter of the largest tumor in the mock-treated group reached 1 cm. The tumors were excised and tumor weight was measured. No visible signs of toxicity were observed in the animals during the treatment period. The animals were maintained

at the animal facility of the Karolinska Institutet, Southern Campus, according to rules and regulations of European standard.

RNA isolation and quality control

Total RNA was prepared using Gentra RNA purification system as directed by the supplier (Ambion, Austin, TX). The samples were treated with Deoxyribonuclease I Amplification Grade to eliminate DNA (Invitrogen Life Technologies, Carlsbad, CA). RNA quality was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA).

cDNA synthesis and real-time quantitative RT-PCR

First strand cDNA synthesis was carried out according to the protocol for Omniscript Reverse Transcription (Qiagen, GmbH, Hilden, Germany). One or two micrograms of RNA were used in the reactions for RT-PCR or PCR, respectively. The Beacon Designer 3 program (Biosoft International, Palo Alto, CA) was employed for design of CB1, CB1a and CB2 primers. The following primers were used: CB1 forward-TCATTAAGACGGTGTTTGATTCT, CB1 reverse-CGTGTCGACGGTCCTTAC TC, CB2 forward-TGACTATGACCTTCACAGCCTCT, CB2 reverse-AGGACCCACATGATGCCAG, CB1a forward-GACATCA AAGGAGAATGAGGAGAAC and CB1a reverse-GAGGGACA GGACTGCAATGG. The primers for β -actin were described previously in Ref. 27. Primers were synthesized by Eurogentec (Southampton, Hampshire, UK). The quantification of CB1, CB1a and CB2 compared to β -actin was carried out with an iCycler iQ (Bio-Rad Laboratories, Sundbyberg, Sweden). The iCycler iQ reaction detection system software from the same company was used for data analysis. cDNA was amplified using the qPCR Kit Platinum SYBR Green qPCR SuperMix-UGD with FITC (Invitrogen) according to the manufacturer's instructions. The samples were divided into triplicates in a 96-well PCR plate (Abgene, Hamburg, Germany) and run at 95°C for 10 min followed by 40 cycles, each cycle consisting of 15 sec at 95°C and 1 min at 55°C . Threshold (Ct) cycle numbers were obtained from amplification of β -actin, CB1, CB1a and CB2. The PCR products were verified by sequencing. ΔCt values were calculated by subtracting the Ct value of β -actin from the Ct value of CB1, CB1a or CB2. The relative fold increase (RFI) of CB1, CB1a and CB2 was calculated as follows. The ΔCt for controls (reactive lymph nodes) and patient samples was first determined. The $\Delta\Delta\text{Ct}$ value was calculated by subtracting the ΔCt value for pooled controls from the ΔCt value for the sample. The RFI of CB1, CB1a or CB2 was calculated by the equation: $\text{RFI} = 2^{-\Delta\Delta\text{Ct}}$. To use this calculation, the PCR efficiencies of the target and control assays must be similar. This was achieved by adjusting primer concentrations. The criterion for using the ΔCt method was fulfilled because by graphing serial dilutions of input cDNA of a random sample against ΔCt values (CB1- β -actin, CB1a- β -actin or CB2- β -actin) the slope of the line was ≤ 0.1 (data not shown).

PCR for CB1a and CB1b

Expression of CB1a and CB1b was investigated using 2 rounds of PCR as described by Ryberg *et al.*¹⁰ The following forward CB1-splice (5'-CCTAATCAAAGACTGAGGTTATGAAGTC-3') and reverse CB1-splice (5'-AGTTCTCCACACTGGATGTT-3') primers were used in a PCR mixture of 50 μl , containing 50 ng cDNA from MCL patient samples. Platinum Taq polymerase (Invitrogen) was used according to the manufacturer's instructions. Amplification was carried out in a PTC-200 (MJ Research Waltham, MA) for 40 cycles: 95°C for 30 sec, 57°C for 45 sec, and 68°C for 1 min. The resulting products were resolved on 2% agarose gels and a major band at 324 bp corresponding to the human unspliced CB1 receptor was detected. The 0.12–0.25-kb gel area was excised and melted (at 95°C for 5 min) and used as template for further PCR amplification. This second round of PCR was performed using the same conditions as described above with

