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VALIDATION OF NOVEL THERAPEUTIC TARGET MOLECULES IN PEDIATRIC SARCOMAS

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SUSANNE MONIKA OESCH
Dipl. Natw., ETH Zürich

born 21.04.1980

citizen of Balgach (SG)

accepted on the recommendation of

Prof. Dr. Wilhelm Krek, examiner
Prof. Dr. Matthias Peter, co-examiner
Prof. Dr. Beat Schäfer, co-examiner

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1 Summary

Rhabdomyosarcoma is the most common pediatric soft tissue sarcoma and is thought to originate from muscle precursor cells. It can histologically be divided into two main subgroups, namely embryonal and alveolar rhabdomyosarcoma (eRMS and aRMS). eRMS is the more frequent subtype with a 5 year-survival rate of over 80%, whereas aRMS occurs more seldom and due to its higher metastatic potential is associated with lower survival rate of only 54%. To achieve better treatment of aggressive aRMS, novel targeted therapeutic interventions, which exploit abnormalities of cancer cells specifically, should be developed. Most aRMS cases carry specific chromosomal translocations between either t(2;13)(q35;q14) or t(1;13)(p36;q14) encoding the chimaeric transcription factors PAX3/FKHR or PAX7/FKRH, respectively. To study genes deregulated due to these translocations or additional genetic aberrations, a microarray analysis was performed, which revealed an expression signature specific for the translocation-positive rhabdomyosarcoma compared to the other subgroups and non-transformed control tissue. This signature is composed of several hundred genes highly up-regulated in translocation-positive samples, among them the gene for cannabinoid receptor 1 (CB1). CB1 activation by cannabinoids has been shown to lead to apoptosis in a series of tumors, such as leukaemia, glioma, breast cancer and skin carcinoma. Based on these facts, it is the aim of our project to investigate whether CB1 can also serve as a potential drug target for the treatment of aRMS as well as to study the physiological role of CB1 in aRMS.

First, we demonstrated that treatment of aRMS cells with cannabinoid receptor agonists, such as HU210, THC, or Met-F-AEA, was able to specifically reduce the viability of CB1-positive aRMS cells (Rh4) compared to CB1-negative eRMS cells (RD) and non-cancer control cells (MRC-5). This effect of HU210 is mediated by the activation of CB1, as addition of a CB1 specific antagonist (AM251) was able to reverse this effect. Caspase-3 assays and PARP-cleavage analysis indicated that the reduced viability upon cannabinoid treatment is caused by apoptosis. Upstream of the apoptotic cascade, treatment of aRMS cells with HU210, THC, or Met-F-AEA leads to a fast dephosphorylation of AKT, which is followed by an up-regulation of the

cellular stress-associated transcription factor p8 (candidate of metastasis). Down-regulation of p8 by siRNA could rescue cells of HU210-induced cytotoxic effects reflecting the importance of this factor in the mediation of the signalling cascade studied. Additionally, treatment of xenografts with HU210 led to significant suppression of tumor growth also in vivo.

Hence, we conclude that cannabinoid receptor agonists might have a therapeutic potential in aRMS and therefore should be considered as effective agents for the future treatment of aRMS.

2 Zusammenfassung

Das in der Kindheit am häufigsten vorkommende Weichteilsarkom ist das Rhabdomyosarkom, wobei man annimmt, dass es aus Muskel-Vorläuferzellen entsteht. Auf Grund histologischer Analysen kann diese Tumorerkrankung in zwei wichtige Untergruppen unterteilt werden: Embryonales und alveoläres Rhabdomyosarkom (eRMS und aRMS). Die häufiger vorkommende embryonale Gruppe hat eine Fünf-Jahres-Überlebensrate von etwa 80%, während der alveoläre Typ seltener diagnostiziert wird, dagegen aber wegen seines vermehrten Auftretens von Metastasen eine geringere Überlebensrate von 54% hat. Um bessere Behandlungen für das aggressive aRMS zu erreichen, wären neuartige und gezieltere medizinische Interventionen, welche spezifisch die Abnormalitäten von Krebszellen ausnutzen, von grosser Bedeutung.

Im Falle von aRMS stellen chromosomale Translokationen, die charakteristisch für diese RMS-Untergruppe sind, eine solche Abnormalität dar. Diese Translokationen ereignen sich entweder zwischen den Chromosomen 2 und 13 oder zwischen den Chromosomen 1 und 13, was zur Entstehung der Fusionsproteine PAX3/FKHR oder PAX7/FKHR führt. Diese Fusionsproteine bestehen jeweils aus zwei Transkriptionsfaktoren, welche als chimäre Transkriptionsfaktoren zu einer deregulierten Genexpression in aRMS-Tumorzellen beitragen. Um diese veränderte Genexpression zu untersuchen, wurde eine Microarrayanalyse durchgeführt, die eine spezifische Expressions-Signatur für die Translokations-positiven Tumore aufgezeigt hat, welche bei eRMS-Biopsien und gesundem Kontrollgewebe nicht auftritt. Unter den mehreren hundert Signatur-Genen der Translokations-positiven aRMS-Fälle befindet sich das Gen für den Cannabinoid Rezeptor 1 (CB1). Von anderen Tumorarten, wie z.B. Leukämie, Glioma, Brustkrebs oder Hautkrebs ist bekannt, dass die Aktivierung des CB1 in diesen Krebszellen zu Apoptose führt. Basierend auf diesem Wissen ist es die Absicht dieses Projektes, den CB1 als Ziel für neue therapeutische Ansätze in aRMS zu untersuchen. Des Weiteren wird eine Aufklärung der physiologischen Rolle des CB1 in aRMS angestrebt.

Zu Beginn dieser Arbeit konnten wir zeigen, dass die Behandlung von aRMS-Zellen (Rh4) mit den Cannabinoid-Rezeptor-Agonisten HU210, THC und Met-F-AEA spezifisch zu einer verringerten Überlebensrate führte, da dieser Effekt bei der Behandlung von Kontrollzellen, wie eRMS-Zellen (RD) und Fibroblasten (MRC-5), in einem siginfikant geringeren Ausmass beobachtet wurde. Wie sich mit HU210 erkennen liess, wird dieser überlebens-mindernde Effekt spezifisch durch die Aktivierung von CB1 erzielt, da eine pharmakologische Inhibition mittels eines spezifischen CB1-Antagonisten (AM251) zu einer signifikanten Erhöhung der Überlebensrate der aRMS-Zellen nach Agonist-Behandlung führte. Eine Analyse der zellulären Caspase-3-Aktivität und der Spaltung von PARP nach Cannabinoid-Zugabe deutete auf Apoptose als überlebens-reduzierenden Mechanismus hin. Bevor die Zellen in Apoptose gehen, kommt es durch die Behandlung mit HU210, THC und Met-F-AEA zu einer schnellen Dephosphorylierung von AKT, welche von einer erhöhten Transkription des stress-assoziierten Proteins p8 gefolgt wird. Die Beobachtung, dass die Reduktion der p8-Expression durch siRNA die Sensitivität der aRMS-Zellen gegenüber HU210-Behandlung verminderte, reflektiert die Wichtigkeit dieses Faktors in der Signaltransduktionskette, welche zum Cannabinoid-induzierten aRMS-Zelltod führt. Zudem bewirkte die *in vivo* Behandlung von aRMS-Xenograft-Tumoren mit HU210 eine signifikante Verminderung des Tumorwachstums.

Auf Grund dieser Resultate folgern wir, dass Cannabinoid-Rezeptor-Agonisten ein Potenzial zur Bekämpfung von aRMS bergen und daher möglicherweise als zukünftige therapeutische Massnahme bei diesen Tumoren dienen könnten.

3 Abbreviations

AKT	V-AKT murine thymoma viral oncogene homolog 1
aRMS	alveolar rhabdomyosarcoma
BSA	bovine serum albumin
CB1	cannabinoid receptor 1 (gene CNR1)
CB2	cannabinoid receptor 2 (gene CNR2)
CML	chronic myeolid leukemia
DMEM	Dulbecco's Minimum Essential Medium
EGFR	epidermal growth factor receptor
eRMS	embryonal rhabdomyosarcoma
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FGFR	fibroblast growth factor receptor
FKHR	forkhead-related
GSK-3	glycogen synthase kinase 3
HU210	synthetic CB agonist (11-hydroxy-Δ8-tetrahydrocannabinol)
IGF-IR	insulin-like growth factor receptor I
i.p.	intra peritoneal
Ki67	cellular marker for proliferation
Met-F-AEA	stable analog of endogenous anandamide (2-methyl-2'-F-anandamide)
MTT assay	cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MYL	myosin light chain 1
NOG mice	NOD/LtSz-scid IL2Rgammanull mice
ns	statistically non significant
PARP	poly(ADP-ribose)polymerase
PAX	paired box protein
PDGFR	platelet derived growth factor receptor
PFA	paraformaldehyde
PKC412	staurosporine derivative, broad spectrum kinase inhibitor
OS	overall survival

P8	transcription factor (also NUPR1 = nuclear protein 1, or COM1 = candidate of metastasis 1)
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RMS	rhabdomyosarcoma
SD	standard deviation
SE	standard error
THC	natural plant-derived CB agonist (∆9-tetrahydrocannabinol)
TNNC2	troponin C type 2
tposRMS	translocation-positive rhabdomyosarcoma
VEGFR	vascular endothelial growth factor receptor

4 Introduction

4.1 Cancer general

Despite many years of intensive research and promising progress in oncology, cancer still remains a life-threatening disease occurring in one out of three persons during lifespan. There are more than 100 types of cancer, all of which have distinct phenotypic and molecular features demanding different treatment-strategies. Consequently, it is very important to classify the disease correctly in order to choose the correct therapy and to enlarge tumor-type specific knowledge. In general, tumors can be classified into four categories which are determined by their tissue of origin. Carcinomas arise from epithelial cells and account for about 80% of all cancers. Malignant alterations of the lymphoid system give rise to leukemias and lymphomas. Tumors deriving from mesenchymal tissue such as bone, muscle, or fat are termed sarcomas. The fourth and last major grouping consists of neuroectordermal tumors such as glioblastoma or neuroblastomas arising from various components of the central and peripheral nervous system.

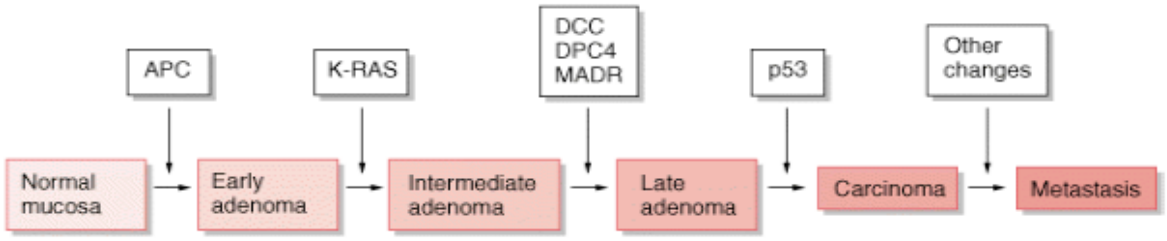

Figure 1: Serial mutations lead to tumor formation as exemplified here with colon cancer (1).

Cancer is thought to be a genetic disease as it relies on mutations in genes called proto-oncogenes or tumor suppressor genes governing functions in the regulation of cell proliferation, cell cycle and cell death – all crucial cellular events for the control of cell fate. Once a healthy cell of any part of the body experiences a certain number of mutations, cellular changes can take place and over time render the cell into a cancer cell characterised by unlimited proliferation, loss of differentiation, and capability to migrate and invade other tissues (Fig. 1). Such genetic changes can be evoked by exposition to carcinogenic compounds, among them certain chemicals, tobacco and alcohol, or radiation but also diet, lifestyle, and viral infections play a

non-neglectable role contributing to carcinogenesis. Further risk factors for the development of cancer include heritable genetic predispositions and increased age, as genetic mutations accumulate over time.

4.2 Treatment strategies

Treatment strategies against cancer comprise surgery, chemotherapy or radiation therapy. While surgery and radiation therapy are very local approaches and can only be used in the case of solid tumors, chemotherapy has a systemic impact and is thus the best strategy to avoid or treat metastatic spread. However, as it unspecifically represses all fast dividing cells in the body mostly by interfering with DNA replication, its use is limited by severe side effects. Often, a combination of treatment options is given and has helped dramatically to increase survival rates of cancer due to constant medical improvement. Still, development of targeted therapeutic interventions specifically exploiting features of cancer cells could further ameliorate clinical outcome.

Recent advances in biochemistry and molecular biology helped dramatically elucidating molecular abnormalities of cancer cells. In general, typical alterations are up-regulation or misfunction of receptors, kinases or signalling proteins as well as increased levels of secreted growth factors and pro-angiogenic molecules promoting a perfect environment for unlimited growth. Based on this knowledge, novel medical approaches include antibodies against diverse receptors expressed on the cell surface such as VEGFR, a receptor involved in angiogenesis, ErbB2, or EGFR, receptors which are both frequently found on breast cancer cells (see table 1). While antibodies are restricted to cell-surface proteins as they can not pass through the cell membrane, easily diffusible small molecule inhibitors are also able to target intracellular proteins (table 2). For instance, such compounds have been developed against BCR/ABL, which is characteristic for CML, or against the intra-cellular domain of the EGFR. As experience with specifically targeted therapies is as young as 10 years and many clinical trials are still ongoing, their impact on clinical outcome remains yet unclear although studies about targeted therapies of breast cancer and

CML are very promising and suggest to further intensify research of targeted treatment options in cancer therapy.

Table 2. Monoclonal Antibodies for Cancer Treatment

Drug	*Target*	*Antibody type*	*FDA-approved indications*	*Toxicities, side effects, and precautions*	*Monitoring*
Alemtuzumab (Campath)	CD52	Humanized, unconjugated	Chronic lymphocytic leukemia	Hematologic toxicity; opportunistic infections; rash Live vaccines should be avoided Herpes and *Pneumocystis* prophylaxis recommended	CBC; CD4 counts
Bevacizumab (Avastin)	VEGF	Humanized, unconjugated	Colorectal cancer, non-small cell lung cancer (nonsquamous)	Gastrointestinal perforation; wound healing complications; hemorrhage; arterial and venous thromboembolism; proteinuria; hypertension Discontinue use several weeks before elective surgery; do not restart until surgical incision has healed	Urinalysis; blood pressure
Cetuximab (Erbitux)	EGFR	Chimeric, unconjugated	Colorectal cancer, head and neck cancers	Acneiform rash; diarrhea; hypomagnesemia; nausea and vomiting; interstitial lung disease (rare)	Electrolyte levels; signs of inflammatory and infectious sequelae in patients with dermatologic toxicity; signs of pulmonary toxicity
Gemtuzumab ozogamicin (Mylotarg)	CD33	Humanized, toxin conjugate (calicheamicin)	Acute myeloid leukemia	Severe myelosuppression; hepatotoxicity	CBC; electrolyte levels; liver chemistries
^{90}Y-Ibritumomab tiuxetan (Zevalin)	CD20	Murine, radioisotope conjugate (yttrium-90)	Non-Hodgkin's lymphoma	Severe, prolonged myelosuppression; severe mucocutaneous reactions (e.g., Stevens-Johnson syndrome); risk of secondary malignancies (e.g., acute myeloid leukemia) Radiation safety precautions required for one week after administration*	CBC; pretreatment antibody titers in patients who have received other murine-based radioimmunotherapy regimens
Panitumumab (Vectibix)	EGFR	Human, unconjugated	Colorectal cancer	Acneiform rash; diarrhea; hypomagnesemia; hypocalcemia; nausea and vomiting; interstitial lung disease (rare)	Electrolyte levels; signs of inflammatory and infectious sequelae in patients with dermatologic toxicity; signs of ocular toxicity (e.g., conjunctivitis, ocular hyperemia, increased lacrimation, eye or eyelid irritation)
Rituximab (Rituxan)	CD20	Chimeric, unconjugated	Non-Hodgkin's lymphoma, rheumatoid arthritis	Lymphocytopenia; HBV reactivation; severe muco-cutaneous reactions (e.g., Stevens-Johnson syndrome) Live vaccines should be avoided	CBC; signs of active HBV infection or hepatitis in patients who are HBV carriers
^{131}I-Tositumomab (Bexxar)	CD20	Murine, radioisotope conjugate (iodine-131)	Non-Hodgkin's lymphoma	Hypothyroidism; severe, prolonged myelosuppression; nausea and vomiting; secondary malignancies (e.g., acute myeloid leukemia) Radiation safety precautions required for one week after administration*	CBC; thyroid function tests; pretreatment antibody titers in patients who have received other murine-based radioimmunotherapy regimens
Trastuzumab (Herceptin)	HER2/neu	Humanized, unconjugated	Breast cancer with HER2/neu overexpression	Cardiomyopathy (especially if coadministered with anthracycline chemotherapy); cytopenias; rash	Electrocardiography; left ventricular ejection fraction

NOTE: *All monoclonal antibodies are administered intravenously. Infusion reactions may occur with all monoclonal antibodies (more often with murine and chimeric antibodies) and are not listed as toxicities.*

FDA = U.S. Food and Drug Administration; CD = cluster of differentiation; CBC = complete blood count; VEGF = vascular endothelial growth factor; EGFR = epidermal growth factor receptor; HBV = hepatitis B virus.

**—Radiation precautions include careful disposal of body fluid-contaminated material, condom use for sexual relations, and hand washing.*

Table 1: List of FDA-approved monoclonal antibodies for cancer treatment (2).

Table 3. Small Molecule Inhibitors for Cancer Treatment

Drug	*Target*	*FDA-approved indications*	*Toxicities, side effects, and precautions*	*Monitoring*
Bortezomib (Velcade)	26S proteasome	Multiple myeloma, mantle cell lymphoma (a subtype of non-Hodgkin's lymphoma)	Peripheral neuropathy; myelosuppression; rash; constipation; diarrhea; edema; nausea and vomiting	Signs and symptoms of peripheral neuropathy; CBC
Dasatinib (Sprycel)	BCR-ABL, SRC family, c-KIT, PDGFR	Chronic myeloid leukemia, acute lymphocytic leukemia	Rash; diarrhea; pleural effusion; fluid retention; mucositis; myelosuppression; QT interval prolongation	CBC; ECG; liver chemistries; weight; signs and symptoms of fluid retention
Erlotinib (Tarceva)	EGFR	Non-small cell lung cancer, pancreatic cancer	Acneiform rash; diarrhea; loss of appetite; nausea and vomiting; fatigue; conjunctivitis; elevated liver chemistries	Liver chemistries; signs of inflammatory or infectious sequelae in patients with dermatologic toxicity
Gefitinib (Iressa)	EGFR	Non-small cell lung cancer	Acneiform rash; diarrhea; loss of appetite; interstitial lung disease (rare); elevated liver chemistries	Liver chemistries; signs of inflammatory or infectious sequelae in patients with dermatologic toxicity
Imatinib (Gleevec)	BCR-ABL, c-KIT, PDGFR	Acute lymphocytic leukemia, chronic myeloid leukemia, gastrointestinal stromal tumor, hypereosinophilic syndrome, systemic mastocytosis	Rash; weight gain; edema; pleural effusion; cardiac toxicity (depression of LVEF); nausea and vomiting; arthralgias and myalgias; myelosuppression	CBC; liver chemistries; weight; signs and symptoms of fluid retention
Lapatinib (Tykerb)	HER2/neu, EGFR	Breast cancer with HER2/neu overexpression	Cardiac toxicity (depression of LVEF; QT prolongation); acneiform rash; palmar-plantar erythrodysesthesia (hand-foot syndrome); diarrhea; nausea and vomiting; elevated liver chemistries	LVEF; ECG; electrolyte levels; liver chemistries
Sorafenib (Nexavar)	BRAF, VEGFR, EGFR, PDGFR	Renal cell cancer, hepatocellular carcinoma	Hypertension; alopecia; bleeding; rash; palmar-plantar erythrodysesthesia (hand-foot syndrome); hypophosphatemia; diarrhea; nausea and vomiting; elevated amylase and lipase levels; myelosuppression; wound-healing complications Discontinue treatment temporarily for surgical procedures	Blood pressure; dermatologic toxicity (including palmar-plantar erythrodysesthesia [hand-foot syndrome]); amylase, lipase, and phosphate levels; CBC
Sunitinib (Sutent)	VEGFR, PDGFR, c-KIT, FLT3	Renal cell cancer, gastrointestinal stromal tumor	Nausea and vomiting; yellow discoloration of skin; hypothyroidism; depression of LVEF; adrenal function abnormalities; diarrhea; myelosuppression; mucositis; elevated lipase and creatinine levels; elevated liver chemistries; increased uric acid levels	Adrenal function in patients with trauma or severe infection, or in those undergoing surgery; blood pressure; ECG; LVEF; CBC; electrolyte levels (magnesium and potassium); phosphate levels; signs and symptoms of pancreatitis; thyroid function tests

NOTE: *All small molecule inhibitors are administered orally except bortezomib, which is administered intravenously. Most small molecule inhibitors undergo metabolism by cytochrome P450 enzymes and are therefore subject to multiple potential interactions (e.g., with anticonvulsants, azole antifungals, dexamethasone, isoniazid [Nydrazid], macrolide antibiotics, nefazodone [Serzone, brand no longer available in the United States], protease inhibitors, rifampin [Rifadin], St. John's wort, verapamil [Calan], and warfarin [Coumadin]).*

FDA = U.S. Food and Drug Administration; CBC = complete blood count; BCR-ABL = breakpoint cluster region-Abelson; PDGFR = platelet-derived growth factor receptor; ECG = electrocardiography; EGFR = epidermal growth factor receptor; LVEF = left ventricular ejection fraction; VEGFR = vascular endothelial growth factor receptor.