TABLE 1 – EXPRESSION OF CB1 AND CB2 mRNA MEASURED AS RFI¹ OF LYMPHOMA ENTITIES² RELATIVE TO POOLED CONTROLS

Number	CB1		CB2		Entity ²
	Mean value	Range	Mean value	Range	
KG35	2,0	1,8–2,3	0,9	0,6–4,1	SLL
KG36	4,1	3,9–4,4	0,5	0,2–0,7	SLL
KG37	0,5	0,2–0,7	0,1	0,1–0,4	SLL
KG38	9,4	9,2–9,7	2,7	2,4–2,9	SLL
KG39	7,3	7,1–7,6	0,5	0,3–0,8	SLL
KG40	117,2	116,9–117,5	1,9	1,4–2,5	SLL
KG41	158,3	158,1–158,6	13,9	13,6–14,1	SLL
KG42	9,2	8,9–9,4	21,5	21,3–21,7	SLL
KG43	0,8	0,6–1,1	2,3	2,0–2,5	SLL
KG44	145,7	145,3–146,0	0,4	0,3–0,5	SLL
KG16	33,9	33,7–34,2	10,0	9,8–10,3	DLBCL
KG17	2,9	2,6–3,1	1,8	1,5–2,0	DLBCL
KG18	17,0	16,8–17,3	10,6	10,4–10,9	DLBCL
KG19	16,8	16,6–17,1	20,3	20,0–20,5	DLBCL
KG20	47,6	47,4–47,9	1,1	0,8–1,3	DLBCL
KG21	3,5	3,2–3,7	0,5	0,2–0,7	DLBCL
KG22	1,4	1,2–1,6	0,2	0,1–0,3	DLBCL
KG23	51,6	51,4–51,9	197,6	197,4–197,9	DLBCL
KG24	1,2	1,0–1,4	0,4	0,1–0,6	DLBCL
KG25	1,4	1,2–1,7	0,5	0,2–0,7	DLBCL
KG26	0,5	0,3–0,8	0,1	0,2–0,3	DLBCL
KG27	1,5	1,2–1,7	0,2	0,0–0,5	DLBCL
KG28	0,1	0,1–0,1	0,3	0,1–0,6	DLBCL
KG29	0,5	0,3–0,8	3,2	2,9–3,4	DLBCL
KG30	28,3	28,1–28,6	5,1	4,9–5,4	DLBCL
KG31	3,5	3,3–3,7	0,1	0,1–0,4	DLBCL
KG32	72,2	71,9–72,4	37,9	37,6–38,1	DLBCL
KG33	0,3	0,2–0,3	0,1	0,1–0,2	DLBCL
KG34	5,3	5,0–5,5	0,2	0,1–0,4	DLBCL
KG1	2,9	2,6–3,1	5,3	5,2–5,3	FL
KG2	69,7	69,5–70,0	32,4	32,2–32,6	FL
KG3	6,0	5,8–6,3	2,9	2,6–3,1	FL
KG4	57,9	57,7–58,2	1,4	1,2–1,7	FL
KG5	6,4	6,1–6,6	1,0	0,8–1,3	FL
KG6	1,3	1,1–1,6	1,5	1,2–1,7	FL
KG7	2,5	2,2–2,7	0,8	0,6–1,1	FL
KG8	8,5	8,3–8,8	1,9	1,6–2,1	FL
KG9	0,2	0,1–0,3	1,1	0,9–1,4	FL
KG10	15,7	15,5–16,0	9,6	9,3–9,8	FL
KG11	8,0	7,8–8,3	9,3	9,0–9,5	FL
KG12	0,5	0,2–0,7	0,1	0,1–0,1	FL
KG13	11,4	11,1–11,6	0,3	0,0–0,5	FL
KG14	2,7	2,5–3,0	1,1	0,8–1,3	FL
KG15	59,3	59,1–59,5	117,5	117,3–117,8	FL
KG45	9,9	9,6–10,1	4,4	4,1–4,6	MZL
KG46	42,1	41,9–42,4	24,5	24,3–24,8	MZL
KG47	11,6	11,4–11,8	0,1	0,1–0,1	MZL
KG48	10,9	10,6–11,1	0,1	0,1–0,3	MZL
KG49	0,4	0,2–0,7	0,2	0,2–0,3	MZL
KG50	0,3	0,0–0,5	1,5	1,3–1,7	MZL
KG51	1,4	1,2–1,7	0,0	0,0–0,3	MZL
KG52	30,1	29,9–30,4	2,3	2,1–2,6	MZL
KG53	2,4	2,3–2,4	1,8	1,8–1,9	MCL
KG54	16,6	16,5–16,7	3,2	3,1–3,3	MCL
KG55	8,5	8,3–8,6	1,9	1,6–2,2	MCL
KG56	8,5	8,2–8,7	2,4	2,3–2,5	MCL
KG57	13,9	13,7–14,1	2,6	2,4–2,7	MCL
KG58	0,9	0,6–1,1	0,1	0,0–0,5	IC
KG59	3,2	2,9–3,4	33,7	33,4–33,9	IC
KG60	224,2	224,0–224,4	0,4	0,1–0,6	BL
KG61	1,8	1,5–2,0	1,5	1,3–1,8	pre B-ALL
KG62	17,4	17,2–17,7	4,2	4,0–4,5	PC
Reactive 1	1,6	1,5–1,7	0,9	0,8–0,9	Ctrl1
Reactive 2	0,6	0,5–0,8	0,9	0,9–1,0	Ctrl2
Reactive 3	1,3	1,2–1,3	1,5	1,2–1,7	Ctrl3
Reactive 4	1,2	0,9–1,5	0,9	0,8–1,0	Ctrl4
Reactive 5	0,5	0,4–0,6	0,9	0,8–1,0	Ctrl5
Pooled Ctrl.	1,0	0,7–1,3	1,0	0,8–1,2	

¹RFI, relative fold increase.—²Lymphoma entities: SLL, small lymphocytic lymphoma; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; MZL, marginal zone lymphoma; MCL, mantle cell lymphoma; IC, immunocytoma; BL, Burkitt lymphoma; pre B-ALL, precursor B cell acute lymphoblastic lymphoma/leukemia; PC, plasmocytoma.

4 μ l of the melted material as template. The resulting PCR product was again resolved on a 2% agarose gel. The PCR products were excised and purified using the GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare, United Kingdom) prior to verification by sequencing.

Western blotting

Cell extracts were prepared using ice-cold lysis buffer (50 mM Hepes, 500 mM NaCl, 0.05% Tween 20 and 1% NP40) to which protease inhibitor cocktail p8340 (Sigma, St. Louis, MO) had been added. Tumor pieces were disrupted for protein extraction using a Sample Grinding Kit (GE Healthcare, Piscataway, NJ). Protein concentration was determined by means of the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Proteins were resolved by 10% SDS/PAGE and transferred onto PVDF membrane, which was incubated with a rabbit polyclonal antibody to CB1 (Cayman Chemicals Ab 101500, Ann Arbor, MI). Antibodies to actin (Santa Cruz) were used as loading controls. Antibody binding was detected by enhanced chemiluminescence using Supersignal West Pico (Pierce) chemiluminescent substrate.

Immunohistochemistry for CB1

All stainings were semiautomated and performed on a Bond Max robot by using the Vision BiosystemsTM bond Polymer Refine, and Bond DAB Enhance, as recommended by the manufacturer (Leica Microsystems, Wetzlar, Germany). Primary polyclonal antibody to CB1 (Cayman, Catalog no. 10006590) was diluted to 1:100 with Bond primary Ab Diluent (AR 9352, Vision Biosystems, Newcastle, UK). Pretreatment protocol for antigen retrieval was citrate buffer pH 7.2 at 60°C overnight.

Caspase-3 assay

DEVD-dependent caspase activity was measured using a Caspase-3/CPP32 Fluorimetric Assay (MCL International, Woburn, MA) according to the manufacturer's instructions. The assay is based on fluorimetric detection of cleavage of the substrate DEVD-AFC. Uncleaved substrate emits blue light (λ_{max} 400 nm), whereas free AFC emits yellow-green light fluorescence (λ_{max} 505 nm). In brief, cells from cell lines were washed and resuspended in AIM-V medium (Invitrogen). Further, cells were pretreated with the CB1 and CB2 inhibitors SR1 or SR2, respectively, for 30 min prior to incubation with 10 μ M of R(+)-MA for 24 hr. Cells were then harvested, lysed and incubated with buffer containing DEVD-AFC for 2 hr at 37°C. Emission was measured using a fluorimeter at 400 nm excitation and 505 nm emission.

Cell death ELISA

Cell death ELISA (Roche, Mannheim, Germany) is a quantitative sandwich ELISA that detects histone and intranucleosomal DNA fragmentation by binding to 2 different monoclonal antibodies. It allows specific determination of mononucleosomes and oligonucleosomes in the cytoplasmatic fraction in cell lysates. The antihistone-biotin antibody binds to histones H1, H2A, H2B, H3 and H4. The anti-DNA-POD antibody reacts with double or single stranded DNA in the cytoplasm. In brief, cells were washed and resuspended in AIM-V medium. Following pretreatment with the inhibitors SR141716 or SR144528 for 30 min, the cells were incubated with 10 μ M R(+)-MA for 24 hr. Cells were harvested and lysed with lysis buffer. The cell lysate was allowed to bind to the enzyme immunoassay plate for 2 hr together with immunoreagent containing anti-DNA-POD and antihistone-biotin and incubation buffer. Thereafter, ABTS substrate was added for 20 min. Adding stop solution terminated the reaction, and the emission was determined at 405 nm.

Mitotic index

For each tumor, a total of 5 \times 6,000 cells were evaluated in different areas of the tissue sections at 400 \times magnification using a microscope grid and the number of mitoses was counted. The mi-

totic index is expressed as the percentage of cells in mitosis of the total number of cells.

Statistical analysis

Caspase-3 assay and cell death ELISA were evaluated using the Kruskal-Wallis test comparing control and treated cells. *p*-Values are presented in figure legends. The software Statistica (Statsoft AB, Tulsa, OK) was used. Correlation analysis was performed using SAS software (version 9.1; SAS Institute, Cary, NC).

Ethical permission

This study was approved by the ethics committees at Karolinska Institutet (Forskningsetikommitté syd and Stockholms Södra Djurförsöksetiska nämnd).

Results

Expression of CB1 and CB2 mRNA in non-Hodgkin lymphomas of B cell type

In the present study, we have analyzed CB1 and CB2 mRNA expression in non-Hodgkin lymphomas of the B-cell type (*n* = 62) (Table I). For some lymphoma entities, such as BL and pre B-ALL, only few frozen samples were available. We still choose to include those entities to have a representation, as broad as possible, of the spectrum of B cell lymphomas.

Using quantitative real-time PCR, we found that the majority of the lymphoma samples expressed higher levels of CB1 (51/62, 80%) and CB2 (38/62, 60%) compared with pooled control tissue from 5 reactive lymph nodes (Table I). With the exception of MCL, all the other lymphoma entities showed a highly variable expression of cannabinoid receptors. Cannabinoid receptor expression did not correlate with morphological variants of DLBCL or with FL tumor grade (data not shown). Importantly, while some tumors expressed lower levels of cannabinoid receptors than reactive lymphoid tissue, no single lymphoma entity uniformly lacked expression of cannabinoid receptors.

Since studies in the T cell line Jurkat have suggested that signaling through CB2 might upregulate functional CB1 receptors,²⁸ we analyzed a possible correlation between expression levels of CB1 and CB2 in the B cell lymphomas. However, statistical analysis in our series of lymphomas showed only a moderate correlation between expression of CB1 and CB2 (correlation coefficient: 0.52 and *p* < 0.001).

mRNA expression of the splice variant CB1a in MCL and other B cell lymphomas

To detect differences in the expression of CB1 transcript variants in MCL, primers that would give rise to products of 324, 157 and 225 bp corresponding to CB1, CB1a and CB1b were used (Fig. 1a). An expanded set of MCL samples (MCL 6-17, *n* = 12) expressed full length CB1 (Fig. 1b, top). However, no products corresponding to CB1a or CB1b were detected after a first round of PCR (Fig. 1b, top) similar to previous reports demonstrating low levels of these transcripts in brain tissue.¹⁰ The region between 125 and 250 bp was therefore excised, the gel was melted, and an aliquot was used as a template in a second round of PCR. CB1a was now clearly detected in 8 out of 12 patient samples, while no expression of CB1b was observed (Fig. 1b, bottom). We further investigated the expression CB1a by quantitative real-time PCR in MCL and the whole panel of lymphoma samples representing different entities. CB1a could be detected in 27 of the 62 samples (44 %) at expression levels ranging from 1 to 227 times the expression in control lymph node (Table II). No correlation between expression of CB1 and CB1a was observed (Fig. 1b and Table II).

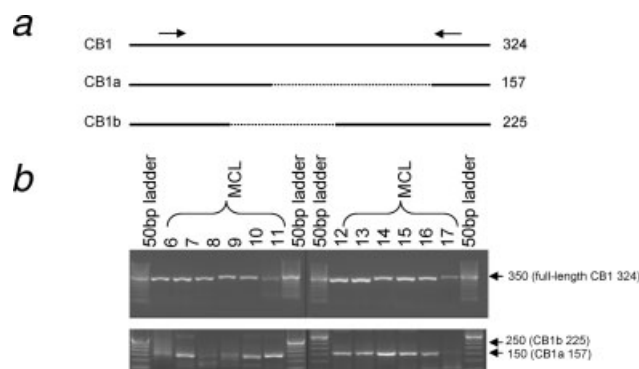


FIGURE 1 – Detection of full-length CB1 and CB1a in cDNA derived from MCL. (a) Schematic picture of the length of the PCR products representing the different transcripts. Primers are indicated with arrows. (b) A 324-bp product corresponding to full-length CB1 was detected from a first round of PCR (upper lane). A region between 120- and 250-bp was excised and melted, and used as template for further PCR amplification. A 157-bp product corresponding to CB1a was detected from a second round of PCR (lower lane). CB1b, predicted product 225 bp, was not expressed.

Protein expression and distribution of CB1 in non-Hodgkin lymphomas of B cell type

The data from the quantitative real-time PCR was validated by Western blot (Fig. 2a) and immunohistochemistry (Fig. 2b) in patient samples from different entities, expressing varying CB1 mRNA levels. Immunostainings demonstrated CB1 expression not only in the lymphoma cells and but also in a few nonmalignant cells such as endothelium (Fig. 2b). In some lymphomas, CB1 expression was highly variable within the tumor cell population (Fig. 2b).

R(+)-MA induces cell death via CB1 and CB2 in CLL

Pharmacological doses of cannabinoids have been reported to reduce cancer growth in cell lines, in xenografted cancers and in a pilot study on human glioblastoma multiforme.^{29–43} We previously reported that treatment with micromolar doses of cannabinoid receptor ligands such as the endocannabinoid anandamide induces apoptosis in MCL.^{6,7} There is very limited knowledge on the effects of cannabinoids in other lymphoma entities. We, therefore, investigated levels of CB receptors on well-characterized cell lines, derived from plasma cell leukemia (SK-MM-2), BL (Raji and Namalwa), CLL (MEC1 and MEC2), and MCL (Rec-1) (Fig. 3a), and the cells were exposed to the metabolically stable analog of anandamide R(+)-MA. To assess the relative contribution of CB1 and CB2, cells were pretreated with receptor antagonists SR141716 (SR1) and SR144528 (SR2), acting on CB1 and CB2 respectively. R(+)-MA induced caspase-3 activity (Fig. 3b) and cell death (Fig. 3c) in cell lines from MCL and CLL but not in BL cell lines (expressing low levels of CB2) or in the plasma cell line SK-MM-2 (expressing low levels of CB1). In the MCL and CLL cell lines, the R(+)-MA-induced cell death was abrogated by pretreatment with either of the receptor antagonists SR1 or SR2, suggesting that both CB receptors are needed for the induction of cell death.