Table 2: List of FDA-approved small molecule inhibitors for cancer treatment (2).

4.3 Cancer in childhood

Childhood cancers may arise in the first two decades of life and account for about 1% of all malignancies. Nevertheless, cancer is the most common cause of disease-related death in children of industrial nations. Interestingly, most tumors diagnosed in children originate from tissues other than those in adults (Fig. 2). As outlined above, about 80% of adult cancers are carcinomas deriving from epithelial structures. In contrast, carcinomas only account for 2% of all tumors in children, whereas

leukemias, neuronal tumors and sarcomas occur at a much higher rate. Childhood cancer seems to be a developmental disease since tumors most commonly arise from embryonic mesenchyme and arise subsequently during the first years of life. Therefore, it is hypothesized that genetic aberrations, emerging during embryogenesis, may lead to abnormal cell signalling governing differentiation and proliferation. A block in differentiation and an induction of uncontrolled cell growth may consequently lead to the formation of a tumor precursor cell. Frequently, pediatric cancers display tumor-specific chromosomal translocations as they can be found in leukemias and sarcomas. They are strongly associated with particular tumor subtypes and therefore, their detection may be helpful for diagnosis and prognosis. On the molecular level, chromosomal translocations give rise to chimeric fusion proteins often involving kinases or transcription factors, which potentially contribute to tumorigenesis by altering cellular signalling. The best example for this phenomenon is probably the CML-typical translocation product BCR/ABL, which encodes for a hyperactive kinase.

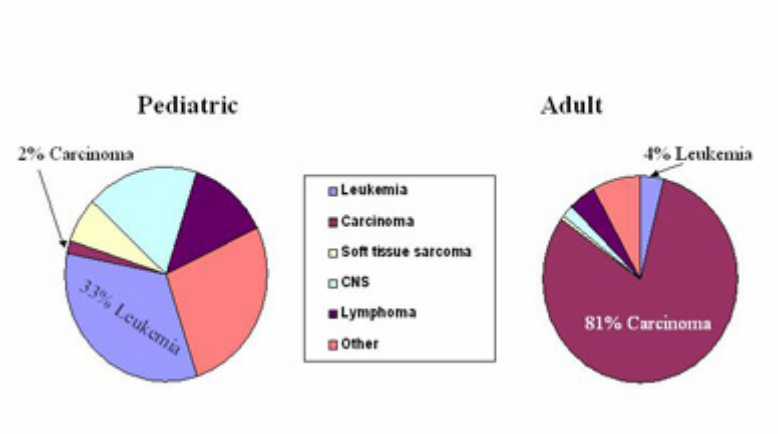

Figure 2: Distribution of cancer categories in children and adults (3)

4.4 Rhabdomyosarcoma

Another pediatric tumor being associated with chromosomal translocations is rhabdomyosarcoma (4), the most common soft tissue sarcoma in childhood accounting for 4-8% of all pediatric tumors. This solid tumor can occur at virtually any site of the body and the median age at the time-point of diagnosis is 5 years (5). It is thought to originate from muscle precursor cells due to resemblance of RMS tumors to primitive skeletal muscle and expression of some transcription factors normally

involved in myogenic differentiation, such as paired box proteins (PAX) and myogenic regulatory factors (MRFs) like MyoD, Myf5, Myf6, and myogenin. Most cases are sporadic tumor incidences, although there is some evidence for inherited predisposition to RMS, which is observed in Li-Fraumeni, Beckwith-Wiedmann, and Nevoid Basal Carcinoma syndromes. In these syndromes, inherited mutations of the tumor suppressor p53, CDKN1C, and PTCH, respectively, may predispose to rhabdomyosarcomagenesis (6, 7).

Based on histological properties, RMS can be divided into four subtypes: embryonal, alveolar, and pleomorphic RMS. The clinical diagnosis of these subgroups is sometimes challenging (8), yet it is essential for prognosis and correct choice of the treatment strategy. Therefore, besides histological evaluation of tumor samples, diagnosis is frequently performed by using real-time PCR for the detection of translocation products, by immunohistochemistry for the staining of subgroups-specific markers or even by global expression profiling techniques (9-12).

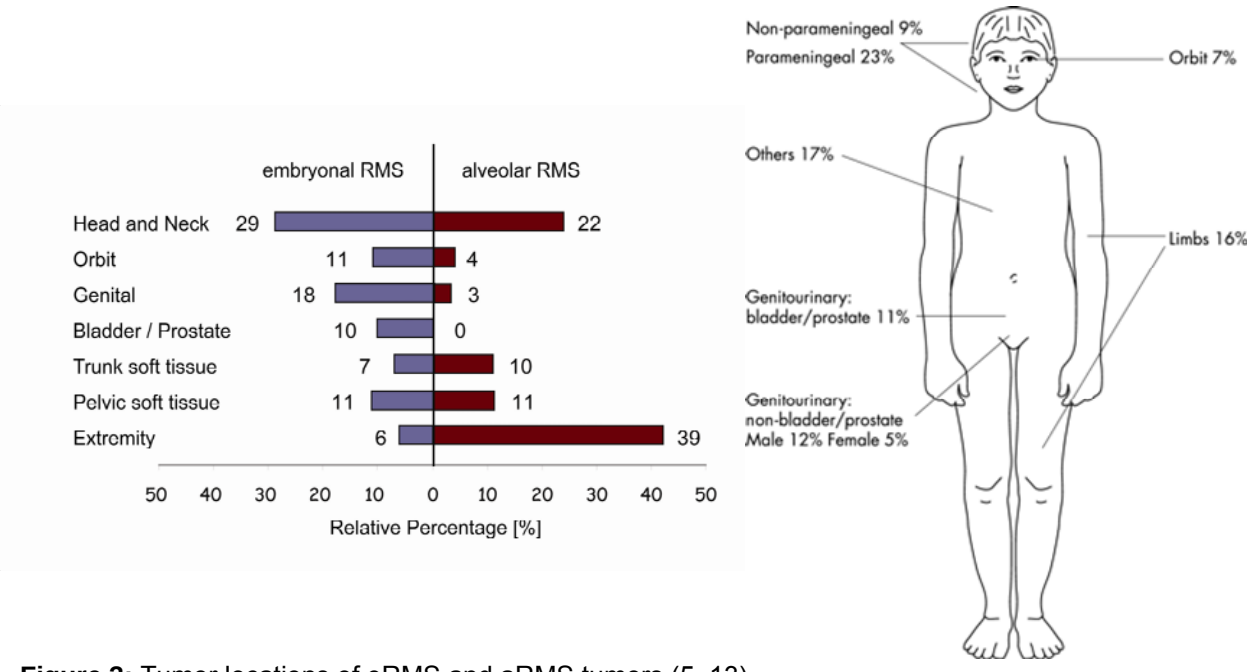

Figure 3: Tumor locations of eRMS and aRMS tumors (5, 13).

4.4.1 Embryonal RMS (eRMS)

The embryonal subtype (eRMS) accounts for about 60% of all RMS cases with highest incidence between ages 0-4 years. eRMS has a good prognosis (4) as it often occurs at favourable sites, including the genitourinary tract, the gastrointestinal tract, and the head and neck region (Fig. 3). Moreover, this subtype is known to exhibit a decreased metastatic potential. eRMS tumors can be histologically characterized by primitive spindle cells arranged at low density (Fig. 4).

Genetically, eRMS tumors lack specific chromosomal translocations, but exhibit high expression levels of PAX3 and PAX7 (14). Moreover, most cases of eRMS exhibit loss of heterozygosity (LOH) at chromosome region 11p15.5 presumably encoding a tumor suppressor (15).

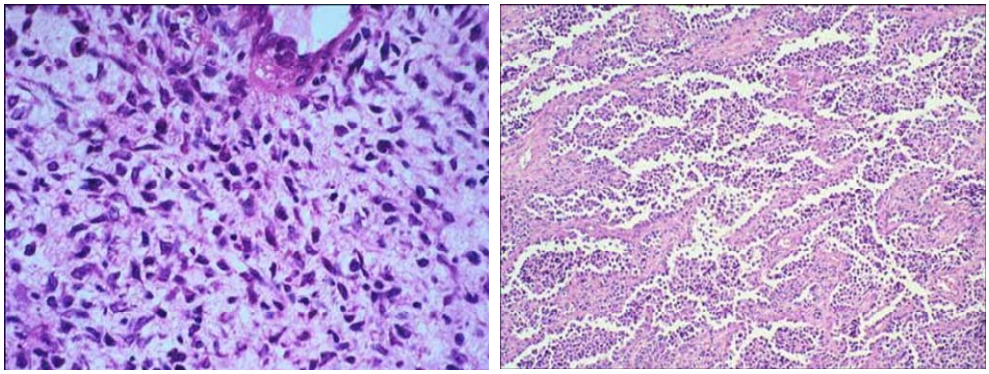

Figure 4: Microscopic view of eRMS (left panel) and aRMS (right panel)(16).

4.4.2 Alveolar RMS (aRMS)

On the other hand, the less frequent alveolar RMS (aRMS) subtype occurs in rather older children, is often observed in extremities (Fig. 3), and has a worse clinical outcome than eRMS associated with its increased metastatic potential. aRMS tumors are histologically characterized by densly packed small round or oval tumor cells, which are loosely, noncohesively arranged to aggregates separated by strands of fibrous tissue (Fig. 4).

About 80% of all aRMS cases bear typical translocations between either t(2;13)(q35;q14) or t(1;13)(p36;q14) encoding for fusions proteins PAX3/FKHR or PAX7/FKHR, respectively (17). Interestingly, all of the involved fused proteins are transcription factors belonging either to the family of paired box (PAX) transcription factors or to the family of forkhead transcription factor proteins. While PAX3 and PAX7 are crucially implicated in the development of skeletal muscle, FKHR plays a role in the regulation of cell cycle progression, cellular metabolism, and apoptosis (18). Recently, the translocation t(2;2)(q35;p23) has been discovered, which fuses the PAX3 gene with the nuclear receptor coactivator NCOA1 gene (19).

4.4.3 Pleomorphic RMS (pRMS)

This type of RMS is very unusual and accounts for only 1 – 3% of all diagnosed RMS tumors. Although, RMS generally belongs to the group of pediatric cancers, this subtype almost solely affects adults between 30 – 50 years of age and has a rather poor treatment outcome.

4.5 Abberrant cellular signalling pathways in RMS

In RMS, a diverse set of mutations, translocations and amplifications have been detected. Hence, many alterations in cell signalling pathways contribute to the formation and maintenance of RMS tumors. Such alterations affect proto-oncogenes and tumor suppressor genes including p53, N-myc, ras, gli, mdm2, c-met and others (20, 21). Furthermore, RMS is known to establish autocrine growth factor loops of EGF, bFGF, TGF-β, IGF-II, NGF, VEGF, CTGF, leading to the constitutive activation of their specific growth factor receptor signalling pathways (22-25). The persistent activation of these growth factor signalling cascades may contribute to a series of tumor-promoting effects, such as the inhibition of differentiation, stimulation of proliferation and evasion of apoptosis. Additionally, there is evidence for an autorcrine loop of myostatin, a negative regulator of muscle growth, which may keep RMS tumor cells undifferentiated (26).

4.5.1 Deregulated expression of PAX genes in RMS

As mentioned before, PAX3 or PAX7 proteins as well as their fusion proteins are characteristic features of RMS. Abberant expression or function of members belonging to the familiy of paired box transcription factors is reported for many different cancer types and therefore seems to have an oncogenic impact (27). In fact, in 1993 the oncogenic potential of PAX genes was described for the first time by Maulbecker and Gruss, who recognized that induction of PAX gene expression in mouse embryonic fibroblasts leads to cell transformation and *in vivo* tumor growth (28). Therefore, the role of PAX3 and 7 will be addressed in the next section regarding their contribution to rhabomyosarcomagenesis.

During development, PAX3 and at lower levels PAX7 are expressed in skeletal muscle progenitor cells driving the expression of myogenic regulatory factors (MRFs), such as MyoD and Myf5, which results in the differentiation of skeletal muscle at their final location. When healthy muscle precursor cells enter the myogenic program, the expression of PAX3 and PAX7 is suppressed and thus both are undetectable in adult skeletal muscle cells (29). In adults, these PAX genes are only found to be expressed in muscle satellite cells, where PAX3 promotes proliferation of previously activated satellite cells in response to muscle injury, whereas PAX7 is required for the maintenance of their undifferentiated state (30).

Controversly, in RMS tumors, which express various proteins involved in muscle cell development and differentiation, such as PAX3, PAX7, and MRFs, differentiation into myotubes and myofilaments is blocked, suggesting that RMS origins from muscle precursor cell exhibiting interrupted myogenesis.

eRMS tumors lacking translocations display deregulated expression of PAX3 and to a higher level of PAX7 (31). As PAX7 is greatly overexpressed in comparison to PAX3, it is thought to contribute to the tumorigenic phenotype of eRMS. Due to PAX7s role as an inhibitory factor of apoptosis and differentiation in muscle satellite cells, this cell type could be the origin of eRMS tumor formation (32, 33).

On the other hand, in translocation-bearing aRMS tumors, the aberrant expression of PAX3/FKHR or the less frequently occurring PAX7/FKHR is considered as the oncogenic driving force. Notably, the aRMS typical fusion-proteins act as chimeric transcription factors containing the DNA binding domain of PAX3 or PAX7 and the trans-activation domain of FKHR (Fig. 4)(34). As reported for PAX3/FKHR, this makes it a more potent transcriptional activator because of its transactivation domain deriving from FKHR, which was shown to have a higher potency than the PAX3 transactivation domain (35, 36). Evidence for the transforming capacity of PAX3/FKHR is derived from chicken embryo fibroblasts, where only PAX3/FKHR but not PAX3 was able to induce transformation (37), and moreover from experiments with cultured fibroblasts, where the fusion-protein inhibited differentiation more efficienltly than its wild-type counterpart PAX3 (38).

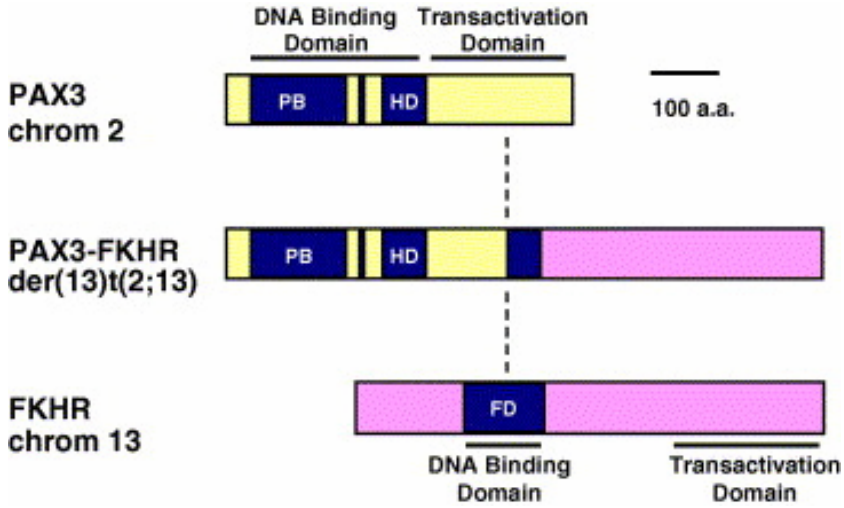

Figure 5: Schematic of the fusion protein PAX3/FKHR (34)

Furthermore, down-regulation of PAX3/FKHR in aRMS cells *in vitro* as well as *in vivo* led to induction of apoptosis (39) or suppression of tumor growth reflecting a crucial role (40) of the fusion-protein in tumor survival. Contribution of PAX3/FKHR to formation of metastasis was also reported by correlating the expression of the chimeric transcription factor with the highly metastatic phenotype of aRMS (41). However, in vivo studies with PAX3/FKHR knock-in mice indicated that PAX3/FKHR alone isn't sufficient for aRMS formation (42) demanding yet other mutations accounting for rhabdomyosarcomagenesis.

Nevertheless, downstream-target genes being activated by PAX3 and PAX3/FKHR are numerous and some of them are know to participate in the maintenance of RMS tumors:

Bcl-2 and BCL-XL

The anti-apoptotic protein BCL-XL is directly activated by PAX3 and its fusion-protein. Hence, affected cells are less susceptible to controlled cell death and apoptosis-induction after PAX3/FKHR down-regulation can be explained by this gene regulation (43).

Hepatocyte growth factor receptor (HGFR, c-Met)

In healthy developing muscle cells, c-Met is transcriptionally regulated by PAX3 and controls migration of these cells. In cancer cells, overexpression or activating mutations in the RTK c-Met causes alterations in regulatory processes such as proliferation, migration, apoptosis, and angiogenesis (44). Due to its overexpression in RMS, c-Met could also contribute to rhabdomyosacomagenesis. This notion gained further support by the observation that c-Met expression in combination with inactivation of the INK4A/ARF locus leads to malignant RMS formation in mice (45).

Insulin-like growth factor receptor I (IGF-IR)

In many cancers, overexpressed IGF-IR acts as an anti-apoptotic protein (46). Interestingly, PAX3- and PAX3-FKHR-induced overexpression of IGF-IR can be linked to an autocrine IGF-II growth factor loop since IGF-IR is activated by IGF-II (47). A recent study showed that anti-IGF-IR antibody (h7C10) had single agent antitumor activity *in vivo* with aRMS xenograft bearing mice (16). *In vitro* analysis revealed a direct and very significant correlation between elevated IGF-IR levels and antiproliferative effects of the anti-IGF-IR antibody.

Platelet derived growth factor (PDGFR)

During development, PDGFR expression is important for the induction of proliferation of muscle precursor cells and is known to be down-regulated after activation of the myogenic differentiation. Therefore, PDGFR is suggested to contribute to cell proliferation in RMS tumors and moreover was found to be strongly correlated to decrease of failure-free survival (48).