Effect of R(+)-MA on tumors in xenotransplanted mice

To determine whether cannabinoids can be used to treat malignant lymphoma *in vivo*, xenograft tumors were established by subcutaneous inoculation of SCID/NOD mice with human MCL cell-line JEKO-1, expressing CB1 and CB2. Treatment with 5 mg/kg of the stable CB1 agonist R(+)-MA by subcutaneous inoculation every 12 hr at the previous injection site was initiated 3 days after tumor cell injection. The tumors in R(+)-MA-treated animals

TABLE II – EXPRESSION OF CB1A mRNA MEASURED AS RFI¹ OF LYMPHOMA ENTITIES² RELATIVE TO POOLED CONTROLS

Number	CB1a	Range	Entity
KG 35	6,9	6,8–7,0	SLL
KG 36	7,8	7,5–8,1	SLL
KG 43	14,6	14,4–14,8	SLL
KG 18	19,2	19,0–19,5	DLBCL
KG 21	2,4	2,1–2,6	DLBCL
KG 22	5,7	5,4–5,9	DLBCL
KG 31	28,2	27,9–28,4	DLBCL
KG 33	1	0,7–1,2	DLBCL
KG 45	28,5	28,4–28,6	MZL
KG 46	228,1	227,9–228,3	MZL
KG 47	22,9	22,7–23,1	MZL
KG 48	10,6	10,3–10,8	MZL
KG 58	11,1	10,8–11,3	IC
KG 62	83,5	83,3–83,7	PC
KG 2	36,8	36,5–37,0	FL
KG 3	45,3	45,0–45,5	FL
KG 4	10,2	9,9–10,5	FL
KG 5	41,3	41,0–41,5	FL
KG 6	19	18,8–19,3	FL
KG 7	5,7	5,4–5,9	FL
KG 8	40,3	40,1–40,5	FL
KG 12	1,7	1,6–1,8	FL
KG 13	21,1	20,9–21,4	FL
KG 14	10,4	10,3–10,5	FL
KG 53	52,6	52,3–52,8	MCL
KG 56	128	127,8–128,2	MCL
Ctrl1	0,9	0,8–1,0	Reactive
Ctrl2	0,6	0,4–0,8	Reactive
Ctrl3	1,1	1,0–1,2	Reactive
Ctrl4	1,5	1,2–1,7	Reactive
Ctrl5	0,9	0,7–1,1	Reactive
Pooled Ctrl	1	0,8–1,2	

¹RFI, relative fold increase. ²Lymphoma entities: SLL, small lymphocytic lymphoma; DLBCL, diffuse large B cell lymphoma; MZL, marginal zone lymphoma; IC, immunocytoma; PC, plasmacytoma; FL, follicular lymphoma; MCL, mantle cell lymphoma.

grew slower and were significantly smaller compared with mock-treated control tumors at termination of the experiment (Fig. 4).

We have previously shown that treatment of MCL *in vitro* with nanomolar doses of cannabinoids during several days leads to decreased growth, while applying micromolar doses of cannabinoids during a short time causes apoptosis.^{6,7} In the xenografted tumors, we found a 25% reduction in the mitotic index ($p < 0.02$) in the tumors from R(+)-MA treated mice (treated mice, average 1.35, control mice average 1.80) but no significant difference in apoptosis as assayed by TUNEL staining (data not shown).

Discussion

Recently, the endocannabinoid system has been recognized as deregulated in various types of epithelial malignancies, and there is an increasing interest in the antineoplastic potential of cannabinoids.^{9,18,19} Most, but not all,¹⁸ of the effects of endogenous and synthetic cannabinoids are mediated *via* the cannabinoid receptors, CB1 and CB2. To investigate whether cannabinoids may be of potential use in lymphoma therapy, we analyzed the expression of these receptors in a panel of malignant B cell lymphomas. Both cannabinoid receptors were expressed in the vast majority of malignant lymphomas investigated, albeit with a large span of expression levels within most entities. The highly variable expression levels in most of the B cell lymphoma entities might explain why these receptors have not previously been recognized as deregulated using array-based gene expression analysis of B cell malignancies. A variation in the mRNA expression of CB1 and CB2 in FL and of CB2 in DLBCL can indeed be found in experiments available in public databases (data from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (FL: GDS1839, DLBCL: GDS75 CB1: Id 34333,

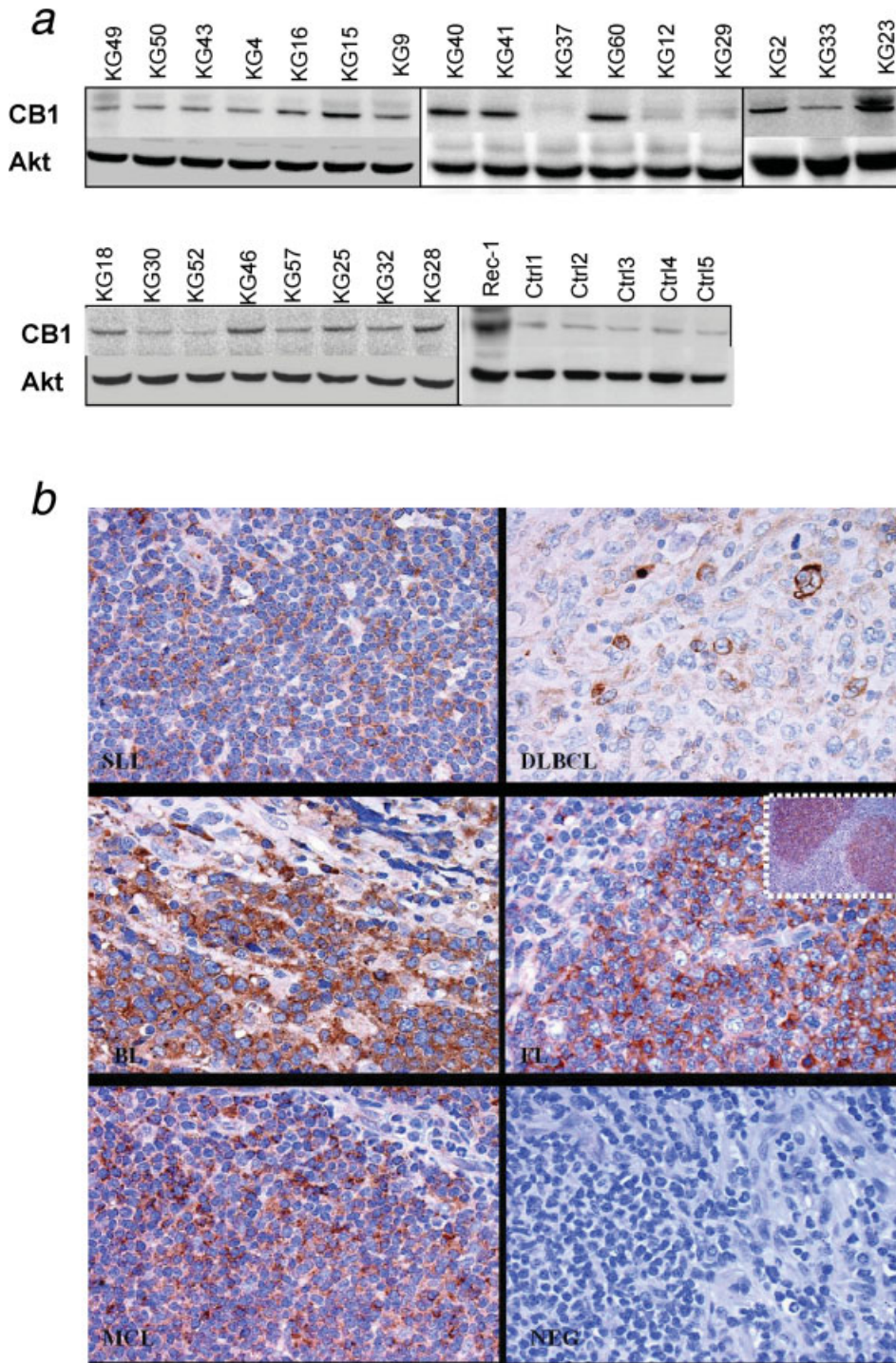


FIGURE 2 – Expression of CB1 protein in non-Hodgkin lymphomas of B cell type. (a) Western blot analysis of CB1 protein expression in tumor extracts. Ninety micrograms of total proteins were loaded per lane. The anti-CB1 antibody 101500 from Cayman Chemicals was used. The membranes were reprobed with antiactin antibody to control for loading. (b) Immunohistochemical staining of CB1 using a rabbit polyclonal antibody directed against the C-terminal of human CB1, 10006590 from Cayman Chemicals, visualizes CB1 in brown while nuclei are stained blue. Lymphomas of the entities SLL/CLL (upper left), DLBCL (upper right), BL (middle left), FL (middle right), and MCL (lower left) are shown. Note the variable staining of tumor cells in this sample of DLBCL and the strong and uniform staining of tumor cells in BL. In FL, strong staining is detected in the neoplastic follicles while the surrounding, nonneoplastic cells are negative (inset). Negative control, with omission of primary antibody, is shown in the low right panel.

CB2: Id 14714). Also in other malignancies, *e.g.*, breast cancer,⁴⁴ brain cancer^{41,45,46} and primary pancreatic cancer,⁴⁷ a high variability in the expression of the cannabinoid receptors within a tumor subtype has been reported. However, there is only limited knowledge on the regulation of cannabinoid receptor expression in tumor tissue. It is, however, known that cannabinoid receptor expression can be increased by signals provided by cells in the tissue microenvironment such as inflammatory stimuli and cytokines.^{28,48}

Also a splice variant of CB1, CB1a, initially detected in brain by Shire *et al.* in 1995⁴⁹ and recently confirmed in a separate study of brain and peripheral tissue¹⁰ was expressed in MCL and other

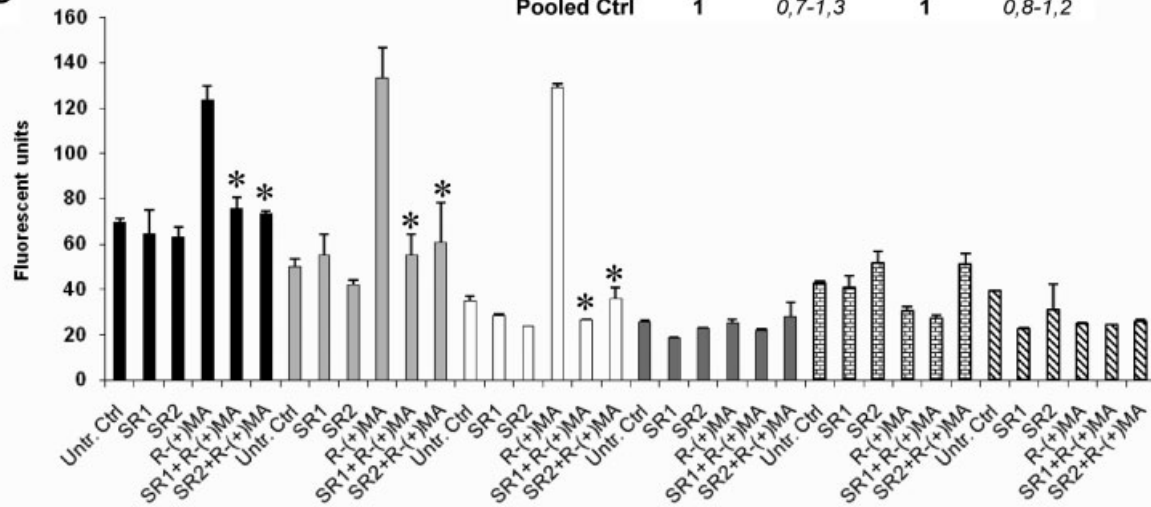
lymphomas. Importantly, the affinity to ligands and the GTP γ S binding activity of the splice variants have been shown to differ from that of full-length CB1. It could, therefore, be speculated that the differential expression of CB1 splice variants add yet another degree of variation in sensitivity to endogenous and synthetic cannabinoids.

In our series of lymphomas, the highest expression of CB1 was seen in 1 case of BL. In this tumor sample and in the BL cell lines, CB2 receptors were expressed at similar or lower levels than in reactive lymphoid tissue. Interestingly, the BL cell lines were resistant to the proapoptotic effects of the cannabinoid receptor agonist

a

	CB1	range	CB2	range
Mec-1	5,5	5,2-5,8	3,7	3,2-4,2
Mec-2	6,4	6,0-6,8	3,5	3,3-3,7
Rec-1	12,6	12,2-13,0	2,5	2,1-2,9
Raji	11,3	10,8-11,4	1,3	1,0-1,6
Namalwa	11,9	11,7-12,1	0,9	0,7-1,1
SKMM	1,6	1,4-1,8	2,2	1,9-2,5
Ctrl1	1,6	1,5-1,7	0,9	0,8-1,0
Ctrl2	0,6	0,4-0,7	0,9	0,9-1,0
Ctrl3	1,3	1,2-1,3	1,5	1,2-1,7
Ctrl4	1,2	0,9-1,5	0,9	0,8-1,0
Ctrl5	0,5	0,4-0,6	0,9	0,8-1,0
Pooled Ctrl	1	0,7-1,3	1	0,8-1,2