4.5.2 PAX3/FKHR activates additional target genes

Additionally, the aRMS-typcial PAX3/FKHR fusion protein can activate yet other genes due to its higher transactivation potency deriving from FKHR as explained above. Especially in regard to the worse clinical prognosis of aRMS cases it is interesting to elucidate alterations in gene expression potentially induced by PAX3/FKHR in comparison to eRMS tumors, which have usually a better disease progression.

Furthermore, genes with high expression in aRMS could be envisaged as future drug targets for novel therapeutic approaches, as explained before, or else serve as diagnostic markers. To approach this idea, a gene expression profiling study was performed in 2004 by Wachtel et al. by comparing translocation-positive aRMS biopsies to translocation-negative RMS cases and healthy muscle cells (19).

Upon statistical evaluation, several transcriptional regulators such as AP2β or enhancer-of-split as well as two proteases called ADAM and elastase, which probably contribute to the enhanced metastatic potential of aRMS, were found to be prominently up-regulated in translocation-positive samples. However, from a therapeutic point of view undoubtedly the most interesting genes with differential expression were found in the group of receptor proteins. In translocation-positive samples, up-regulation of cannabinoid receptor 1 (CB1), FGFR2, FGFR4, α2-adrenergic receptor, as well as the acetylcholine receptor β3 by a factor 10-100-fold was detected. All of these receptors can be targeted with receptor-specific agonists, inhibitors or antagonists, but so far, only activation of CB1 as well as inhibition of FGFR have been shown to have promising anti-neoplastic effects. In the next section we will thus focus on CB1 and its physiological implications.

Noteworthy, several lines of evidence led to the conclusion that CB1 expression is directly regulated by the PAX3/FKHR fusion protein (49-51). While exogenous expression of this fusion-protein leads to gene induction of CB1 in 293T cells (Fig. 6A), its down-regulation in aRMS cells significantly lowers CB1 transcript levels over time (Fig. 6B). Additionally, chromatin immunoprecipitations confirmed the direct binding of Pax3/FKHR to the promoter region of CB1 (49).

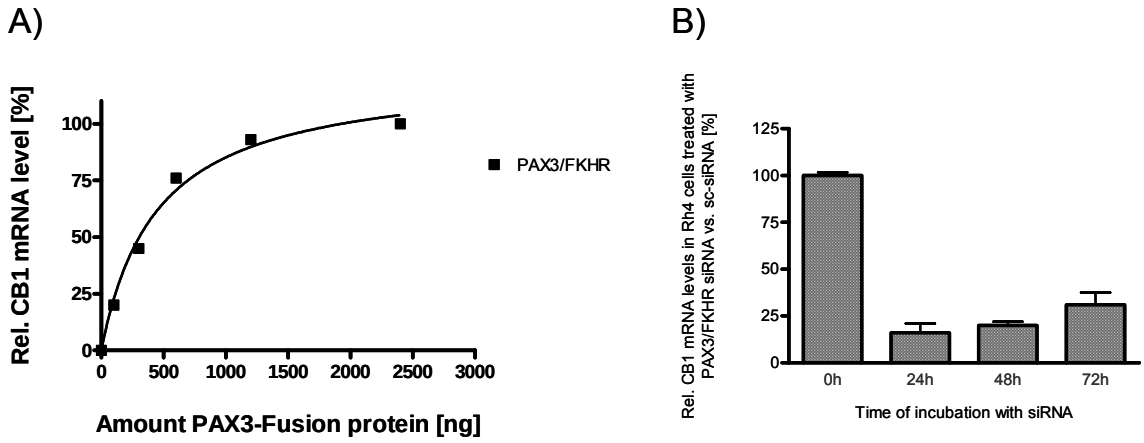

Figure 6: CB1 expression is directly regulated by PAX3/FKHR as demonstrated by exogenous PAX3/FKHR expression in 293T cells (A) and PAX3/FKHR silencing in aRMS cells (B).

4.6 Cannabinoid receptor

Cannabinoid receptors are G-protein coupled receptors, which got their name due to activation by substances called cannabinoids extracted from the plant *Cannabis Sativa*. So far, two cannabinoid receptors have been well characterised and a third one was proposed recently. CB1 is abundantly expressed in the brain and at lower levels in peripheral nerve terminals and various extraneural sites such as spleen, vascular endothelium or testis being mainly responsible for central effects of cannabinoids (52). In contrast, CB2 is almost exclusively detected in the immune system, where it is involved in the regulation of inflammation (53). The novel cannabinoid receptor GPR55, also referred to as CB3, is mainly found in adrenals, spleen and brain (54).

Molecules with affinity to cannabinoid receptors are called cannabinoids, a term which includes cannabinoid receptor agonists as well as antagonists. Generally, these substances are of very lipophilic nature, most of them have mixed affinities for CB1 as well as CB2, and can be assigned to four different structural groups (53)(Fig. 5). The first group contains classical cannabinoids which are tricyclic dibenzopyran derivatives. They occur naturally in the marijuana plant *Cannabis Sativa*, as the famous Δ^9-tetrahydrocannabinol (THC), or are synthetic analogues of the natural compounds, eg. HU210. The second group consists of synthetic non-classical cannabinoids lacking the dihydropyranring of THC. Thirdly, aminoalkylindoles with

representatives as WIN55, 212-2 form a group. The fourth group of cannabinoids are called endocannabinoids, which are eicosanoid compounds rather than cannabinoid compounds having completely different structures than the before mentioned groups. Substances as arachidonoylethanolamide (AEA) occur endogenously in the body and form together with their molecular targets and metabolizing enzymes the endocannabinoid system, which is involved in neuromodulation and regulation of the immune response (55). Confusingly, some of the endocannabinoids also activate vanilloid receptors (VR), which don't belong to the group of cannabinoid receptors. Additionally, cannabinoid receptor antagonists such as SR141716A with selective binding to either CB1 or CB2 have been synthesized being very valuable for determination of receptor specificity.

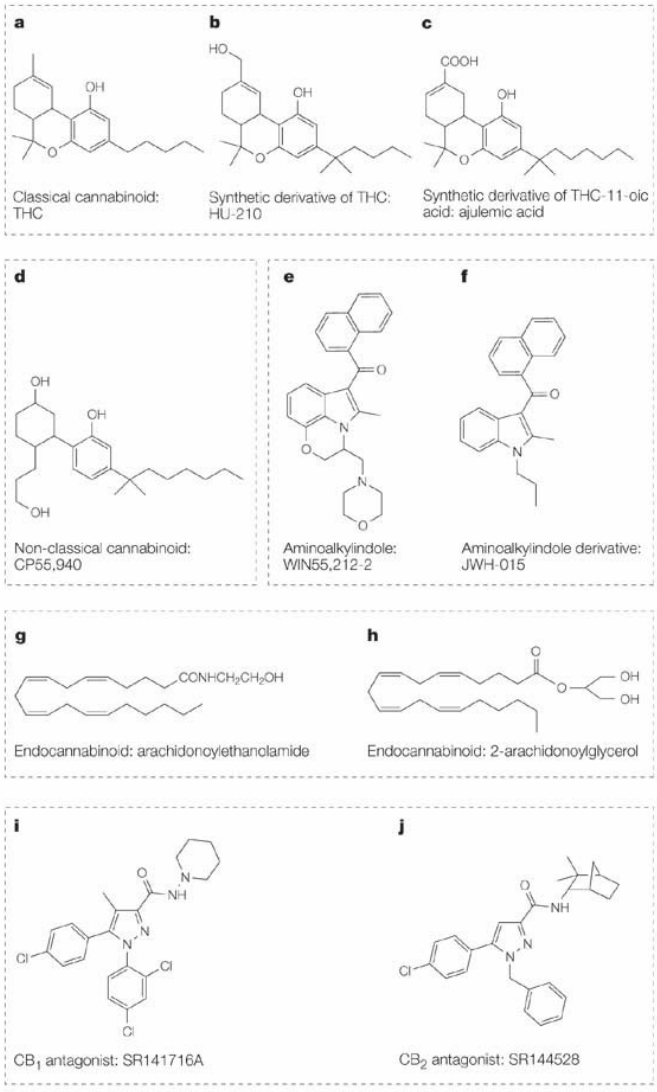

Figure 7: Structure of cannabinoid groups (53)

Physiological effects of cannabinoids are much more diverse than generally assumed and thus could serve as thearpeutic option for various health problems. Apart from their well-known psychoactive action mediated exclusively through CB1 stimulation, cannabinoid receptor agonists enhance appetite, relief pain, inhibit nausea and emesis, inhibit muscle weakness and have positive influences on mood effects such as sedation, anti-depression or hypnosis (56). These properties enable cannabinoid receptor agonists to palliatively counteract side-effects of chemotherapies, which the two cannabinoids dronabinol and nabilone are already approved for. On the other hand, inhibition of the cannabinoid system is exploited to fight obesity as cannabinoids centrally control feeding behaviour through CB1. About two years ago, the specific CB1-antagonist SR141716 (Rimonabant) produced by Sanofi-Aventis was allowed in the European Union and other countries to reduce weight in obese patients, whereas clinical trials with yet other CB1-antagonists are ongoing (57).

Yet other implications for therapeutic use of cannabinoids are neurodegenerative diseases such as Alzheimers (58) and Multiple Sclerosis (59), where the activation of CB2 receptors and the blockade of endocannabinoid-degrading enzymes may potentiate the neuroprotection and trigger anti-inflammatory responses. Supposedly, these anti-inflammatory effects are thanks to the ability of cannabinoids to suppress the expression of cytokines, as well as other pro-inflammatory mediators. In addition, these drugs might also function by increasing the production of anti-inflammatory mediators (53). This way, they might also provide the basis for more effective drugs for the management of chronic inflammatory diseases, eg. Arthritis or Crohn’s Disease.

Most interesting for oncologists of course is the recently emerging role of cannabinods and their receptors in cancer. Evidence exists that loss of CB1 accelerates intestinal tumor growth (60) whereas increased endocannabinoid levels reduce the development of precancerous lesions in the mouse colon (61). Besides that, numerous publications underline the anti-neoplastic action of cannabinoids, which will be further discussed in the next section consisting of a review written together with J.Gertsch (D-CHAB, ETH Zürich).

5 Review

5.1 Cannabinoid Receptor Ligands as Potential Anti-Cancer Agents – High Hopes for New Therapies?

Susanne Oesch[1], Jürg Gertsch[2]

[1] University Children's Hospital Divisions of Clinical Chemistry and Oncology, University of Zürich, Steinwiesstrasse 75, CH-8032 Zürich, Switzerland

[2] Department of Chemistry and Apllied Biosciences, ETH, Wolfgang-Pauli-Str. 10, CH-8093 Zürich, Switzerland

The endocannabinoid system (ECS) is an endogenous lipid signaling network comprising arachidonic acid-derived ligands, cannabinoid receptors, transporters and endocannabinoid degrading enzymes. The cannabinoid CB_1 receptor is expressed in neurons but also co-expressed together with the CB_2 receptor in different peripheral tissues. In the last years, CB receptor ligands, including Δ^9-tetrahydrocannabinol (THC), have been proposed as novel anticancer agents.

The aim of this review is to critically discuss the potential of CB receptor activation as therapeutic anti-cancer strategy with regard to ligand selectivity, tissue specificity, and potency. Intriguingly, antitumor effects mediated by cannabinoids are not confined to inhibition of cancer cell proliferation but also reduced angiogenesis, migration, metastasis, inhibition of carcinogenesis, and attenuation of inflammatory processes. Although in the last decade many new CB_1 and CB_2 receptor selective agents have been described most studies in the area of cancer research have employed non-selective CB ligands. Moreover, many of these ligands exert prominent CB receptor-independent pharmacological effects, such as activation of the GPR55 receptor, PPAR-gamma, and the TRPV channels. At present, the role of

the ECS in tumorigenesis is still poorly understood and the molecular mechanisms of cannabinoid anti-cancer action are being elucidated. Furthermore, in light of the problematic central effects exerted by CB_1 receptor ligands the development of CB_2 receptor-selective anticancer agents should be advantageous. Probably the most interesting question is whether cannabinoids could be useful in combination with established chemotherapeutic agents.

5.1.1 Introduction

The endocannabinoid system (ECS) comprises the two well characterized G-protein coupled receptors (GPCRs) CB_1 and CB_2 (62-64), as well as the putative new CB receptor GPR55 (54, 65), a former orphan GPCR. There is also pharmacological evidence of yet another putative cannabinoid receptor that remains unknown (66). The endogenous ligands that activate CB receptors are arachidonic acid derived derivatives, primarily arachidonoyl ethanolamide (anandamide) and 2-arachidonoyl glycerol (2-AG) (67, 68), which are directly released from cell membranes. These endocannabinoids are pleiotropic lipids and anandamide also activates transient receptor potential vanilloid 1 (TRPV1), binds to peroxisome proliferators-activated receptor (PPAR-gamma) and potentially signals via serotonin 5HT(3) receptors (69-71). Moreover, 2-AG is a potent ligand for GPR55 whose function is yet to be uncovered (54).

Among other effects, endocannabinoids have been reported to modulate cell differentiation, cell signaling, cell migration, and cell fate (72-74). The terpenophenolic phytocannabinoids from *Cannabis sativa* with the prototype cannabinoid Δ^9-tetrahydrocannabinol (THC) (75) were the first CB receptor ligands known and they have served as molecular scaffolds for the chemical development of analogous structures, such as CP55,940 (76). The use of CP55.940 as radioligand has finally helped to clone the CB receptors. It is probably not overstated to claim that without *Cannabis* research the ECS would not have been explored to such an extent as it was in the last two decades. Today, it is increasingly clear that the ECS is not only involved in central nervous system regulation (mainly via CB_1), neuroimmunological processes (both via CB_1 and CB_2), but also in several peripheral physiological processes (57).

It is important to highlight that many of the functions of the ECS which are currently proposed are not yet fully understood. Within the last decade, growing evidence has accumulated that suggests that cannabinoid receptor agonists may have antitumor properties on a variety of cancer types, and the topic has been reviewed recently in several cancer research related journals (55, 77-79). In this review, the recent developments and insights shall be critically discussed with respect to CB receptor

signaling, ligand selectivity, effect specificity in different tissues, and potential therapeutic relevance.

In 1975, Munson et al. for the first time reported that cannabinoids can reduce tumor growth and viability of lung cancer cells *in vitro* as well as *in vivo* experiments (80). After this initial observation another twenty years passed until more detailed investigations yielded further insights on the anticancer mechanisms of cannabinoids. However, only recently the mechanism of action of CB receptor ligands is starting to become uncovered (*vide infra*). While some signaling events involved in the cytotoxic effects exerted by cannabinoids apply for all cellular models other molecular mechanisms are restricted to only a few cell types. Thus, an important question is how CB receptor activation ultimately leads to inhibition and apoptosis of cancer cells? It is currently not clear whether these effects are specific for certain types of cancer cells and whether the *in vitro* data actually correlate with more physiological data, in particular with regard to the concentrations employed.

5.1.2 CB receptor mediated signal transduction events leading to anticancer effects

Despite a growing amount of data on the cellular signaling events triggered by cannabinoids in non-neuronal cells there is no straight-forward mechanism of action which could be postulated. Pharmacological intervention in cancer therapy typically relies on well-defined molecular mechanisms that lead to tumor growth attenuation, such as inhibition of microtubule dynamics, topoisomerase inhibition or DNA intercalation. So far, GPCR signaling in cancer therapy is not a well-designed strategy and there are still many open questions, like signaling dynamics, limited prolongation of effect due to desensitization, specificity of signals, and potentially unwanted effects. However, with a change of paradigm from the “one selective-drug acting on one target” to network pharmacology (81) anticancer strategies involving GPCRs may become more interesting in the future. Fundamental to such developments is a better understanding of GPCR cellular signaling cascades as cancer cells often hijack the normal physiological functions of GPCRs to survive (82). As shown in the following sections, CB receptor signaling events leading to antitumor

effects are complex and largely dependent on tissue type and physiological context (Fig.1).

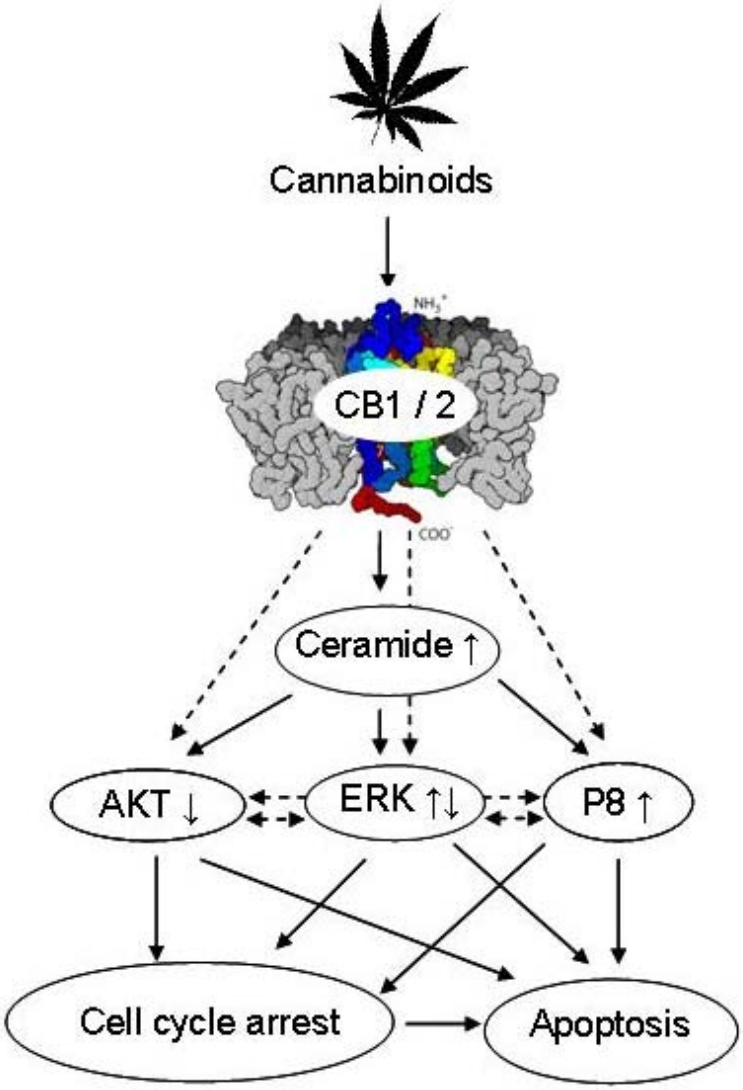

Figure (Review) 1: Major signaling pathways involved in the anticancer effects exerted by CB receptor ligands.

5.1.2.1 Ceramide

Ceramides are composed of sphingosine and fatty acid moieties. Ceramides are commonly found at high concentrations within the cell membrane, derived from sphingomyelin, one of the major membrane lipids. Ceramide is also a lipid messenger specifically released upon activation (e.g. via GPCR activation) and it

also appears to play a key role in mediating different effects on cell survival subsequent to CB receptor activation (83). An acute rise of ceramide by sphingomyelin hydrolysis is observed both in glioma cells and in normal primary astrocytes after cannabinoid challenge presumably mediated through CB_1 (84, 85). In addition, the malignant glioma cells as well as other cancer cells (eg. pancreatic cancer) are subject to a sustained de novo ceramide generation in a CB_1/CB_2-dependent manner resulting in inhibition of AKT (86, 87), which among other cues will finally drive them into apoptosis. More strikingly, it appears that cannabinoids are able to protect astrocytes and other neuronal cells from oxidative stress (88) and other neurotoxic signals (89) through ceramide signaling. Where the exact switch for this differential reaction to the apparently identical stimulus lies remains unknown, although regarding the known signaling events it should be concluded that AKT acts downstream of it.