b



c

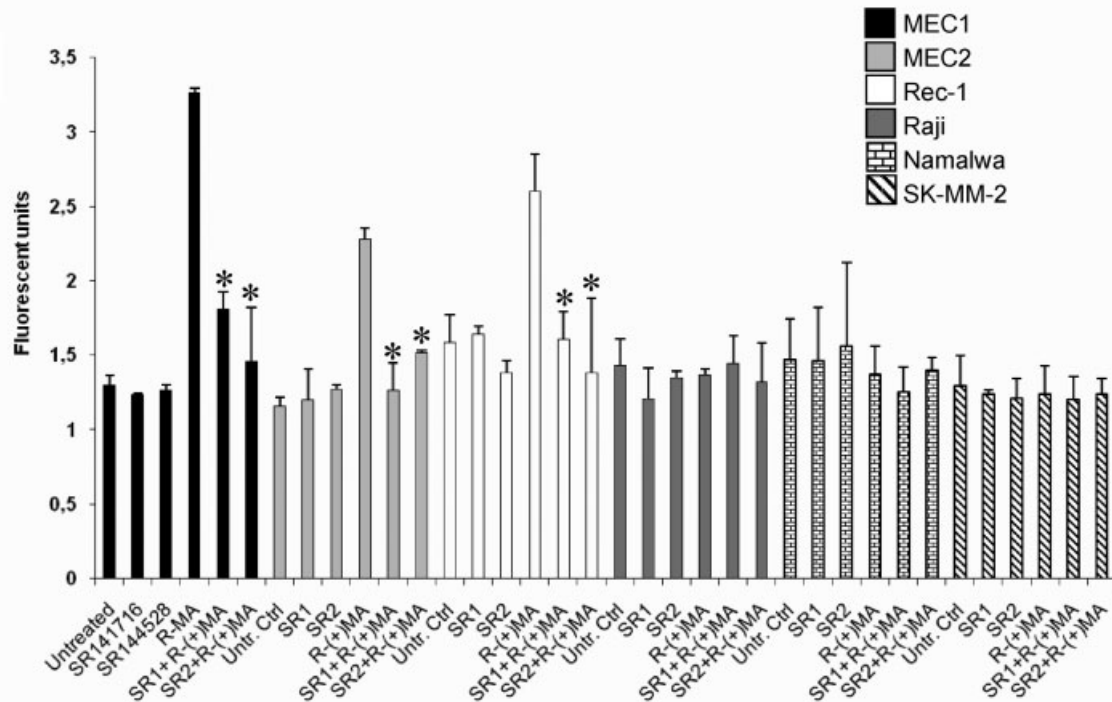


FIGURE 3 – Expression of cannabinoid receptors and response to cannabinoids in B cell lymphoma cell lines. (a) Expression of CB1 and CB2 mRNA in cell lines representing different subtypes of non-Hodgkin lymphomas of B cell type as assayed by quantitative RT-PCR. Values represent relative fold increase in lymphoma cell lines compared to pooled controls. (b) Cannabinoid-induced caspase-3 activity. The caspase-3 fluorimetric assay was performed, and fluorescence units are shown on the y-axis. One of two individually performed experiments is shown. * $p < 0.05$ when agonist treated cells with and without inhibitors were compared, Kruskal-Wallis analysis. B CLL cell lines MEC1 and MEC2, MCL cell line Rec-1, BL cell lines Raji and Namalwa and the plasma cell leukemia cell line SK-MM-2 were resuspended in AIM-V medium without serum and pretreated with the CB₁ antagonist SR141716A (SR1) (10 nM) or the CB₂ antagonist SR144528 (SR2) (10 nM) for 30 min prior to incubation with vehicle or 10 μ M of R+(-)MA for 24 hr. (c) Cannabinoid-induced cell death. The cell death ELISA was performed, and fluorescence units are shown on the y-axis. One of two individually performed experiments is shown. * $p < 0.05$ when agonist treated cells with and without inhibitors were compared Kruskal-Wallis analysis. The cells were incubated in AIM-V medium without serum and pretreated with the CB₁ antagonist SR141716A (SR1; 10 nM) or the CB₂ antagonist SR144528 (SR2; 10 nM) for 30 min prior to incubation with vehicle or 10 μ M of R+(-)MA for 24 hr.

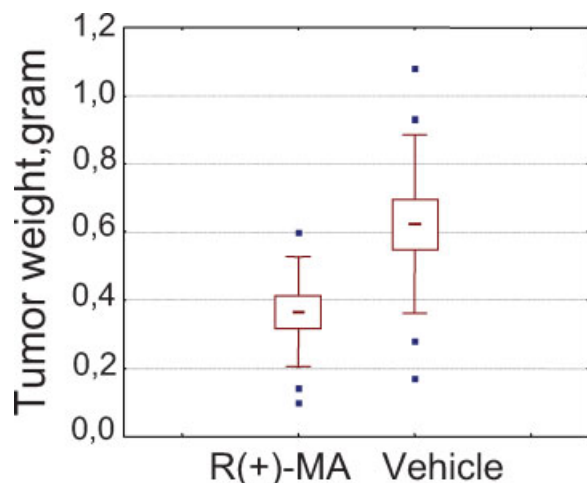


FIGURE 4 – Effect of R(+)-MA on the xenotransplanted mantle cell lymphoma. SCID-NOD mice were injected subcutaneously with 10×10^6 JEKO-1 cells in 0.4 ml PBS. The mice were assigned randomly to 2 groups, each containing 11 and 12 animals, respectively. Mice were treated with R(+)-MA, 5 mg/kg, or vehicle by subcutaneous injection in the tumor area twice a day. Group size: treated, $n = 11$, mock-treated, $n = 12$. Animals were sacrificed at day 33, and a 40% reduction of tumor weight in the R(+)-MA treated animals compared to mock-treated animals was detected. Mean (–), mean \pm SE (box), mean \pm SD (error bars), outliers (dots). Mann–Whitney U Test $p < 0.02$ when treated and mock-treated groups were compared. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

R(+)-MA. In contrast, cannabinoid receptor ligation in the B CLL cell-lines MEC1 and MEC2, expressing higher levels of both CB1 and CB2 compared to reactive lymph nodes, induced caspase-3 activation and apoptosis. This differential response to cannabinoids is in accordance with our earlier study, where cannabinoid agonists induced cell death in MCL by targeting both CB1 and CB2, while cells expressing only CB1 or only CB2 were resistant.⁷ A similar requirement of ligation of both CB1 and CB2 has been observed after treatment of dendritic cells with Δ^9 -tetrahydrocannabinol.⁵⁰

In the present study, we investigated the efficacy of the anandamide analogue R(+)-MA in a mouse model of tumors derived from a human MCL cell line and observed a significant inhibition of *in vivo* tumor growth. This is the first report on cannabinoid-induced decreased growth of tumors of the human lymphoid system *in vivo*. Another stable analog of anandamide, 2-methyl-2-F-anandamide (Met-F-AEA), has earlier been shown to reduce the *in vivo* growth of tumors originating from thyroid epithelial cells.²⁹ When possible mechanisms contributing to the decreased lymphoma growth in response to R(+)-MA were investigated, a 25% ($p < 0.02$) reduction in mitotic index was observed in tumor tissue from treated mice. Thus, inhibition of tumor growth in lymphoma could, at least in part, be mediated through effects on cell cycle regulation. Cell cycle arrest has earlier been reported to inhibit the growth of breast, prostate and melanoma cancer cells.^{31,35,38,39,51} In our study, *in vitro* treatment with R(+)-MA caused induction of apoptosis, while no differences in apoptosis as measured by TUNEL staining was observed after treatment with R-MA *in vivo* (data not shown). Cannabinoids have been reported to exhibit anti-angiogenic effects in malignant glioma and in thyroid cancer.^{32,33} This led us to analyze vascular endothelial growth factor (VEGF) *ex vivo* in the xenografted MCL. However, no difference in VEGF mRNA or protein expression was observed between R(+)-MA and mock-treated tumors (data not shown).

Our results show that CB1 and CB2 are expressed in several entities of non-Hodgkin lymphomas of B cell type. The highly variable expression within well-defined lymphoma entities suggests that cannabinoid receptors may be potential targets for individualized therapeutic interventions. Treatment with the stable endocannabinoid analog R(+)-MA induces apoptosis in tumor cell lines from MCL and CLL, expressing higher levels of both CB1 and CB2 compared with reactive lymphoid tissue. Furthermore, decreased growth of MCL *in vivo* was observed following intratumoral injections of R(+)-MA. Similarly, in studies using animal models or xenografts of breast, lung cancer, skin, prostate and brain cancer, antitumor effects by cannabinoids have been reported (reviewed in Refs. 19 and 20). In a pilot study on patients with glioblastoma multiforme, Δ^9 -tetrahydrocannabinol reduced tumor cell proliferation *in vivo*.³⁴ These cumulative data suggest that targeting of the endocannabinoid system could possibly be part of a future therapy for certain malignant lymphomas as has been suggested for other forms of cancer.

References

- Jares P, Colomer D, Campo E. Genetic and molecular pathogenesis of mantle cell lymphoma: perspectives for new targeted therapeutics. *Nat Rev Cancer* 2007;7:750–62.
- Herreros B, Sanchez-Aguilera A, Piris MA. Lymphoma microenvironment: culprit or innocent? *Leukemia* 2008;22:49–58.
- Kuppers R. Mechanisms of B-cell lymphoma pathogenesis. *Nat Rev Cancer* 2005;5:251–62.
- de Jong D. Molecular pathogenesis of follicular lymphoma: a cross talk of genetic and immunologic factors. *J Clin Oncol* 2005;23:6358–63.
- Wahlin BE, Sander B, Christensson B, Kimby E. CD8+ T-cell content in diagnostic lymph nodes measured by flow cytometry is a predictor of survival in follicular lymphoma. *Clin Cancer Res* 2007;13:388–97.
- Flygare J, Gustafsson K, Kimby E, Christensson B, Sander B. Cannabinoid receptor ligands mediate growth inhibition and cell death in mantle cell lymphoma. *FEBS Lett* 2005;579:6885–9.
- Gustafsson K, Christensson B, Sander B, Flygare J. Cannabinoid receptor-mediated apoptosis induced by R(+)-methanandamide and Win55,212 is associated with ceramide accumulation and p38 activation in Mantle Cell Lymphoma. *Mol Pharmacol* 2006;70:1612–20.
- Islam TC, Asplund AC, Lindvall JM, Nygren L, Liden J, Kimby E, Christensson B, Smith CI, Sander B. High level of cannabinoid receptor 1, absence of regulator of G protein signalling 13 and differential expression of Cyclin D1 in mantle cell lymphoma. *Leukemia* 2003;17:1880–90.
- Di Marzo V, Bifulco M, De Petrocellis L. The endocannabinoid system and its therapeutic exploitation. *Nat Rev Drug Discov* 2004;3:771–84.
- Ryberg E, Vu HK, Larsson N, Groblewski T, Hjorth S, Elebring T, Sjogren S, Greasley PJ. Identification and characterisation of a novel splice variant of the human CB1 receptor. *FEBS Lett* 2005;579:259–64.
- Bouaboula M, Rinaldi M, Carayon P, Carillon C, Delpech B, Shire D, Le Fur G, Casellas P. Cannabinoid-receptor expression in human leukocytes. *Eur J Biochem* 1993;214:173–80.
- Galiegue S, Mary S, Marchand J, Dussosoy D, Carriere D, Carayon P, Bouaboula M, Shire D, Le Fur G, Casellas P. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* 1995;232:54–61.
- Daaka Y, Friedman H, Klein TW. Cannabinoid receptor proteins are increased in Jurkat, human T-cell line after mitogen activation. *J Pharmacol Exp Ther* 1996;276:776–83.
- Noe SN, Newton C, Widen R, Friedman H, Klein TW. Anti-CD40, anti-CD3, and IL-2 stimulation induce contrasting changes in CB1 mRNA expression in mouse splenocytes. *J Neuroimmunol* 2000;110:161–7.
- Noe SN, Newton C, Widen R, Friedman H, Klein TW. Modulation of CB1 mRNA upon activation of murine splenocytes. *Adv Exp Med Biol* 2001;493:215–21.
- Klein TW. Cannabinoid-based drugs as anti-inflammatory therapeutics. *Nat Rev Immunol* 2005;5:400–11.
- Ek S, Hogerkorpe CM, Dictor M, Ehinger M, Borrebaeck CA. Mantle cell lymphomas express a distinct genetic signature affecting lymphocyte trafficking and growth regulation as compared with subpopulations of normal human B cells. *Cancer Res* 2002;62:4398–405.