5.1.2.2 AKT signaling

Akt1, also known as "AKT" or protein kinase B represents a group of 3 enzymes of the serine/threonine-specific protein kinase family. AKT is involved in cellular survival pathways, by inhibiting apoptotic processes (90). Independent of the ceramide-mediated effect on AKT described above, both CB_1 and CB_2 receptors are coupled to Gi/Go proteins in different cell types and they inhibit adenylate cyclase and can activate the phosphatidylinositol 3-kinase (PI3 kinase), which activates the prosurvival protein serine/threonine protein kinase AKT (v-akt murine thymoma viral oncogene cellular homolog) via PIP3 (phosphatidylinositol (3,4,5)-tri-phosphate) (91, 92). Along that line, cannabinoid stimulation of whole brain areas and of healthy non-transformed cells like astrocytes, but also CB_1-positive CHO cells causes AKT activation almost independently of drug concentration (93-95).

In contrast, one of the general pro-apoptotic signaling events after cannabinoid-treatment in cancer cells is the dephosphorylation of AKT1, occurring after both CB_1 and CB_2 receptor activation and probably involving dominant ceramide signaling. PI3K dependent AKT activation can be regulated through the tumor suppressor PTEN (phosphatase and tensin homolog), which works essentially as the opposite of

PI3 kinase mentioned above (96). PTEN acts as a phosphatase to dephosphorylate PtdIns(3,4,5)P3 back to PtdIns(4,5)P2. This removes the membrane-localization factor from the AKT signaling pathway. Without this localization, the rate of AKT activation decreases significantly, as do the all the downstream pathways that depend on AKT for activation. PIP3 can also be de-phosphorylated at position "5" by the SHIP family of inositol phosphatases, SHIP1 and SHIP2. These poly-phosphate inositil phosphatases dephosphorylate PtdIns(3,4,5)P3 to form PtdIns(3,4)P2.

An increasing number of data suggest that AKT inhibition is one of the critical events after cannabinoid administration which determines the cellular downstream effects that ultimately lead to apoptosis. In transformed cancer cells, low nanomolar concentrations of CB agonists lead to AKT phosphorylation through transactivation by EGFR followed by a pro-survival proliferative burst (97), whereas higher concentrations decrease the activation status of AKT, usually culminating in growth arrest or apoptosis (86, 98, 99). Importantly, overexpression of AKT could rescue the cannabinoid-induced apoptosis in melanoma cells reflecting its essential role in the mediation of cannabinoid-induced apoptosis (100).Therefore, the concentration range of cannabinoids and the cellular transformation status appear to critically influence the cytotoxic effects mediated via AKT signaling.

5.1.2.3 ERK

Another well known signaling molecule recruited by cannabinoid receptor agonist treatment of cancer cells is ERK (Extracellular Signal-Regulated Kinase). However, reports about its activation or inhibition by cannabinoids differ between cancer types, indicating a yet unclear and maybe more complex role. After incubation with cannabinoids, cells derived from glioma (101), prostate cancer (98), and breast cancer (102) display a sustained ERK activation, in melanoma cells activation levels of ERK remained unchanged (100), whereas in lung (103) and colon cancer cells a reduced phosphorylation of ERK was observed (99). While inhibition of the usually pro-proliferative signaling molecule ERK is in line with a pro-apoptotic signaling cascade (104), it is more difficult to understand the contribution of activated ERK to the cannabinoid-induced growth inhibition. Evidence exists that ceramide induced by cannabinoid-treatment and inhibition of PKA by Gi-coupled cannabinoid receptor

stimulation can both cause chronic ERK activation, which is published to lead over long time spans to cell cycle arrest and cell death (101, 102). In leukaemia cells Erk1/2 was induced more strongly by CB_2 receptor-selective agonists than in primary leukocytes (105). In the same study it was shown that Erk phosphorylation was context-dependent as lipopolysaccharide-induced Erk1/2 activation could be partially blocked by CB_2 ligands.

5.1.2.4 Sp(G/C)F-1 (P8)

Similarly affected by CB-agonist stimulation is the transcription factor p8 (or candidate of metastasis 1, also referred to as Sp(G/C)F-1), an endoplasmic reticulum associated stress protein able to bind to DNA (106). After treatment with cannabinoids, it is up-regulated, probably in response to de-novo synthesized ceramide, in different cancer cell lines and subsequently leads to co-recruiting of the transcription factors ATF4, TRB3 and CHOP, all three also critically involved in the cellular response to stress-stimuli probably both via CB_1 and CB_2 receptors (87, 107). P8 seems to be a key factor for cellular sensitivity towards cannabinoids as its siRNA-mediated knock-down in glioma cells as well as in breast cancer cells can abolish cytotoxicity of THC and it is further implicated in the potential synergistic effect of chemotherapeutic agents in combination with cannabinoids (87, 108).

5.1.3 Cell cycle arrest and apoptosis

As classical anticancer agents directly inhibit tumor cell growth, also the effects of cannabinoids on cancer cell cycle and apoptosis induction have been investigated in detail. However, there is no emerging general picture, like e.g. the G2/M cell cycle arrest typically observed with tubulin-targeting antimitotic agents, probably because different cell types react differently to distinct concentrations of cannabinoids and there are different stress-related mechanisms of action (55). Moreover, cannabinoids are not very cytotoxic and typically exert their effect in the upper nM and µM range, depending on the initial cell number and respective experiment. While some CB receptor expressing cancer cells survive even higher µM cannabinoid treatment (e.g. HL60 cells) (Gertsch et al., unpublished data) other cancer cells (e.g. Jurkat T-cells

(109)) undergo cell cycle arrest and apoptosis, in part coupled to the signaling pathways described above.

Interestingly, Met-f-AEA, WIN-55,212-2, and THC lead to up-regulation of tumor suppressor genes like p16 (INK4A), p27, and p53 (98, 110, 111) and the oncogene RB is hypophosphorylated (100, 112), which could be due to altered activation levels either of AKT or ERK. Further down the cascade, different cyclins such as D1 and D2 as well as the transcription factor E2F1 are down-regulated, followed by lower activity of cyclin dependent kinases cdk2, 4, 6 (98), and cdc2 (113), finally causing cell cycle arrest at different cell cycle check-points. These events can lead to just lower proliferation of cells or else also prime them for apoptosis.

Additionally, cannabinoid challenge as was demonstrated with THC and WIN-55,212-2 can also prompt cancer cells to undergo apoptosis through the mitochondrial apoptosis pathway, where classically AKT inhibition leads to activation of the pro-apoptotic BCL2-familiy member BAD (99, 114). Upon BAD activation, mitochondrial cytochrome C is released leading to caspase-3 activation and PARP cleavage (113), which will finally drive cells into apoptosis. This putative mechanism may account for cell cycle arrest and induction of apoptosis in adherent cells, but may be significantly different among cell types (*vide infra*).

5.1.4 Anti-angiogenesis effects

All of the growth-inhibitory mechanisms of cannabinoids discussed so far are direct cellular effects. However, cannabinoids can modulate intercellular signaling, leading to modulation of important regulatory factors involved in inflammation and cellular activation and thereby indirectly influence tumor development. In this regard, most prominently ranks the effect of cannabinoid treatment on cancer angiogenesis (115). Different cannabinoid compounds with varying CB_1 and CB_2 receptor affinities were able to decrease the formation of new blood vessels on tumors of different origin (eg. nonmelanoma skin cancer and glioma) by downregulating different essential proangiogenic factors, such as VEGF, PIGF, and Ang2 accompanied by dephosphorylation of the VEGF receptors 1 and 2 (110, 116, 117). These events can

also partially account for a lower rate of metastasis as this process is crucially linked to peri- and intratumoral vascularization (118).

Not only cancer cells are influenced by cannabinoids but also endothelial cell sprouting and vessel formation could be blocked in various angiogenesis assays by application of Met-f-AEA (119). Both the CB_1 and CB_2 receptor agonists ACEA and JWH-015 decreased the weight and vascularization of carrageenan-induced granulomas in rats and reduced mast cell number and activation in granulomatous tissue (120). Interestingly, in this study ACEA and JWH-015 prevented the transcription and expression of rMCP-5, a protein involved in sprouting and advance of new blood vessels. Currently, it is unclear whether these effects are actually mediated via CB receptor signaling or whether other yet unknown mechanisms are involved.

5.1.5 The CB_2 receptor – a proto-oncogene?

Results from another study suggest that cannabinoids stimulate proliferation of neural stem/precursor cells acting on both CB_1 and CB_2 cannabinoid receptors by acting through a phosphoinositide-3 kinase/Akt pathway (121). Both the anti- and pro-proliferative effects exerted via CB receptor activation are intriguing and deserve further investigation. In leukaemia cells, the CB_2 receptor has been suggested to act as proto-oncogene, which under certain circumstances may turn into an oncogene that promotes carcinogenesis. Valk et al., 1999, (122) have identified the new common virus integration site Evi11 and demonstrated that the gene encoding the CB_2 receptor (*Cnr2*) is its potential target, thus suggesting that *Cnr2* could be a proto-oncogene. Subsequent research by the same group demonstrates that the CB_2 receptor acts like an oncoprotein that blocks neutrophilic differentiation when overexpressed in myeloid precursor cells and that hematopoietic precursor cells that express high levels of CB_2 possess increased susceptibility for leukemia development, suggesting that CB_2 and Evi1 might collaborate in leukemogenesis (123). Moreover, the CB_2 receptor appears to mediate this activity through MEK/extracellular signal-related kinase (ERK) and PI3-K pathways (124). This would suggest that blocking rather than activating the CB_2 receptor should be beneficial with regard to leukaemia treatment.

High CB_2 receptor expression in myeloid precursors is also associated with different immunomodulatory effects, such as inhibition of immune cell migration (125) and inhibition of TNF-alpha expression (57, 126). Since both CB_2-selective agonists and inverse agonists are anti-inflammatory *in vivo*, and paradoxically apparently both via CB_2 interaction (57, 126-128), it is presently not clear what should be developed for therapeutic intervention, CB_2-selective agonists or inverse agonists. Moreover, the effects of these ligands may potentially be substantially different between the *in vitro* and *in vivo* situations, and an agonist *in vitro* may act as an antagonist *in vivo* and *vice versa* (128). However, there is still insufficient evidence that the CB_2 receptor actually becomes an oncogene due to mutations or increased expression.

On the other hand, it is already clear that the CB_2 receptor regulates cell growth and differentiation in promyelotic human cells, being a regulator of signal transduction via the oncogenic Erk1/2 pathways and execution of mitogenic signals that are relevant to cell differentiation (*vide supra*). In light of the fact that healthy humans have a high functional surface expression of CB_2 receptors in their monocytes and B cells but a low surface expression in their T-cells *(Gertsch J et al., unpublished results)* it may be possible that *Cnr2* is a proto-oncogene in T-cells but not promyelocytic cells (precursors of monocytes/macrophages). This would be confirmed by the fact that cannabinoids induce apoptosis in Jurkat T-cells but not HL60 cells.

Another factor that complicates studies with the CB_2 receptor is that *Cnr2* has undergone rapid evolution leading to pronounced species differences in ligand receptor interactions (receptor affinities and G-protein recruiting) (129) and efficacy in animal studies can therefore not always be extrapolated to humans. There are still too many uncertainties to draw any conclusion regarding cannabinoid treatment of lymphomas, despite studies showing an overexpression of the CB_2 receptor. Intriguingly, CB_1 agonists can induce apoptosis in mantle cell lymphoma (MCL) via CB_1 receptor activation (130), under conditions where also the CB_2 receptor is activated because in these studies non-selective cannabinoids were used (Table 1; Fig. 2).

Cancer	Cell lines	Effects	Compound	Reverse?	In vivo?	Reference
Glioblastoma multiforme	U251-MG and U87-MG	Cell cycle inhibition	THC	?	no	Galanti, 2007
	SF126, U87, U251, U373-MG, SF188, GBM primary cells	Cell death, reduced proliferation	WIN, THC	SR1, SR2	no	McAllister, 2005
Glioma	U87	ROS, apoptosis	CBD	no	yes	Massi, 2006
	C6, U87mg	Apoptosis	THC	SR1, SR2	yes	Carracedo, 2006
	?	Apoptosis	AEA	no?	no	Contassot, 2004
	C6	Cell death by oxidative stress	THC	SR1	no	Goncharov, 2005
	C6	Apoptosis	WIN	no	no	Ellert-Miklaszewska, 2005
	U87, U373	Anti-proliferative	CBD	SR2	no	Massi, 2004
	H4 Neuroglioma cells	Apoptosis	R-Metanandamide	no	no	Hinz, 2004
	C6	Reduced proliferation	1-AG, phosphate esters of anandamide	no	no	Fowler, 2003
	C6	Apoptosis	THC	no	no	Gomez del Pulgar, 2002
	C6	Anti-proliferative	AEA, 2-AG, JWH105, CP55,	SR1/2, AM251, AM630, capsazepine	no	Jacobsson, 2002
	C6, U87mg	Reduced viability	Ajulemic acid	SR2	yes	Recht, 2001
	C6	Apoptosis	THC	SR1 & SR2 combination	yes	Galve-Roperh, 2000
	C6	Apoptosis	THC	SR1	no	Sanchez, 1998
	C6	Apoptosis	JWH 133	SR2	yes	Sanchez, 2001
	U87-mg, T98G, LN-229, MT310	Reduced viability	C1'-phenyl-substituted Δ(8)-THC	no	no	Krishnamurthy, 2008
Neuroblastoma	B102	Growth-inhibition	THC	no	no	Cabral, 1987
	NB2A, (C6)	Anti-proliferative	THC	no	no	End, 1977
Leukemia	Jurkat	Apoptosis	THC	SR1 & SR2	no	Jia, 2006
	Jurkat, MOLT-4	Apoptosis	CBD	SR2	yes	McKallip, 2006
	Jurkat	Apoptosis	THC	SR2	no	Herrera, 2005
	Jurkat	Apoptosis	THC	no	no	Lombard, 2005
	EL-4, LSA, P815, Jurkat, Molt4	Apoptosis	THC, HU210, Anandamide, JWH015	SR2	yes	McKallip, 2002
	K562	Anti-proliferative	THC	no	no	Dvilansky, 1984
	L1210 murine leukemia	Anti-proliferative	Δ8-tetrahydrocannabinol	no	yes	Tucker, 1977
	L1210 murine leukemia, lewis lung carcinoma	Inhibit DNA-synthesis	THC, Δ8-tetrahydrocannabinol	no	yes	Carchman, 1976
Lymphoma	Rec1, Jeko, JVM-2	Apoptosis	WIN, R(+)-Methanandamide	SR1 & SR2	no	Gustaffson, 2006
	MCL cells from biopsies	Apoptosis	WIN, AEA	no	no	Flygare, 2005
	Rec-1, MEL1, MEL2, Raji, Namalwa, JEKO-1	Apoptosis	R(+)-Methandandamide	SR1 & SR2	yes	Gustafsson, 2008
Lung cancer	A549 and SW-1573	Growth inhbition, anti-angiogenic, anti-migration	THC	no	yes	Preet, 2008
	Lewis lung carcinoma	Anti-proliferative	THC, Δ8-tetrahydrocannabinol, CBD	no	no	Friedmann, 1977
	Lewis lung adenocarcinoma cells	Anti-proliferative	THC	no	no	White, 1976
	L1210 murine leukemia, lewis lung carcinoma	Inhibit DNA synthesis	THC, Δ8-tetrahydrocannabinol	no	yes	Carchman, 1976
	Lewis lung adenocarcinoma cells	Tumor growth reduced	THC, Δ8-tetrahydrocannabinol, CBN	no	yes	Munson, 1975
Colon cancer	SW480, HCT15	Apoptosis	THC	AM251	no	Greenhough, 2007
	?	Cell death	AEA	no	no	Patsos, 2005
	CaCo-2, DLD-1	Reduced proliferation	2-AG, AEA, HU210	SR1 & SR2	no	Ligresti, 2003
	CaCo-2	Anti-proliferative, reduced viability	HU210, AEA, NAGly	no	no	Gustaffson, 2008
Pancreatic cancer	MiaPaCa, Panc-1	Apoptosis	THC, WIN, JWH	SR2	yes	Carracedo, 2006
	MiaPaCa2	Apoptosis	ACEA, AM251, JWH-015, AM630	no	no	Fogli, 2006
Breast cancer	MDA-MB-231, T47D and MCF-7	Cell cycle arrest, lipid rafts critically involved	SR141716	no	no	Sarnataro, 2006
	EVSA-T, MCF-7, MDA-MB-468, MDA-MB-231, SKBr3, T47D	Cell cycle blockage, apoptosis	THC	SR1, better SR2	no	Caffarel, 2006
	HBcc	Reduced proliferation	AEA	no	no	De Petrocellis, 2002
	HBcc	Anti-proliferative	PEA + AEA	SR2	no	Di Marzo, 2001
	HBCC, DU145	Anti-proliferative, PRL / NGF receptors↓	AEA, 2-AG, HU210	SR1	no	Melck, 2000
	MCF-7	Anti-proliferative, PRL / NGF receptors↓	AEA	SR1	no	Melck, 1999
	EFM-19	anit-proliferative	AEA	SR1	no	Bisogno, 1998
	EFM-19, MCF7	anti-proliferative	AEA, 2-AG, HU210, R-Methanandamide	SR1	no	De Petrocellis, 1998
Prostate cancer	LNCaP	Apoptosis	WIN	SR1 SR2	yes	Sarfaraz, 2006
	DU145, LNCaP, PC-3	Apoptosis	WIN	SR1, SR2	yes	Sarfaraz, 2005
	DU145, LNCaP, PC-4	Cell death	AEA	SR1	no	Mimeault, 2003
	PC-3	Cell death	AEA	no	no	Sarker, 2003
	HBCC, DU145	Anti-proliferative, PRL / NGF receptors↓	AEA, 2-AG, HU210	SR1	no	Melck, 2000
	PC3	Apoptosis	THC	no	no	Ruiz, 1999
Cervix carcinoma	CC299, Caski, HeLa	Apoptosis	AEA	VR1 inhbition	no	Contassot, 2004
Melanoma	B16, A375	Apoptosis, anti-angiogenic	WIN, THC, JWH133	SR2	yes	Blazquez, 2006
Skin tumor	PDV.C57, HaCa4	Apoptosis, anti-angiogenic	WIN, JWH133, THC	SR1 & SR2	yes	Casanova, 2003
Thyroid cancer	KiMol, TK-6	Growth inhibition	Met-F-AEA	SR1	yes	Portella, 2003
Thymoma	?	ROS, apoptosis	CBD	no	no	Lee, 2008
Cholangiocarcinoma	Mz-ChA-1, HuH28, HuCC-T1, SG231	Apoptosis (FAS to lipid raft)	AEA	no	no	De Morrow, 2007

Table (Review) 1: Overview of effects of different cannabinoid receptor ligands on cancer cells *in vitro* and *in vivo*, showing cell type, type of effect, cannabinoid compound used, whether the effect could be reversed by CB_1 or CB_2 antagonists, respectively. SR1 stands for SR141716A and SR2 for SR144528.

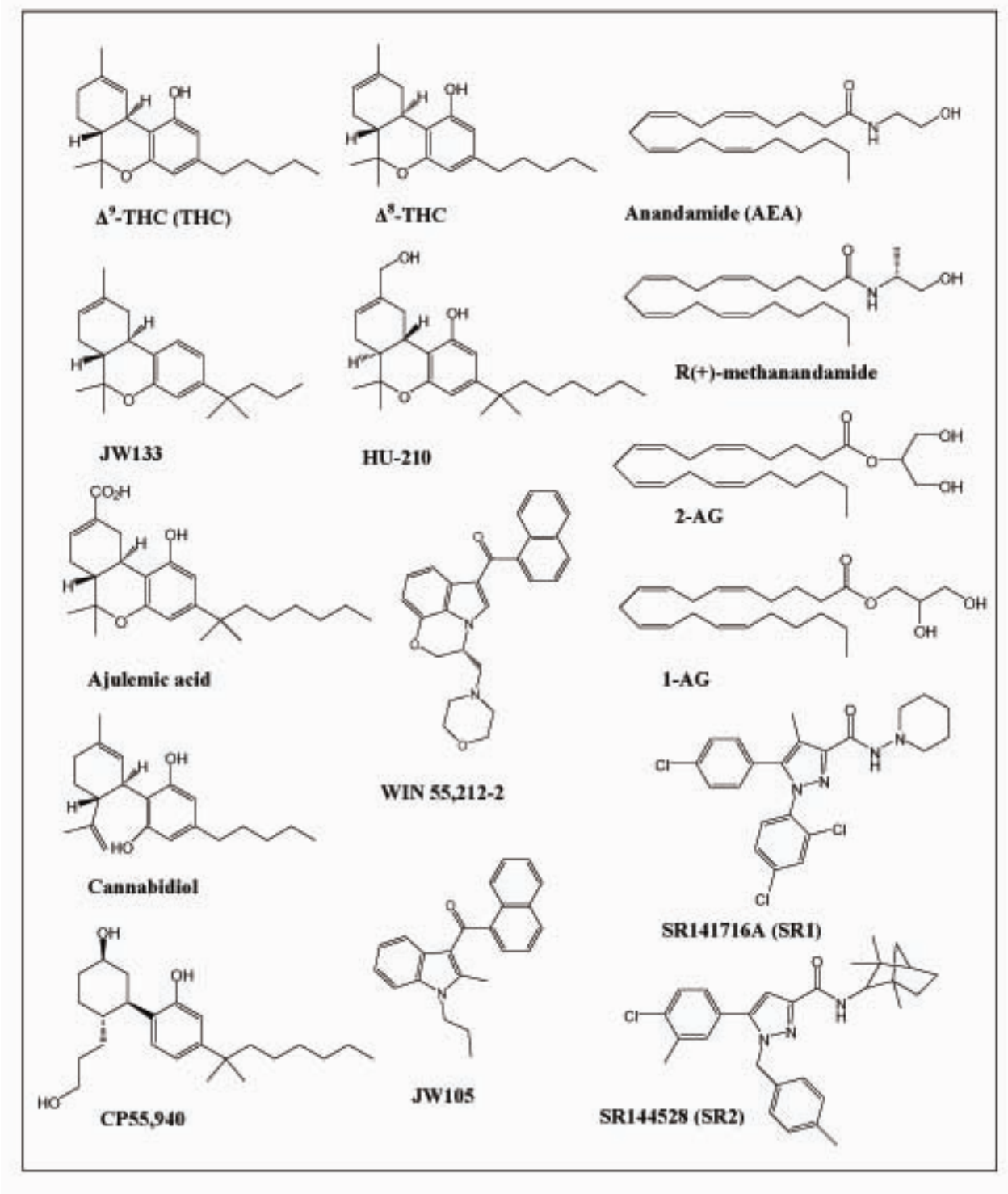

Figure (Review) 2: List of CB receptor ligands used to date in the study of the anticancer effects of cannabinoids. Shown is the ratio of CB receptor affinities as K_i values (nM) (human CB_1 / human CB_2).

5.1.6 CB receptor independent anti-cancer effects of cannabinoids

Not all effects of cannabinoids are mediated through CB receptors and there is also an increasing amount of data showing that many ligands are non-specific to CB receptors. Apparently well-designed selective ligands are specific for a certain target until their non-specificity is shown, often leading to wrong conclusions drawn from pharmacological experiments. The same may be true for CB receptor inverse agonists (i.e. antagonists). Recently, it was shown that the CB_1 selective agonist SR141716A also binds to GPR55 (131). While there are an increasing number of CB_1 and CB_2 receptor selective agonists and antagonists it is not clear whether these compounds have other cellular actions at the pharmacological concentrations used (Table 1). Moreover, studies like the one performed on the murine lymphomas L-4, LSA and P815 with THC treatment may be hard to interpret as THC more strongly activates GPR55 than CB receptors (131), the role of which in cancer remains to be elucidated. Recently, it was shown that GPR55 signals via Rho and activates nuclear factor of activated T-cells (132). This certainly complicates the interpretation of studies performed without using knockout mice as controls.

Among the known non-cannabinoid targets of cannabinoids are the TRPV channels (133), the GPR55 receptor (131), PPAR-gamma (134), and the 5HT(3) receptor (71). Anandamide or similar cannabinoid structures activate the vanilloid receptor (VR1 or TRPV1), which can be blocked with the TRPV1 antagonist capaszepine. In cervical cancer cells with aberrant VR1 expression, rather stimulation of the TRPV1 accounts for apoptosis-inducing effects of anandamide than CB_1 or CB_2 (135), whereas the migration-reducing effects of R(+)-methanandamide could be blocked by antagonists to VR1, CB_1, and CB_2 pointing at the complexity of cannabinoid-evoked signal generation (136).

Recently, De Petrocellis et al. (2008) suggested that phytocannabinoids and cannabis extracts exert some of their pharmacological actions by interacting with TRPA1 and TRPM8 channels, with potential implications for the treatment of pain and cancer (137). Another well-described mechanism for CB-independent action of certain cannabinoid receptor agonists is their binding to some members of the nuclear receptor transcription factor superfamily PPARs (peroxisome-proliferator-

activated receptors) (70, 134), although the extent to which this mechanism might be involved in the effects of cannabinoids as anti-tumour agents remains poorly described.

Finally, the new cannabinoid-like receptor GPR55 with signaling distinct from CB_1 and CB_2 may be a hitherto neglected receptor with regard to the anticancer effects of several cannabinoids because many cannabinoids interact with GPR55 and the receptor appears to be present in numerous cell types (65, 131). GPR55 is activated by a whole range of plant, synthetic and endogenous cannabinoids and is blocked by the non-psychoactive phytocannabinoid, cannabidiol (54, 131). The most striking difference reported so far is maybe the agonist activity of the CB_1 receptor antagonists AM251 and AM281 at GPR55 (54) rendering elucidative studies of cannabinoid receptor specificities into a highly challenging task. To date, nothing has been published about its expression in cancer and therefore putative effects of cannabinoids through GPR55 occurring in tumour cells cannot be ruled out and deserve further attention. Activation of GPR55 by AM251 could also explain the observation that this drug could excert anti-proliferative effects on pancreatic cancer cells in the low µM range (138). Similarly, another article reported cell-cycle arrest after CB_1 antagonist SR141716 treatment on breast cancer cells (139), an effect which could, at least in part, also be mediated by interaction with GPR55.

In hormone-dependent breast and prostate carcinoma types, it was described that cannabinoid treatment with various compounds can decrease expression levels of receptors involved in their pro-proliferative response to the cytokines prolactin, NGF, and androgen (102, 140, 141). Namely, in prolactin-dependent breast cancer, 2-AG and anandamide downregulate the prolactin receptor and the trkNGF receptor, whereas in postate cancer, 2-AG, anandamide and WIN-55,212-2 result in lower levels of the prolactin and the androgen receptor. Apart from this indirect proliferation-inhibiting effect on cytokine levels, certain plant-derived and synthetic cannabinoids lead to an inhibition of the multidrug-transporter ABCG2 and P-glycoprotein (p-gp) in different cell systems (MEF, immortalized renal cells, Caco-2 cells, and rat brain microvessel cells) potentially contributing to a resensitization and accumulation of chemotherapeutic agents such as topotecan or doxorubicin (142-146).

5.1.7 Elevated endocannabinoid levels in tumors – is it good or bad?

There is good evidence that certain tumor cells overexpress endocannabinoids, which are typically released during cellular stress. E.g. in colon tissue anandamide levels are significantly upregulated after malignant transformation (147). Since endocannabinoids activate both CB_1 and CB_2 receptors they could initiate the anticancer signaling pathways described above. This leads to the obvious question why a growing tumor should kill itself by such a mechanism. It has previously been postulated that endocannabinoid tone may be a means of controlling endogenous tumor control (reviewed in (55)). However, despite data from several studies showing anticancer effects mediated by the endogenous ligands anandamide and 2-AG (either directly or by increasing their levels by blocking degradation or transport) it is still not clear whether the ECS is an endogenous anticancer or a pro-carcinogenic system.

The latter was proposed in a recent study employing knockout mice, in which CB_1/CB_2 receptors were suggested to play a key role in UV-induced inflammation and skin cancer development (148). The authors have shown that in the skin UVB activates NF-κB via CB receptors, leading to increased TNF-α expression. As cannabinoids at low concentrations typically inhibit TNF-α expression from immune cells (57, 126) this seems to be rather conflictive. However, several lines of evidence suggest that the ECS in the skin is different from the ECS in the rest of the body and CB_2 receptor agonist seem to be pro-inflammatory in the skin (reviewed in (57)). Somewhat contradictory, CB receptor activation in melanoma has been shown to reduce tumor growth via AKT signaling (100).

In a report by Aguado et al. on glioma stem-like cells it was shown that cannabinoids such as HU210 and JWH133 cause higher expression of glial differentiation markers in a CB_1 and CB_2-dependent manner, respectively (149). Upon engraftment of these more differentiated cells into mice, a lower rate of gliomagenesis was observed in comparison to engrafted control cells, suggesting a potential inhibitory role for cannabinoid receptor agonists in cancer stem cell differentiation. Obviously, the role of the ECS system is not clear it is likely that different tissues employ the ECS differently. While for many tissues (CNS, liver, gut, arteries, etc.) it may be beneficial

to activate the ECS, other tissues may develop pathologies (adipose tissue, skin). As pointed out in a recent review by Di Marzo (57), endocannabinoids may be able to act in opposite directions, depending on the physiological context.

With regard to cancer, it needs to be emphasized that CB receptor expression in cancer cells has largely been determined at the level of mRNA expression and by Western blots (100, 141, 150), thus not taking into account that surface expression may vary and not correlate with gene expression. Unpublished data in our laboratory clearly indicate that many cancer cells lack CB surface expression despite of being positive in RT-PCR and Western Blot analyses. Thus, studies ignoring the fact that in many cancer cell lines CB receptors are not coupled to G-proteins may potentially draw erroneous conclusions.

In spite of the vast amount of publications supporting the approach of cannabinoids as anti-cancer agents, it should be noted that there are some major not negligible drawbacks, such as the apparently pro-survival effects of cannabinoids at low concentrations in cancer cells and their immune-suppressive action. Apparently, concentrations of THC at nanomolar levels, comparable with those detected in the serum of patients after administration of THC, accelerate proliferation of cancer cells instead of inducing apoptosis (97). The same observation holds also true for the *in vitro*-incubation of several cancer cell types with WIN55,212-2 and HU210, an effect that was described to occur through trans-activation of the EGFR leading to activation of the AKT and MAPK signaling pathways (97). In this regard, use of cannabis as it is already approved to ease chemotherapeutic treatment regimens (151, 152) could potentially boost tumour growth and any future therapeutic administration of cannabinoids as anti-cancer agents should be applied locally.

The second critical point confers to the fact that THC alters the immune response by suppressing the cell-mediated TH_1 response, which is of high relevance in the battle against tumour cells (153). Since TNF-α expression is typically inhibited by low cannabinoid concentrations and TNF-alpha itself inhibits tumor growth (154) it is not clear what the effect of cannabinoids on physiological tumor development is. On the other hand, a pro-inflammatory environment can lead to carcinogenesis (154, 155) and cannabinoids may be able to prevent this. With the commonly used xenograft

model, where human cancer cells are engrafted into immunedeficient mice, it is impossible to predict the impact of the cannabinoid treatment on the immune surveillance of the tumor, whereas evidence from a melanoma allograft model shows that the inhibitory effects on tumor growth and formation is independent of immune status of the mice and site of drug injection (100). Thus it can be concluded that the beneficial effects of such a treatment prevail, even though negative long term effects can not be excluded.

5.1.8 Conclusions and Outlook

Cannabinoids may have anticancer effects in the appropriate context but their effects may not be sufficiently radical for chemotherapy. To date, THC and its trans-isomer dronabinol (Marinol®) and the synthetic derivative nabilone (Cesamet®) are successfully used as adjuvants to chemotherapheutic treatment because they prevent nausea, vomiting and stimulate appetite (156). Currently, there is no clinical data indicating that co-treatment with these cannabinoids actually results in improved or reduced anti-cancer efficacy of the actual chemotherapeutic agents. Such clinical comparisons would be very interesting. Based on current knowledge, the ECS may be a system that, under the appropriate conditions, produces synergy with established chemotherapeutic agents. More research should be directed towards the potential synergism and antagonism of cannabinoids in chemotherapy. Despite several promising reports from studies with cannabinoids in animal xenograft models, a limited amount of data exist with humans (*vide infra*) and a therapeutic benefit therefore remains speculative.

Moreover, there are numerous apparently non-toxic natural products that potentially exert anti-tumor effects, many of which have been confirmed in animal models (157), but few have been put to test in a clinical setting. There are limited financial resources to put to test apparently promising anticancer agents in clinical studies. While data obtained in different cellular and animal models suggest that cannabinoid ligands could be useful to treat cancer, the abundance of CB receptors in different tissues could clearly be a problem with regard to potentially unwanted effects. Interestingly, most studies have been carried out with ligands that target both CB_1 and CB_2 receptors in a non-selective manner (Fig. 1).

In light of a potential therapeutic application the unwanted psychotropic effects mediated via CB_1 could be problematic. On the other hand, there is still a limited amount of data on CB_2 receptor-selective anticancer effects (by either agonists or antagonist) and the potential therapeutic relevance remains unclear. Future studies using either CB_1 or CB_2 receptor-selective ligands in combination with animal models in which CB receptors have been genetically deleted should be useful. Conclusions drawn from experiments with CB receptor antagonists may be misleading because these ligands may potentially interact with other targets, such as GPR55 (vide supra).

Maybe more promising is the potential of the ECS in the suppression of cancer development. It is tempting to speculate that the ECS is involved in carcinogenesis and tumorigenesis in certain tissues as it potentially modulates the chemical microenvironment. Along that line, two recent studies have shown that loss of CB_1 led to an increase in carcinogenesis of colon cancer (60) and enhanced endocannabinoid tonus prevented colon cancer (61), thus pointing to a suppression of colon carcinogenesis by the ECS and the CB_1 receptor. Also the CB_2 receptor has been suggested to exert beneficial regulatory effects in the gut, such as attenuation of inflammation and probably colon cancer (158).

To provide stronger evidence, future research will have to uncover potential novel ways of chemoprevention by cannabinoids. More than 10 years ago, Sidney et al. (1997) (159) concluded that “not only is the evidence linking *Cannabis* smoking to cancer negative, but the largest human studies cited indicated that cannabis users had lower rates of cancer than non-users. What's more, those who smoked both cannabis and tobacco had lower rates of lung cancer than those who smoked only tobacco-a strong indication of chemoprevention.” Although this statement was recently challenged by a study performed by Aldington et al. (2007) (160) in which *Cannabis* smoking was shown to increase the risk for lung cancer, it is not certain that cannabinoids are responsible for this correlation (161).

On the contrary, the increasing amount of data showing an attenuation of tumor growth by both orally and locally administed cannabinoids in different animal models raises high hopes for potentially new treatments, in particular in combination with

established chemotherapeutic agents. What remains inconclusive is the exact mode of action of cannabinoids and the role of classical CB receptors, the potential involvement of GPR55, and the other non-cannabinoid targets. In conclusion, prior to the development of anticancer agents acting via the ECS a better understanding of the underlying physiological processes of the ECS in malignancy would be desirable.

6 Aim

Survival rates of RMS patients have been steadily improved over the last decades using conventional chemotherapeutics by intensifying therapy modalities. However, the increase of survival rate has reached a plateau and the 40% of 5-year overall survival for aRMS cases still leaves room for further improvement with other therapeutic interventions. Hence, targeted therapies are an urgent need, which can be met by better understanding of the molecular mechanisms contributing to this cancer type. A gene expression study on translocation-positive aRMS biopsies identified different potential target genes, among them the gene for the cannabinoid receptor 1 (CB1), which has already been described as an effective therapeutic target in other cancer types.

Based on this knowledge, the purpose of this research project was the investigation of CB1 as a potential drug target for the treatment of translocation-positive aRMS. Towards this aim, diverse cannabinoid receptor agonists were studied for their viability-reducing effect on aRMS cells using eRMS cells and non-transformed fibroblast cells as controls. Signalling pathways and mechanisms contributing to this effect were investigated as well as receptor-specificity of the treatment was addressed by means of chemical CB1 receptor antagonists. Additionally, a xenograft mouse model was applied to confirm the *in vivo* potential of cannabinoids as therapeutic agents for aRMS and to validate the cellular mechanisms observed *in vitro*. Eventually, this study should give insight whether cannabinoid receptor agonists could serve as targeted therapeutic intervention for aRMS, as it was proposed in other cancer types, by employing *in vitro* and *in vivo* assessments of the anti-neoplastic effect.

7 Results

7.1 Paper: Targeting CB1 in Rhabdomyosarcoma

(Submitted to Molecular Cancer Therapeutics)

CB1 is a potential drug target for treatment of translocation-positive rhabdomyosarcoma

Susanne Oesch[1], Dagmar Walter[1], Marco Wachtel[1], Kathya Prêtre[1], Maria Salazar[2], Manuel Guzmán[2], Guillermo Velasco[2], Beat W. Schäfer[1*]

[1]University Children's Hospital, Division of Oncology, 8008 Zürich, Switzerland

[2]Departement of Biochemistry and Molecular Biology 1, School of Biology, Complutense University, 28040 Madrid, Spain

[*]Corresponding Author: Beat.Schaefer@kispi.uzh.ch

7.1.1 Author contributions

Susanne Oesch:	Performed and analysed all experiments. Wrote the manuscript.
Marco Wachtel:	Provided data for Fig. 1A.
Dagmar Walter, Kathya Prêtre:	Supported the treatment of mice resulting in Fig. 6A.
Maria Salazar, Guillermo Velasco, Manuel Guzman:	Gave Susanne Oesch the opportunity to perform some experiments in their laboratory as part of a collaboration. They contributed also with critical reading of the manuscript.
Beat Schäfer:	Group leader, involved in the conception of the project and data interpretation. Corrected the writing of the manuscript, approved the final version of the manuscript and provided the financial support.

7.1.2 Abstract

Gene expression profiling has revealed that the gene coding for cannabinoid receptor 1 (CB1) is highly up-regulated in rhabdomyosarcoma biopsies bearing the typical chromosomal translocations PAX3/FKHR or PAX7/FKHR. Since cannabinoid receptor agonists are capable of reducing proliferation and inducing apoptosis in diverse cancer cells such as glioma, breast cancer and melanoma, we evaluated whether CB1 is a potential drug target in rhabdomyosarcoma. Our study shows that treatment with the cannabinoid receptor agonists HU-210 and THC lowers the viability of translocation-positive rhabdomyiosarcoma cells through induction of apoptosis. This effect relies on inhibition of AKT signalling and induction of the stress-associated transcription factor p8. SiRNA-mediated down-regulation of p8 rescued cell viability upon cannabinoid treatment. Finally, treatment of xenografts with HU210 led to a significant suppression of tumor growth in vivo. These results support the notion that cannabinoid receptor agonists could represent a novel targeted approach for treatment of translocation-positive rhabdomyosarcoma.