18. Flygare J, Sander B. The endocannabinoid system in cancer-Potential therapeutic target? *Semin Cancer Biol*; in press.
19. Bifulco M, Di Marzo V. Targeting the endocannabinoid system in cancer therapy: a call for further research. *Nat Med* 2002;8:547-50.
20. Guzman M. Cannabinoids: potential anticancer agents. *Nat Rev Cancer* 2003;3:745-55.
21. Bifulco M, Laezza C, Pisanti S, Gazzero P. Cannabinoids and cancer: pros and cons of an antitumour strategy. *Br J Pharmacol* 2006;148:123-25.
22. Herrera B, Carracedo A, Diez-Zaera M, Guzman M, Velasco G. p38 MAPK is involved in CB2 receptor-induced apoptosis of human leukaemia cells. *FEBS Lett* 2005;579:5084-8.
23. McKallip RJ, Lombard C, Fisher M, Martin BR, Ryu S, Grant S, Nagarkatti PS, Nagarkatti M. Targeting CB2 cannabinoid receptors as a novel therapy to treat malignant lymphoblastic disease. *Blood* 2007;100:627-34.
24. Rayman N, Lam KH, Van Leeuwen J, Mulder AH, Budel LM, Lowenberg B, Sonneveld P, Delwel R. The expression of the peripheral cannabinoid receptor on cells of the immune system and non-Hodgkin's lymphomas. *Leuk Lymphoma* 2007;48:1389-99.
25. Jaffe ES, Harris NL, Stein H, Vardiman JW, eds. World Health Organization classification of tumours: pathology and genetics of tumours of haematopoietic and lymphoid tissues. Lyon: IARC Press, 2001.
26. Rimokh R, Berger F, Bastard C, Klein B, French M, Archimbaud E, Rouault JP, Santa Lucia B, Duret L, Vuillaume M, Coiffier B, Bryon P-A, Magaud JP. Rearrangement of CCND1 (BCL1/PRAD1) 3' untranslated region in mantle-cell lymphomas and t(11q13)-associated leukemias. *Blood* 1994;83:3689-96.
27. Rundlof AK, Fernandes AP, Selenius M, Babic M, Shariatgorji M, Nilsson G, Ilag LL, Dobra K, Bjornstedt M. Quantification of alternative mRNA species and identification of thioredoxin reductase 1 isoforms in human tumor cells. *Differentiation* 2007;75:123-32.
28. Borner C, Holtt V, Sebald W, Kraus J. Transcriptional regulation of the cannabinoid receptor type 1 gene in T cells by cannabinoids. *J Leukoc Biol* 2007;81:336-43.
29. Bifulco M, Laezza C, Portella G, Vitale M, Orlando P, De Petrocellis L, Di Marzo V. Control by the endogenous cannabinoid system of ras oncogene-dependent tumor growth. *FASEB J* 2001;15:2745-7.
30. Bifulco M, Laezza C, Valenti M, Ligresti A, Portella G, Di Marzo V. A new strategy to block tumor growth by inhibiting endocannabinoid inactivation. *FASEB J* 2004;18:1606-8.
31. Blazquez C, Carracedo A, Barrado L, Real PJ, Fernandez-Luna JL, Velasco G, Malumbres M, Guzman M. Cannabinoid receptors as novel targets for the treatment of melanoma. *FASEB J* 2006;20:2633-5.
32. Blazquez C, Casanova ML, Planas A, Del Pulgar TG, Villanueva C, Fernandez-Acenero MJ, Aragonés J, Huffman JW, Jorcano JL, Guzman M. Inhibition of tumor angiogenesis by cannabinoids. *FASEB J* 2003;17:529-31.
33. Casanova ML, Blazquez C, Martinez-Palacio J, Villanueva C, Fernandez-Acenero MJ, Huffman JW, Jorcano JL, Guzman M. Inhibition of skin tumor growth and angiogenesis in vivo by activation of cannabinoid receptors. *J Clin Invest* 2003;111:43-50.
34. Guzman M, Duarte MJ, Blazquez C, Ravina J, Rosa MC, Galve-Roperh I, Sanchez C, Velasco G, Gonzalez-Feria L. A pilot clinical study of Delta9-tetrahydrocannabinol in patients with recurrent glioblastoma multiforme. *Br J Cancer* 2006;95:197-203.
35. Laezza C, Pisanti S, Crescenzi E, Bifulco M. Anandamide inhibits Cdk2 and activates Chk1 leading to cell cycle arrest in human breast cancer cells. *FEBS Lett* 2006;580:6076-82.
36. Ligresti A, Bisogno T, Matias I, De Petrocellis L, Cascio MG, Cosenza V, D'Argenio G, Scaglione G, Bifulco M, Sorrentini I, Di Marzo V. Possible endocannabinoid control of colorectal cancer growth. *Gastroenterology* 2003;125:677-87.
37. Ligresti A, Moriello AS, Starowicz K, Matias I, Pisanti S, De Petrocellis L, Laezza C, Portella G, Bifulco M, Di Marzo V. Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. *J Pharmacol Exp Ther* 2006;318:1375-87.
38. Melck D, De Petrocellis L, Orlando P, Bisogno T, Laezza C, Bifulco M, Di Marzo V. Suppression of nerve growth factor Trk receptors and prolactin receptors by endocannabinoids leads to inhibition of human breast and prostate cancer cell proliferation. *Endocrinology* 2000;141:118-26.
39. Melck D, Rueda D, Galve-Roperh I, De Petrocellis L, Guzman M, Di Marzo V. Involvement of the cAMP/protein kinase A pathway and of mitogen-activated protein kinase in the anti-proliferative effects of anandamide in human breast cancer cells. *FEBS Lett* 1999;463:235-40.
40. Portella G, Laezza C, Laccetti P, De Petrocellis L, Di Marzo V, Bifulco M. Inhibitory effects of cannabinoid CB1 receptor stimulation on tumor growth and metastatic spreading: actions on signals involved in angiogenesis and metastasis. *FASEB J* 2003;17:1771-3.
41. Sanchez C, de Ceballos ML, del Pulgar TG, Rueda D, Corbacho C, Velasco G, Galve-Roperh I, Huffman JW, Ramon y Cajal S, Guzman M. Inhibition of glioma growth in vivo by selective activation of the CB(2) cannabinoid receptor. *Cancer Res* 2001;61:5784-9.
42. Sarfaraz S, Afaq F, Adhami VM, Malik A, Mukhtar H. Cannabinoid receptor agonist-induced apoptosis of human prostate cancer cells LNCaP proceeds through sustained activation of ERK1/2 leading to G1 cell cycle arrest. *J Biol Chem* 2006;281:39480-91.
43. Sarfaraz S, Afaq F, Adhami VM, Mukhtar H. Cannabinoid receptor as a novel target for the treatment of prostate cancer. *Cancer Res* 2005;65:1635-41.
44. Caffarel MM, Sarrio D, Palacios J, Guzman M, Sanchez C. Delta9-tetrahydrocannabinol inhibits cell cycle progression in human breast cancer cells through Cdc2 regulation. *Cancer Res* 2006;66:6615-21.
45. Ellert-Miklaszewska A, Grajkowska W, Gabrusiewicz K, Kaminska B, Konarska L. Distinctive pattern of cannabinoid receptor type II (CB2) expression in adult and pediatric brain tumors. *Brain Res* 2007;1137:161-9.
46. Held-Feindt J, Dorner L, Sahan G, Mehdorn HM, Mentlein R. Cannabinoid receptors in human astroglial tumors. *J Neurochem* 2006;98:886-93.
47. Carracedo A, Gironella M, Lorente M, Garcia S, Guzman M, Velasco G, Iovanna JL. Cannabinoids induce apoptosis of pancreatic tumor cells via endoplasmic reticulum stress-related genes. *Cancer Res* 2006;66:6748-55.
48. Maresz K, Pryce G, Ponomarev ED, Marsicano G, Croxford JL, Shriver LP, Ledent C, Cheng X, Carrier EJ, Mann MK, Giovannoni G, Pertwee RG, et al. Direct suppression of CNS autoimmune inflammation via the cannabinoid receptor CB1 on neurons and CB2 on autoreactive T cells. *Nat Med* 2007;13:492-7.
49. Shire D, Carillon C, Kaghad M, Calandra B, Rinaldi-Carmona M, Le Fur G, Caput D, Ferrara P. An amino-terminal variant of the central cannabinoid receptor resulting from alternative splicing. *J Biol Chem* 1995;270:3726-31.
50. Do Y, McKallip RJ, Nagarkatti M, Nagarkatti PS. Activation through cannabinoid receptors 1 and 2 on dendritic cells triggers NF-kappaB-dependent apoptosis: novel role for endogenous and exogenous cannabinoids in immunoregulation. *J Immunol* 2004;173:2373-82.
51. De Petrocellis L, Melck D, Palmisano A, Bisogno T, Laezza C, Bifulco M, Di Marzo V. The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation. *Proc Natl Acad Sci USA* 1998;95:8375-80.