7.1.3 Introduction

Rhabdomyosarcoma is the most common soft-tissue sarcoma in children, representing 5-8% of all childhood malignancies (162). It is believed to originate from muscle precursor cells and histology recognizes two major subtypes: The embryonal subtype (eRMS) accounts for about 60% of RMS cases and has a rather good prognosis (4). The alveolar subtype (4) is less frequent, more aggressive, usually presents with metastasis and is thus associated with rather poor treatment outcome. While no consistent genetic alterations have been identified so far in eRMS, about 80% of aRMS patients display typical chromosomal translocations t(2;13)(q35;q14) or t(1;13)(p36;q14) encoding for fusion proteins PAX3/FKHR or PAX7/FKHR, respectively (17). These chimaeric transcription factors are oncogenic and presumably act mainly through their capability to enhance transcription of PAX3 or PAX7 target genes. To gain insight into molecular changes elicited by these transcription factors and to find new potential therapeutic targets for treatment of aRMS, gene expression analysis was performed in a range of RMS biopsies by several research groups (19, 163, 164). These studies consistently revealed a gene expression signature of up-regulated genes in translocation-positive versus translocation-negative samples. Interestingly, translocation-negative aRMS clustered together with eRMS samples in these analyses. Hence, at the molecular level RMS can be divided into translocation-positive RMS (tposRMS) and translocation-negative RMS (tnegRMS).

The gene expression signature of tposRMS contains a number of receptor molecules that might be potentially amenable as drug targets. Among these there are receptors such as c-met that have already been validated as therapeutic target (165). One of the top-ranking genes in this signature is the cannabinoid receptor 1 (CB1) that is strongly expressed in tposRMS. However, no studies have been undertaken yet to test whether CB1 might serve as a future target for therapeutic intervention in this tumor. Evidence exists since 1975 that cancer cell growth can be inhibited by treatment with cannabinoid receptor agonists, as first described by Munson et al. in Lewis lung carcinoma cells (80). Since then, additional cancer cell types such as glioblastoma (85), breast carcinoma (166) or melanoma (100) were reported to be sensitive to the anti-proliferative action of cannabinoids. In general, the anti-tumoral

actions of diverse cannabinoid receptor agonists are mediated through the cannabinoid receptor types CB1 and CB2 as reviewed in Guzman et al. (56).. Notably, not only *in vitro* cell culture systems are subject to this treatment response, but also *in vivo* experiments using either xenografts or syngeneic mouse models have demonstrated the potential of cannabinoids as anti-cancer agents, without observing major psychoactive or immune-suppressive effects (80, 101). Recently, the first clinical study using THC in severe cases of glioblastoma has been reported (167).

At the molecular level, cannabinoids trigger changes in various signalling pathways in cancer cells. One of the primary events after cannabinoid treatment is a sustained *de-novo* synthesis of the lipid second messenger ceramide, which in turn is followed by inhibition of AKT signalling (86). Strikingly, both of these signalling events mark a major difference between tumor cells and healthy non-transformed cells, which undergo AKT activation without *de-novo* synthesis of ceramide after cannabinoid stimulation (93). In parallel to AKT inhibition, alterations in ERK signalling have been reported. However, depending on tumor type either inhibition (99) or sustained activation (102) have been reported. Recently, the stress-associated transcription factor p8 was found to be critically involved in cannabinoid-induced apoptosis of cancer cells (87) as its down-regulation could rescue viability of various cancer cells (107, 108). At the end of the signalling cascade, tumor cells either undergo cell cycle arrest (111, 113) or apoptosis (85) (114).

To improve the treatment outcome of the aggressive tposRMS subtype, novel targeted therapies are urgently needed. Therefore, this study aimed to characterize the effects of cannabinoids on tposRMS cells *in vitro* as well as *in vivo*. Our results demonstrate that the CB1 receptor could represent a potential molecular target for future therapeutic approaches in tposRMS.

7.1.4 Materials and methods

Cannabinoids

HU210 was purchased from Tocris (Northpoint, UK), Met-F-AEA from Cayman (Ann Arbor, MI, USA), AM251 from Sigma-Aldrich (Buchs, Switzerland) and THC from The Health Concept (Richelbald, Germany). All substances were solved in DMSO. For in vitro experiments, they were applied at final DMSO concentrations of maximally 0.05% (v/v). For in vivo experiments, HU210 was prepared at 0.25% DMSO (v/v) and diluted in PBS supplemented with 5mg/ml BSA.

Cell culture

Rh4 tposRMS cells were kindly provided by P.Houghton (St Jude Children's Research Hospital, Memphis, TN, USA). RD eRMS, and MRC-5 lung fibroblast cells were obtained from ATCC (LGC Promochem, Molsheim Cedex, France). All cells were routinely maintained in DMEM supplemented with 10% FCS. When performing viability or signalling experiments, cells were plated at a density of 17.000 cells/cm^2, let to adhere over-night, and transferred to serum-free medium 6 hours before starting drug treatments.

Cell viability and apoptosis detection

Cell viability was evaluated in 96-well plates using Methylthiazolyldiphenyl-tetrazolium bromide (MTT) from Sigma-Aldrich (Buchs, Switzerland). Apoptosis was either analysed with CaspGLOW red active caspase-3 staining kit (Biovision, USA) allowing labelling of apoptotic cells with a fluorescent caspase-3 substrate and subsequent detection by fluorescence microscopy. Alternatively, the apoptosis-indicating ratio of cleaved to uncleaved PARP protein was determined densitometrically by western blotting.

Reverse transcriptase-polymerase chain reaction

RNA was extracted with the RNeasy Mini Kit (Qiagen, Basel, Switzerland) including a DNase digestion step with RNase-free DNase (Qiagen). One ug of total RNA was reverse transcribed with random hexamer primers using the High capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Rotkreuz, Switzerland). PCR was performed with primers for hCB1 (5'-CGTGGGCAGCCTGTTCCTCA-3' and 5'-

CATGCGGGCTTGGTCTGG-3') and for GAPDH (Microsynth, Balgach, Switzerland) using the following parameters: After initial denaturation at 94°C for 5 min., a cycle (40x) with 94°C for 30 sec, 55°C for 30 sec., and 72°C for 45 sec. was performed, followed by a final extension at 72°C for 5 min..

Quantitative real-time PCR

qRT-PCR was performed under universal cycling parameters on an ABI7900HT instrument using commercially available Mastermix and target probes for CB1, p8, and GAPDH (all from Applied Biosystems, Rotkreuz, Switzerland). Cycle threshold (C_T) values were normalized to GAPDH. Relative expression levels of the target genes among the different samples were calculated using the $\Delta\Delta C_T$ method.

Gene silencing

Rh4 cells were transfected with 10nM siRNA against human p8 (Qiagen, Basel, Switzerland) or scrambled siRNA (Ambion, Austin, TX) using GeneEraser (Stratagene, La Jolla, CA). One day after siRNA-transfection, equal numbers of cells were plated for subsequent viability experiments. p8 down-regulation efficiency was verified by means of quantitative RT-PCR.

Western Blot Analysis

For detection of intracellular signalling proteins, whole cell extract was produced with a lysis buffer consisting of 50mM Tris pH 7.5, 1% Triton X-100, 1mM EGTA, 50mM NaF, 10mM sodium β-glycerophosphate, 5mM sodium pyrophosphate, 1mM sodium orthovanadate, 1% PMSF, 0.1% β-mercaptoethanol, and protease-inhibitor cocktail complete (Roche, Rotkreuz, Switzerland) according to standard protocols. Samples were sonicated and equal amounts of protein were used for western blotting with the NuPAGE system (Invitrogen, Basel, Switzerland). Antibodies used for detection included rabbit antibodies raised against: CB1 (1:1000; Affinity Bioreagents, Golden, CO), PARP, phospho-AKT(Ser473), phospho-AKT (Thr308), AKT-total, phospho-ERK (Thr202/Tyr204), ERK-total, phospho-GSK-3-alpha/beta (Ser21/9), and GSK-3-beta (all 1:1000; from Cell Signaling Technology, Boston, MA). Detection of actin with a rabbit antibody (1:2000; Sigma-Aldrich, Buchs, Switzerland) was used to control for equal protein loading. As secondary antibody, an anti-rabbit antibody conjugated to

HRP (1:2000; Pierce, Rockford, IL) was used. Detection was performed with ECL technology (Amersham, Buckinghamshire, UK).

Confocal microscopy

Cells on coverslips were fixed with PFA and incubated with anti-CB1 antibody (1:500; Affinity Bioreagents, Golden, CO) in PBS / 2.5% goat serum for 0.5 hours at 37°C. For visualization, a secondary anti-rabbit antibody labelled with Alexa Fluor 594 (1:500; Molecular Probes, Leyden, Netherlands) was used. Control immunostainings using the secondary antibody alone were performed in parallel. Confocal fluorescence images were acquired using Laser Sharp 2000 software (Biorad, Hercules, CA) and a Confocal Radiance 2000 coupled to an Axiovert S100 TV microscope (Carl Zeiss, Oberkochen, Germany).

Immunohistochemical staining

Tumors were fixed, embedded in paraffin and sectioned into 2um slices. Immunohistochemical stainings for Ki67 (Lab Vision Corporation, Fremont, CA), and cleaved capase-3 (Cell Signaling Technology, Boston, MA) were performed on the Ventana Benchmark automated staining system (Ventana medical systems, Tucson, AZ).

Tumorigenicity assay

7.5 x 10^6 Rh4 cells in 100µl PBS were injected subcutaneously into the flank of NOD/LtSz-scid IL2Rgammanull (NOG) mice (Jackson Laboratory, Bar Harbor, ME). When tumors reached a size of 150mm^3, mice were randomly assigned to treatment and control groups and injected peritumorally for 13 days with 0.2mg/kg HU210 or vehicle (DMSO) alone. Tumor growth was monitored daily with external caliper, and the tumor volume was calculated as $(4\pi/3)*((\text{width} + \text{length})/4)^3$. Animals were sacrificed one day after the last treatment..

Statistical analysis

Statistical analysis was performed with two-tailed t-test with the statistics programm SPSS. For analysis of tumor growth a longitudinal analysis was performed by comparing linear regressions of two groups.

7.1.5 Results

7.1.5.1 The CB1 receptor is upregulated in translocation-positive RMS

Gene expression profiling of RMS biopsy samples shows a signature for tposRMS (19) that includes as one of the top-ranking genes CNR1, encoding the CB1 receptor (Fig. 1A). In contrast, transcript levels of the related CNR2 gene, encoding the CB2 receptor, were only detected slightly above background in the same microarray analysis. Among the commonly used tposRMS cell lines, Rh4 cells most accurately reflect the translocation-specific gene expression signature and therefore this cell line was selected as model system for this study. In addition, expression of CB1 is 430-fold higher than expression of CB2 inRh4 cells as analyzed by quantitative RT-PCR (data not shown). To validate the up-regulation of CB1 specifically in Rh4 cells on both RNA and protein level, we first applied conventional RT-PCR which revealed higher expression of CB1 mRNA in Rh4 cells than in control cell lines MRC-5 (fibroblast), and RD (eRMS) (Fig. 1B). Indeed, expression in Rh4 cells was 1000-fold higher than in the controls when assessed quantitatively. Further, CB1 was expressed in Rh4 cells also at the protein level as shown by Western blot (Fig. 1C). Last, confocal microscopy of cultured Rh4 and RD cells (Fig. 1D) showed higher immunofluorescence staining intensities for CB1 in Rh4 cells. Hence, expression of CB1 is evident both on mRNA and on protein level in tposRMS cells, confirming the previous findings using gene expression profiling.

7.1.5.2 Cannabinoids reduce the viability of tposRMS cells in vitro

After validating expression of CB1 in tposRMS cells, we next assessed cell viability after treatment with different cannabinoid receptor agonists. Treatment with the mixed cannabinoid receptor agonist HU-210 (Fig. 2A) or with the main active component of marijuana (Δ^9-tetrahydrocannabinol, THC; Fig 2C) reduced in a dose-dependent manner the viability of Rh4 cells but not of tnegRMS cells (RD) or control non-tranformed fibroblasts (MRC-5) which express lower levels of the CB1 receptor. A similar result was obtained with the anandamide related compound Met-F-AEA (Fig. 2D).

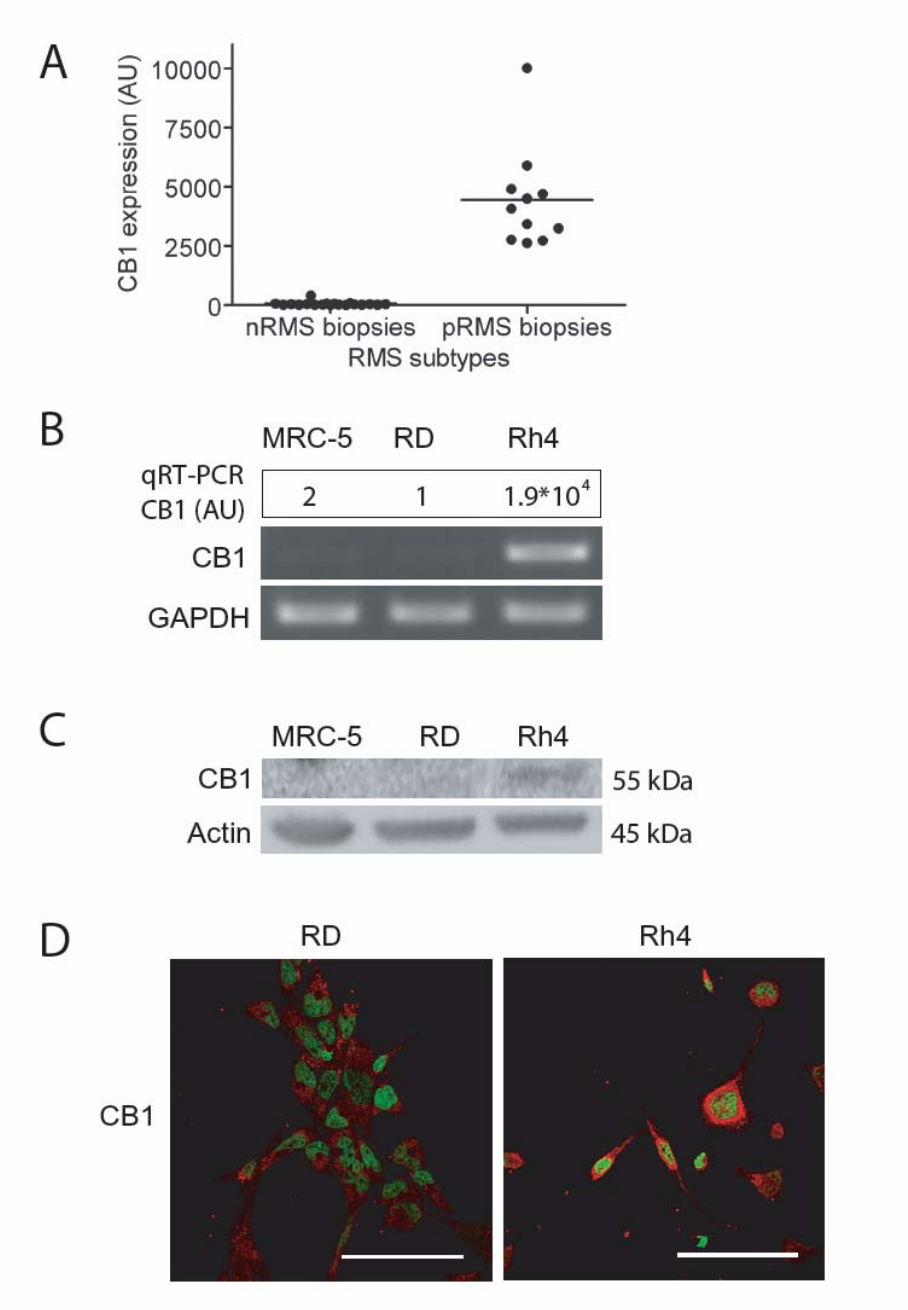

Figure (Paper) 1: CB1 expression in tposRMS cells. A: Gene expression values of CB1 are shown in arbitrary units. Samples analysed by microarray gene expression profiling were translocation-negative (tnegRMS) versus translocation-positive (tposRMS) biopsy samples. B: Quantitative and normal RT-PCR with primers for CB1 and for GAPDH were performed with cDNA of cell lines MRC5 (fibroblast), RD (tnegRMS), and Rh4 (tposRMS) cells. Quantitative results are indicated in arbitrary units. C: CB1 levels of MRC5, RD, and Rh4 cells are shown as determined by Western Blotting. D: Immunofluorescence stainings with anti-CB1-antibody on RD and Rh4 cells are depicted (scale bar 100µM).

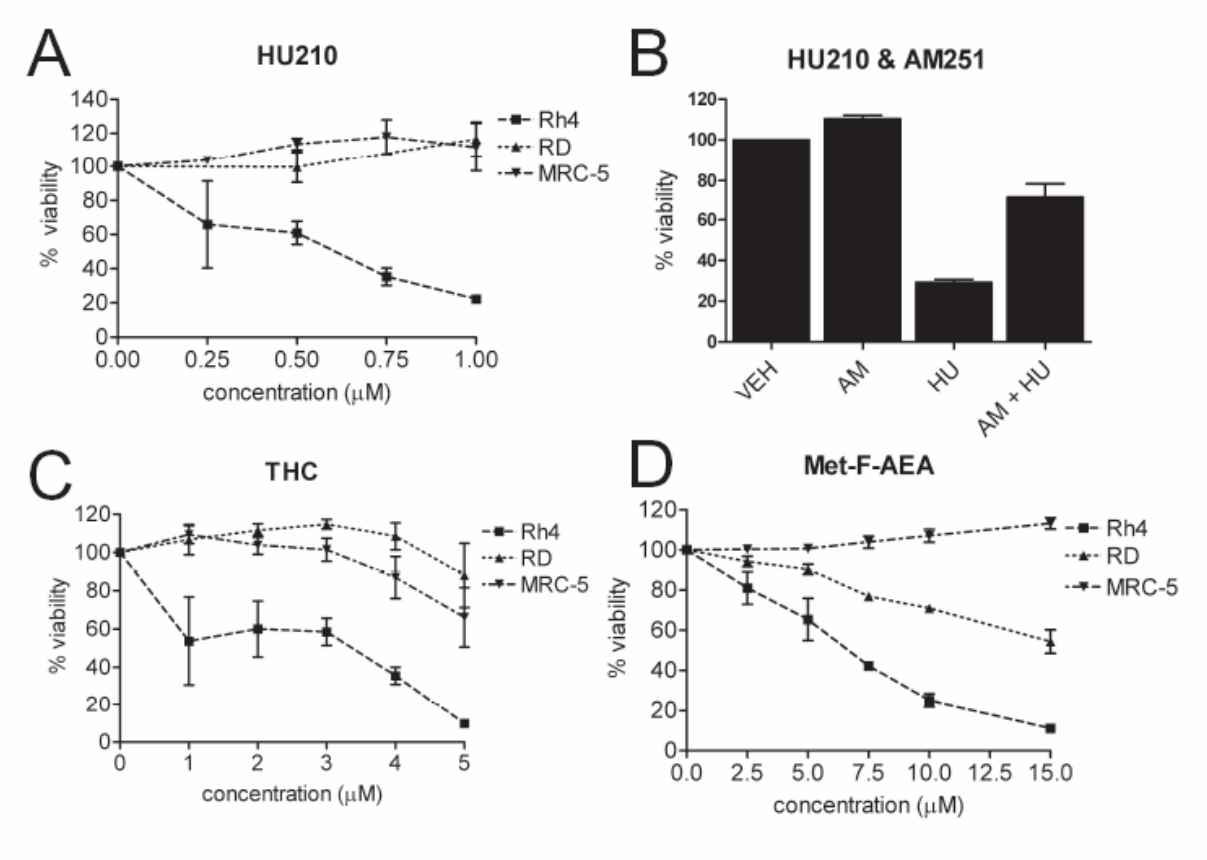

Figure (Paper) 2: Cannabinoids reduce viability of tposRMS cells. A: Different cell lines Rh4 (tposRMS), RD (tnegRMS), and MRC5 (fibroblasts) were incubated with increasing concentrations of HU210 for 72 hours. Subsequent viability measurements by means of MTT are shown (n=3, +/- SE; significance at 1.25μM: $p<0.005$). B: Viability measurements are shown for Rh4 cells pre-incubated with vehicle or with 0.5μM AM251 before undergoing subsequent treatment with 1μM HU210 for 24hrs (n=3, +/- SE, significance: $p<0.05$). C-D: Dose-dependent viability of Rh4, RD, and MRC5 cells after treatment with THC for 24 hours or Met-F-AEA for 48 hours was measured (n=3, +/- SE, significance at 5μM THC and 10μM Met-F-AEA: $p<0.05$).

In addition, pharmacological blockade of the CB1 receptor significantly restored cell viability from 29.2% (+/- 2.4SD) to 71.9% (+/- 11.2 SD) (Fig. 2B), supporting the notion that the observed reduction in cell viability was specifically mediated through the CB1 receptor.

7.1.5.3 Cannabinoids induce apoptosis in tposRMS cells

To determine whether the decreased cell viability after cannabinoid treatment in tposRMS cells is due to apoptosis, caspase-3 activation and PARP cleavage were analyzed. First, Rh4 cells were treated with 1.25µM HU210 and cell extracts analyzed by immunoblotting for PARP cleavage at different time points (Fig. 3A). Already 6h after start of the treatment, PARP cleavage could be observed and after 24hrs, almost no uncleaved protein was detectable. Pre-treatment of Rh4 cells with 0.5µM of the CB1 specific antagonist AM251 significantly rescued cleavage of PARP protein (Fig. 3B and 3C). At a concentration of 1.25µM HU210, for example, the ratio of cleaved to uncleaved PARP protein could be rescued from 1.13 (+/- 0.05 SD) to 0.40 (+/- 0.03 SD).
Additionally, caspase-3 activation after 24 hrs of HU210 treatment was measured in Rh4 cells (Fig. 3D). A concentration-dependent increase of cells positively stained for active caspase-3 was detected with close to 100% apoptotic cells at 1.25µM of HU210. In line with the previous results we also observed that both THC (2 and 4µM) and Met-F-AEA (5 and 10µM) treatment induced cleavage of PARP protein after 4-5 hours of incubation (Fig. 3F). Therefore, treatment of tposRMS with cannabinoids induces apoptosis in tposRMS cells.

7.1.5.4 Cannabinoids inhibit Akt signalling

Earlier studies investigating effects of cannabinoids on cancer cells could demonstrate alterations in AKT and ERK signalling upon drug treatment. Based on this, we next studied AKT and ERK signalling in tposRMS cells after cannabinoid treatment.

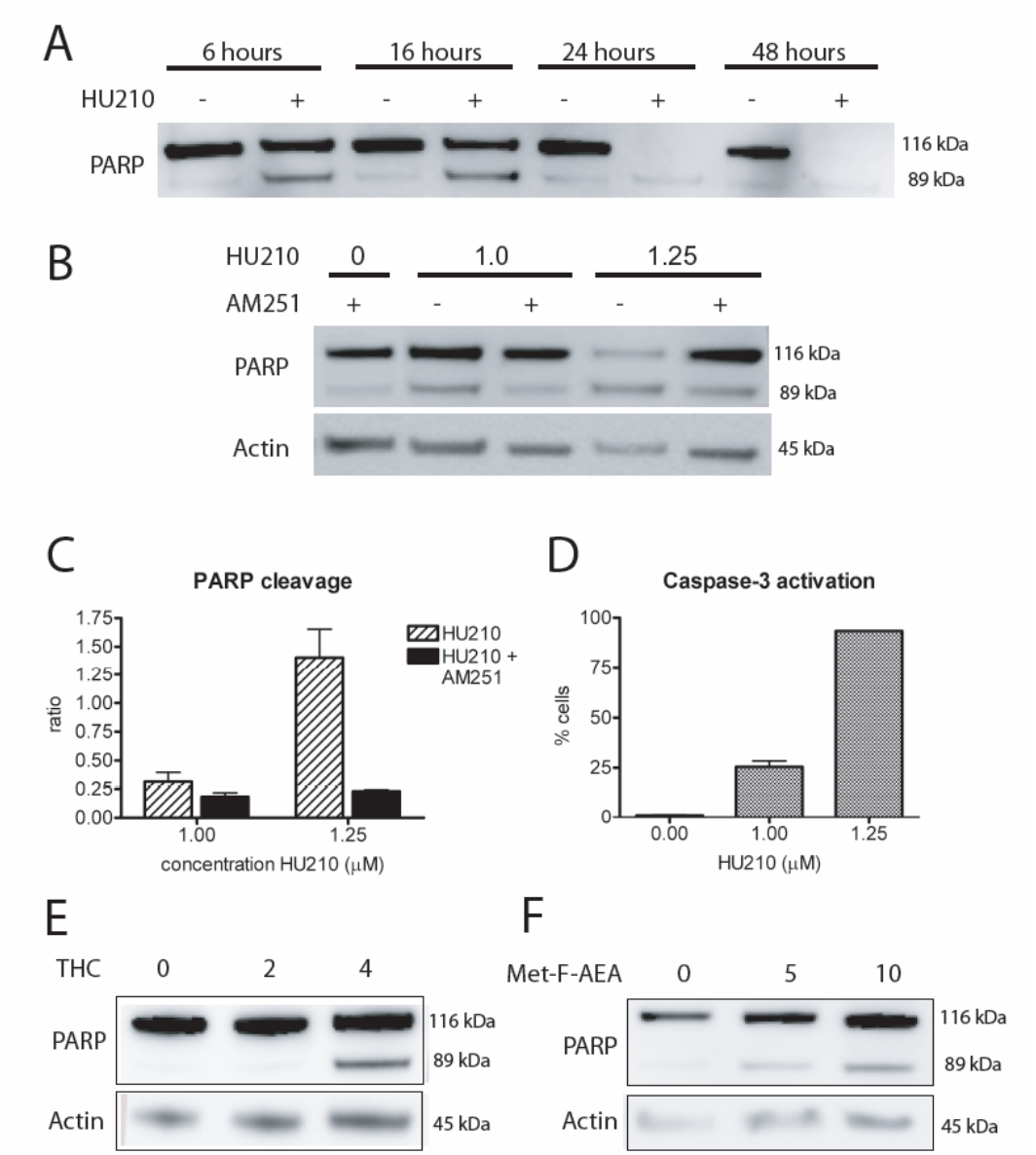

Figure (Paper) 3: Cannabinoids induce apoptosis in tposRMS cells. A: Rh4 cells were treated for 6, 16, 24, and 48 hours with either 1.25μM HU210 or DMSO. Subsequently, Western blotting was performed with an anti-PARP antibody. B: After pre-incubation of Rh4 cells with 0.5μM of CB1 antagonist AM251, HU210 was added at concentrations 1 and 1.25μM HU210 for 20 hours. Cell lysates were probed with anti-PARP (upper panel) and anti-actin (lower panel) by immunoblotting. C: Densitometric quantification of the ratio of cleaved to uncleaved PARP product of (B) is shown (values +/-SE, n=2). D: Percentage of cells staining positively for pro-apoptotic caspase-3 is shown as evaluated after 20 hours of HU210 treatment of Rh4 cells (values +/- SE, n=3, significance: $p<0.005$). E: Rh4 cells were either treated with 2 and 4μM of THC or 5 and 10μM of Met-F-AEA for 24 hours. Protein extract was analysed for PARP and actin by Western Blotting (here shown a representative blot).

Rh4 cells were incubated with 1.25µM HU210 for 30 min and 2 h prior to cell lysis. A rapid decrease in pospho-AKT at Ser473 was detected indicating inhibition of AKT activity (Fig. 4A). Under the same experimental conditions, phosphorylation of ERK was found to increase in tposRMS cells after drug treatment in comparison to vehicle-treated cells, at both Thr202/Tyr204 (Fig. 4B). Further analysis 2h after drug treatment showed that also phosphorylation by PDK1 on Thr308 on AKT. In addition, the AKT-down-stream target GSK3β became dephosphorylated (Fig. 4C and D). Notably, THC (2 and 4µM) as well as Met-F-AEA (5 and 10µM) triggered dephosphorylation of AKT at Ser473 (Fig. 4E and F), only slightly delayed compared to HU210. No difference for ERK phosphorylation was observed with either of these two substances (data not shown). In summary, all three cannabinoid agonists lead to inhibition of AKT signalling in tposRMS cells, while ERK activation was only seen after treatment with HU210. These experiments suggest that the AKT pathway is likely to mediate the action of cannabinoids in our tumor model.

7.1.5.5 Cannabinoids reduce viability via upregulation of transcription factor p8

p8 is a transcription factor involved in cellular stress responses as it is activated in response to several cellular injuries through pathways implicated in growth inhibition (106, 168). Furthermore, p8 mediates apoptosis upon cannabinoid treatment of different cancer cells as demonstrated in glioblastoma (87), pancreatic cancer (107), and breast cancer (108) cells. Therefore, we tested the involvement of p8 in the antiproliferative action of HU210, THC, and Met-F-AEA in our model. p8 levels were assessed in mRNA isolated 16hrs after addition of drugs to tposRMS cells. A clear dose-dependent increase in p8 transcripts up to 6.5-fold was observed for all cannabinoids used compared to vehicle-treated control samples (Fig. 5A).

To validate the requirement of p8 upregulation for induction of apoptosis, p8 expression was specifically down-regulated by treatment with siRNA. Levels after treatment were on average down to 16% (+/- 5.5% SD) of remaining expression 48hrs after transfection.

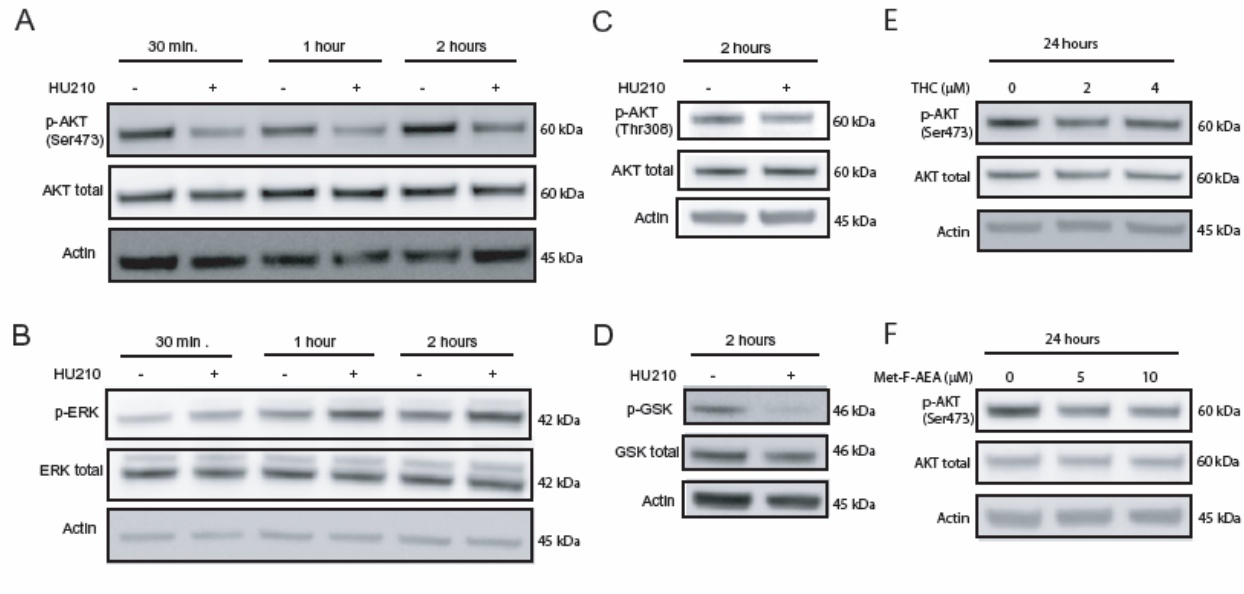

Figure (Paper) 4: Cannabinoid receptor agonists affect AKT and ERK signalling in tposRMS cells. A-B: Rh4 cells were incubated with 1.25µM HU210 for 30 min., 1 hour, and 2 hours and cell lysates prepared. Then, Western Blotting was performed with antibodies against phospho-AKT (Ser473) (A), and against phospho-ERK (B). C-D: Anti-phospho-GSK-3-alpha/beta (Ser21/9) (C), and anti-phospho-AKT (Thr308) (D) were probed on extracts of cells treated for 2 hours with 1.25µM of HU210. E-F: Phosphorylation status of AKT at Ser473 was analysed by Western Blotting after treatment of Rh4 cells with 2 and 4µM THC (E) or 5 and 10µM Met-F-AEA (F) for 24 hours.

Upon incubation with 1.25µM of HU210 for 48hrs, viability of scrambled-transfected cells was reduced to 37% (+/- 9.4 SE), whereas cells with lower p8 transcript levels showed a rescue in viability up to 63% (+/- 8.5 SE) (Fig. 5B). This suggests that an increase in p8 levels after cannabinoid treatment is an important component of the molecular response. These experiments indicate that p8 mediates, at least in part, the reduction in viability observed after cannabinoid treatment.

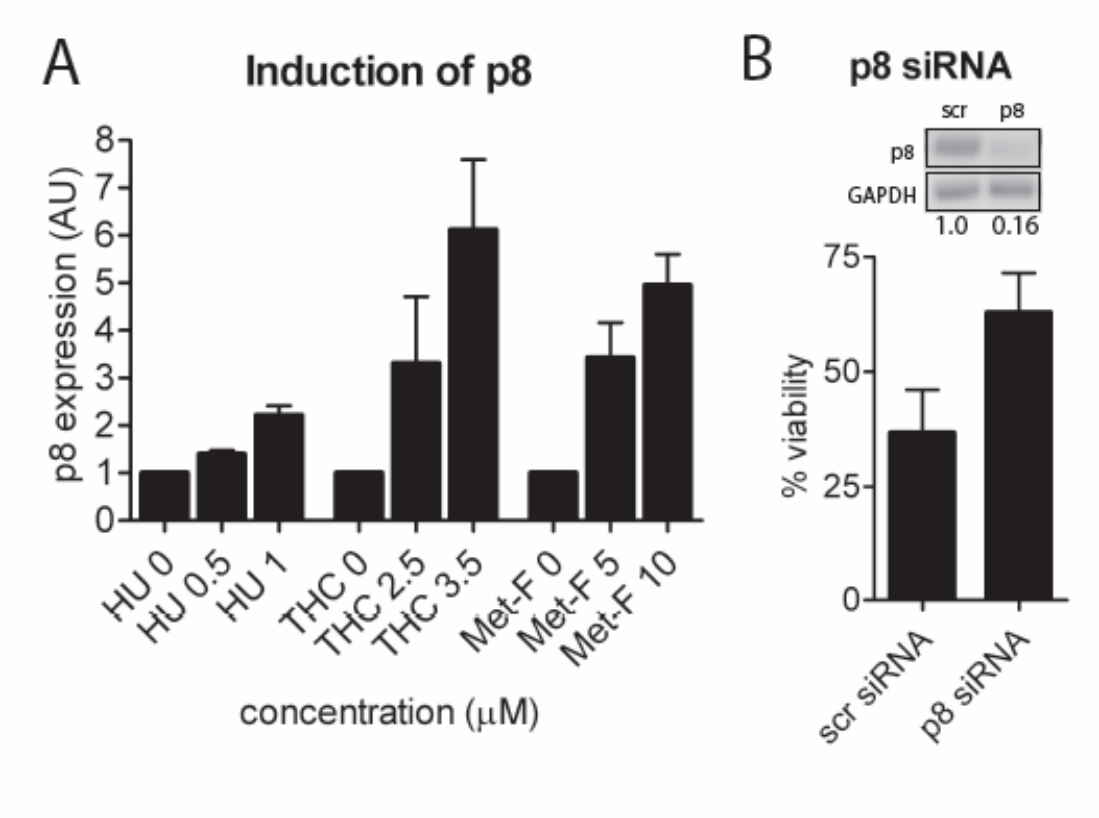

Figure (Paper) 5: Induction of pro-apoptotic p8. A: Rh4 cells were treated with 0.5 and 1μM of HU210, 2.5 and 3.5μM of THC (B), or 5 and 10μM of Met-F-AEA for 16 hours. RNA was extracted and analysed for p8 transcripts with qRT-PCR (n=3, +/-SE, $p<0.05$). Values were normalized to GAPDH. B: P8 was down-regulated by means of siRNA. A representative RT-PCR and quantitative values (in arbitrary units, normalized to scrambled siRNA transfected control cells) are indicated in the upper panel. Viability after HU210 (1.25μM) treatment was assessed at 48hours with MTT (n=3, +/-SE, $p<0.05$).

7.1.5.6 HU210 reduces tumor growth of tposRMS xenografts

To test whether HU210 might have a therapeutic effect on tposRMS tumors in vivo, tumor xenografts were generated by subcutaneous injection of Rh4 cells into immune-deficient NOG mice. Tumors were treated peritumoral with HU210 daily for 13 subsequent days. We observed significantly reduced tumor growth in HU210-treated compared to vehicle-treated animals. To gain further insight into the molecular events leading to this observation, animals were euthanized after the last day of treatment. Tumors were excised and paraffin-embedded sections were either hematoxilin-eosin (H/E) stained or subsequently immunohistochemically analyzed with antibodies against the proliferation marker Ki67 and the apoptosis indicator

cleaved caspase-3. H/E-stained sections from HU210-treated animals displayed a high number of cell-free patches filled with connective tissue, which are probably remainings of previously apoptotic or necrotic areas. In agreement with this, a moderate increase of apoptotic cells, which was variable across tumors, was detected in HU210-treated mice compared to vehicle-treated animals (data not shown). On the other hand, no difference in the staining pattern for Ki67 was observed between treatment modalities (data not shown). In conclusion, HU210 is capable of reducing aRMS xenograft growth by inducing apoptosis, whereas proliferation of tumor cells remained unaffected.

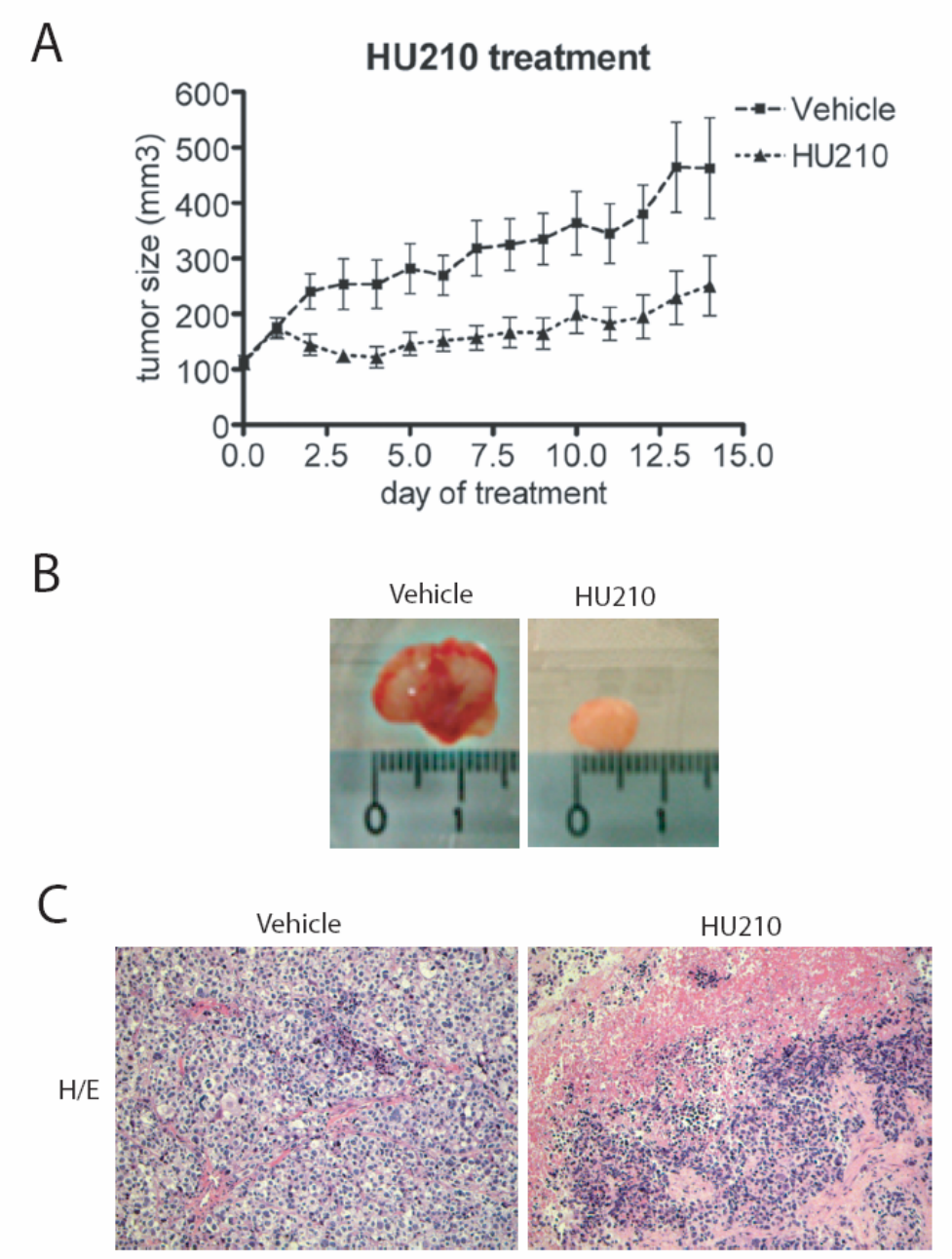

Figure (Paper) 6: HU210 reduces tumor growth in vivo. A: NOG mice were injected with 7.5 x 10^6 tposRMS (Rh4) cells subcutaneously into the flank. After reaching a tumor size of 100 – 150mm^3, animals were assigned randomly to either the vehicle (n=7) or the HU210 (n=6) group. Treatment was given daily by injecting either 0.2ug/kg HU210 or DMSO in PBS peritumorally for 13 days, while tumor growth was monitored daily and mice were sacrificed on the day after the last treatment. A: Tumor growth over time is shown for HU210-treated in comparison to vehicle-treated animals (+/- SE, $p<0.001$). B-C: Representative sections of tumors from vehicle and HU210-treated animals were stained with H/E. Original magnification 100x.

7.1.6 Discussion

Evidence from *in vitro* and *in vivo* experiments suggests that cannabinoid receptor agonists can reduce tumor growth and induce apoptosis in several tumor types, including melanoma, breast and prostate cancer, colon cancer, leukaemia, and glioma. However, to our knowledge the response to cannabinoid treatment has not been studied in sarcomas yet. Here, we investigated effects of cannabinoid receptor agonists in the sarcoma tposRMS, which we not only confirmed to express high levels of CB1 mRNA, but also demonstrate expression on the protein level by Western blot and immunohistochemistry.

In vitro, cannabinoid receptor agonists HU210, THC, and Met-F-AEA exerted an anti-proliferative and pro-apoptotic action on tposRMS cells through activation of the CB1 receptor. Specificity of this effect for CB1 was shown by two means: First, cell viability by this treatment is much less affected in fibroblasts or tnegRMS control cell lines which express only low levels of CB1. Second, the CB1 specific antagonist AM251 was able to reduce apoptosis significantly and thereby partially restore cell viability. TposRMS cells were most sensitive to submicromolar concentrations of HU210 and was comparable to those observed for THC and Met-F-AEA in other cancer cells such as pancreatic cancer (107), breast cancer (113), or colon cancer (150) cells.

Key events contributing to cannabinoid-triggered induction of apoptosis in tposRMS cells are reduction in AKT signalling and up-regulation of the transcription factor p8. While cancer cells such as melanoma (100), colon cancer (99), and glioma (86) also experience dephosphorylation of AKT after cannabinoid-stimulation, non-transformed CB1-expressing cells such as neurons react with increased phosphorylation of AKT under the same circumstances (93, 95). The key event responsible for this fundamental difference is still unknown, however de novo ceramide synthesis (83) seems to be important for induction of apoptosis in cancer cells. Apart from AKT, the transcription factor p8 was recently shown to be up-regulated by cannabinoid receptor agonists and this event seems to be crucial for sensitivity to cannabinoid receptor agonists (87). Only cannabinoid-sensitive glioma cell clones showed significant upregulation of p8 after drug treatment and knockdown of this gene could

rescue cell viability in different cannabinoid-treated cancer cells, such as glioma (87), breast cancer (108) or pancreatic cancer (107). Also in tposRMS cells examined here, p8 is critical in the pro-apoptotic signalling after cannabinoid treatment since inhibition of its accumulation by means of RNA interference significantly rescued cell viability. In contrast, results for the ERK pathway are not consistent in our tumor model. This is similar to other reports and seems to be tumor type specific. In addition stimulation or inhibition of this signalling pathway might also depend on the type of agonist used, as shown in this study. Nevertheless, the ERK pathway is less likely to play an important role in the pro-apoptotic effect of cannabinoids in tposRMS. These observations prompted us to further examine the efficiency of HU210 for treatment of a tposRMS xenograft mouse model.

So far, HU210 has been efficiently used in animal models to investigate neurogenesis (169) and multiple sclerosis (170) and was recently shown to prevent formation of preneoplastic lesions in mouse colon (61). However, HU210-treatment of xenograft bearing mice has not been reported so far. Here, we observed significantly reduced tumor-growth in HU210-treated animals in comparison to vehicle-treated animals without overt psychoactive signs. Growth reduction seen was comparable to other xenograft models treated with cannabinoids, such as treatment of pancreatic cancer tumors with THC or JWH-133, for example (107). A moderate increase in the number of apoptotic cells was observed in HU210-treated xenograft sections, however we can not exclude that other mechanisms could additionally account for reduction in tumor growth. However, analysis of transcript levels of myogenic differentiation markers, such as myosin light chain or troponin C2 (50) did not significantly differ between treatment modalities, ruling out the possibility that cannabinoids induce differentiation in tposRMS cells as observed after inhibition of PAX3/FKHR function.

In comparison to other drug classes such as the broad-spectrum kinase inhibitor PKC412 investigated in our laboratory (51), HU210 treatment as single agent appears less efficient in tumor growth reduction. Nevertheless, potential use of cannabinoids as therapeutic intervention for tposRMS should still be pursued, possibly in combination with conventional chemotherapies, kinase inhibitors, or other targeted agents. Several reports indicate synergistic activity of cannabinoid receptor

agonists in combination with well-established anti-neoplastic substances. THC was reported to act synergistically with suboptimal doses of Doxorubicin or Cisplatin (87), and synergism between HU210 and 5-Fluorouracil was recently reported as well (150).

In summary, our results support and extend the previously demonstrated anti-tumor activities of cannabinoid receptor agonists by showing pro-apoptotic effects of HU210, THC, and Met-F-AEA on tposRMS cells in vitro and, for the first time, demonstrate that HU210 has tumor growth inhibiting properties in vivo. This could represent one possible novel treatment strategy that might improve outcome in this pediatric tumor.

7.1.7 Acknowledgments

The authors' work is supported by OncoSuisse (grant numbers 01473-02-2004 and 01944.08-2006). Furthermore, we cordially thank Cristina Blazquez and Tania Aguado for their support and Beat Bornhauser for the breeding of the NOG mice.

7.2 Additional results

7.2.1 HU210 induces apoptosis *in vivo*

In order to check whether HU210 also induces apoptosis in vivo as was observed in cell culture, paraffin-embedded sections were analysed for apoptotic cells with an antibody against cleaved caspase-3. A moderate increase of apoptotic cells, which was variable across tumors, was detected in HU210-treated mice compared to vehicle-treated animals. On the other hand, no difference in the staining pattern for the proliferation marker Ki67 was obtained between treatment modalities (Fig. 6A). In conclusion, HU210 is capable of reducing tposRMS xenograft growth by inducing apoptosis, whereas proliferation of tumor cells remained unaffected.

7.2.2 Differentiation is not involved in the tumor growth reduction

The transcription factor p8 is critically involved in the mediation of the pro-apoptotic effect of HU210 and other cannabinoids on tposRMS Rh4 cells in vitro. In vivo analysis of p8 transcript levels in tumor biopsies could not validate this finding, as p8 is only upregulated to a non-significant extent in HU210-treated animals versus vehicle treated mice (Fig. 6B). Further mechanisms contributing to tumor growth reduction include differentiation of tumor cells. To address this question, mRNA levels of myogenic markers Myl1 and TNNC2, which are known to reflect RMS differentiation (50), were measured in excised tumors. However, as only a non-significant increase of both transcript levels was observed in HU210-treated sapmles, this mechanism can be ruled out (Fig. 6B).

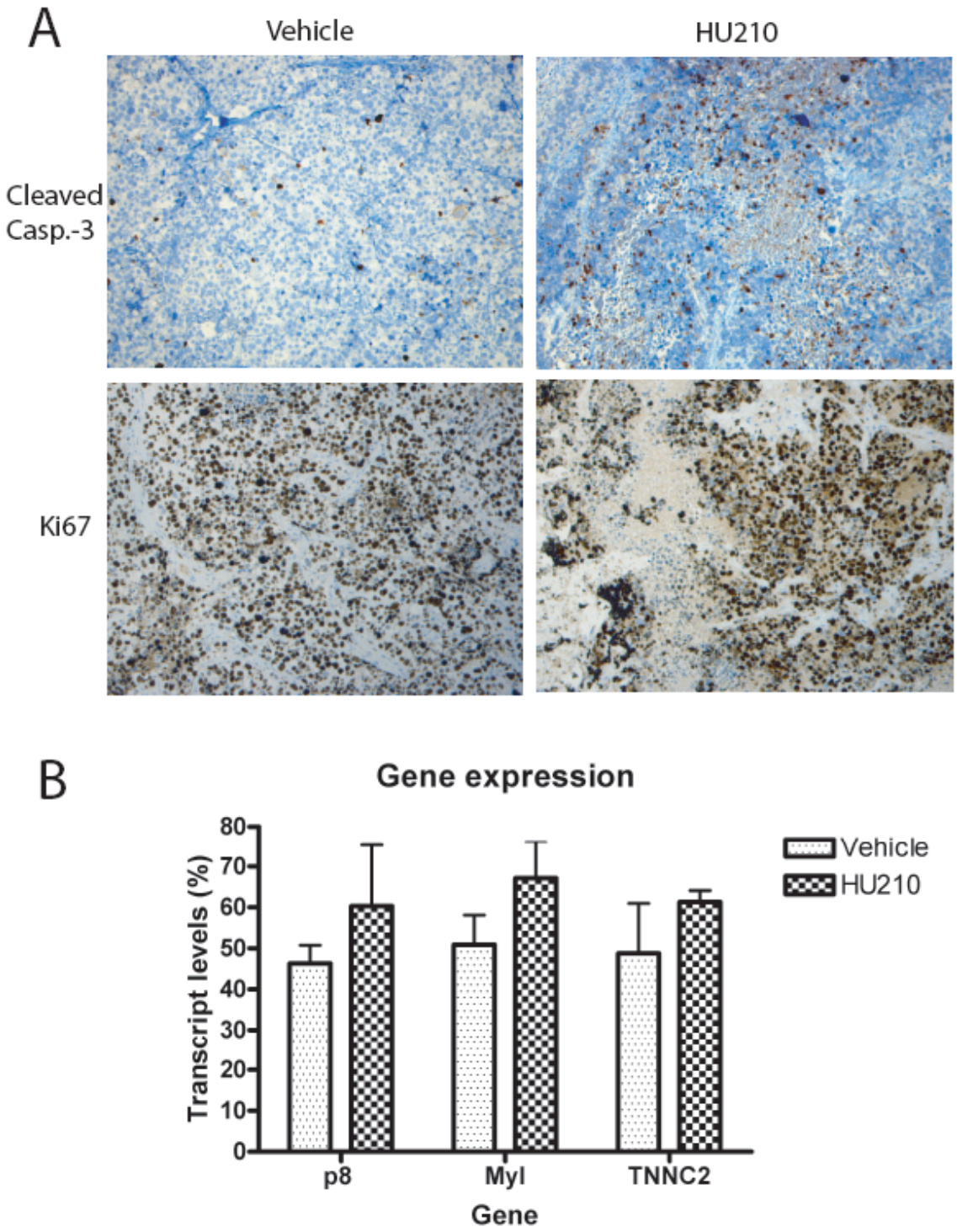

Figure 8: NOG mice bearing tposRMS tumors were treated for 13 days with HU210 or vehicle. Tumor biopsies were obtained one day after the last treatment. A: Stainings for cleaved caspse-3 or Ki67 are shown. B: Percentage of transcript levels of p8, Myl, and TNNC2 in comparison to the tumor with the highest level are shown as determined with qRT-PCR on tumor tissue of vehicle (n=7) or HU210 (n=6)-treated animals (values +/-SE, ns).

8 Discussion

Eventhough survival rates of pediatric cancers have risen over the last decades, treatment of pediatric cancers still remains a task to be improved due to many unwanted late implications drastically reducing life quality of patients. Current multimodal treatment approaches comprising surgery, radiation, and chemotherapy often cause developmental disturbances, problematic fertility, or even secondary tumors at higher age (Fig. 7)(171). Therefore, new approaches should be found, which don't base on targeting general cellular mechanisms such as replication or mitosis, but rather specifically inhibit proliferation of cancer cells. Towards this aim, elucidation of molecular tumor characteristics can help to find appropriate drug targets, which can be highly different among tumor types.

Table 3. Relative Risk of Selected Severe (Grade 3) or Life-Threatening or Disabling (Grade 4) Health Conditions among Cancer Survivors, as Compared with Siblings.

Condition	Survivors (N=10,397)	Siblings (N=3034)	Relative Risk (95% CI)
	percent		
Major joint replacement*	1.61	0.03	54.0 (7.6–386.3)
Congestive heart failure	1.24	0.10	15.1 (4.8–47.9)
Second malignant neoplasm†	2.38	0.33	14.8 (7.2–30.4)
Cognitive dysfunction, severe	0.65	0.10	10.5 (2.6–43.0)
Coronary artery disease	1.11	0.20	10.4 (4.1–25.9)
Cerebrovascular accident	1.56	0.20	9.3 (4.1–21.2)
Renal failure or dialysis	0.52	0.07	8.9 (2.2–36.6)
Hearing loss not corrected by aid	1.96	0.36	6.3 (3.3–11.8)
Legally blind or loss of an eye	2.92	0.69	5.8 (3.5–9.5)
Ovarian failure‡	2.79	0.99	3.5 (2.7–5.2)

* For survivors, major joint replacement was not included if it was part of cancer therapy.
† For both groups, this category excludes basal-cell and squamous-cell carcinoma (grade 2). For siblings, this category includes a first cancer.
‡ Values are for women only.

Figure 9: Consequences of anti-cancer therapies in children (Oeffinger. 2004).

Our study focused on novel drug targets for the treatment of alveolar rhabdomyosarcoma, a childhood cancer with 5-year overall survival of about 57% (*Dantonello, 2008).* Typically, this disease cases bear translocations encoding for chimaeric transcription factors PAX3/FKHR or PAX7/FKHR which lead to up-

regulation of the cannabinoid receptor 1 (CB1)(19, 49). This receptor represents an excellent targeting option for novel therapeutic approaches, as its transcript levels are virtually absent in control tissue such as skeletal muscle or fibroblasts, and further has been described to lead to reduced proliferation upon activation in cancer cells.

In line with these initial reports, we could demonstrate induction of apoptosis by several cannabinoid receptor agonists in Rh4 cells, showing the typical gene signature triggered by the chromosomal translocations. As outlined in the discussion part of the paper, concentrations of cannabinoids used in this work are well comparable with other cancer types (113, 150) and *in vivo* experiments could demonstrate the efficacy of cannabinoid treatment in aRMS. Still, lots of questions regarding mechanisms of action and feasibility of such a treatment for aRMS and for cancer in general remain and will thus be further discussed in the following sections.

8.1 Fate decision: Role of AKT signalling

One mechanism critically involved in the viability-reducing action of cannabinoids on aRMS cells is the inhibition of AKT, a signalling molecule contributing to survival and proliferation upon its activation. AKT dephosphorylation occurs very fast after drug treatment in aRMS cells, suggesting that this cell signalling event lies at the beginning of the cellular cascade leading to cell death. Importantly, cannabinoid receptor agonists elicit differential effects on phosphorylation of AKT depending on transformation status of the stimulated cells. Treatment of non-transformed cells such as neurons, astroglias, or cells stably transfected with CB1 (eg. CHO expressing CB1) causes AKT activation (93, 95), rather leading to a pro-survival burst. For instance, cannabinoid treatment can protect neuronal cells from oxidative stress (88). Controversly, cancer cells (eg. glioma, melanoma, colon cancer) treated with cannabinoids experience an inhibition of AKT phosphorylation, which thus could be one of the most critical cellular events on the way to apoptosis (86, 99). Proof of the importance of AKT inhibition in the pro-apoptotic signalling of cannabinoids was given by Blazquez et al., who could rescue cell viability of WIN55,212-2-treated melanoma cells by transfecting the cells with a constantly active AKT-construct (100).

This transformation-dependent reaction to cannabinoids either leading to pro-survival or pro-apoptotic cell signalling allows these compounds to be highly cancer-specific cytotoxic agents. While most other targeted anti-neoplastic strategies rely on inhibition of aberrantly over-expressed proteins on cancer cells, cannabinoid receptor agonists exert viability-reducing effects through differential signal integration between healthy and cancer cells after receptor activation. Therefore, receptor expression still remains a prerequisite for successful therapy, but it must not be exclusively restricted to the targeted cancer cells.

Apparently, there must be a decisive step in cannabinoid-receptor mediated signalling up-stream of AKT, as this signalling molecule only accomplishes orders generated elsewhere. The lipid signal-mediator ceramide might play a fate-deciding role as its sustained de-novo synthesis has been demonstrated to occur only in malignant cells leading to AKT dephosphorylation while its acute rise by sphingomyelin hydrolysis occurs in both healthy and cancerous cells (83). But how can the time-consuming and long-term *de novo* biosynthesis of ceramide account for an effect already observable after 10 min., as it was the case in melanoma cells (100) as well as in rhabdomyosarcoma cells? And which are the additional cues leading to *de novo* ceramide synthesis limited to transformed cell types after cannabinoid receptor agonist stimulation?

Based on this argumentation, yet other mechanisms should be implicated in the sensitivity of cancer cells towards cannabinoids. It is tempting to speculate that autocrine effects, different lipid raft distribution or even expression of yet unknown additional cannabinoid ligand binding receptors as the novel cannabinoid receptor GPR55 could contribute to mixed effects on ceramide and AKT signalling examined after cannabinoid challenge. Hart et al. already reported an autocrine transactivation of EGFR to be inovlved in pro-survival signalling after nano-molar concentrations of cannabinoids in cancer cells (97), while lipid raft disruption can influence ligand binding at CB1 as well as modulating down-stream signalling (139, 172, 173). Hence, biological mechanisms mediating the above explained differential response to cannabinoids remain yet to be uncovered after having achieved a good basis by former elucidation of down-stream signalling events in several CB-expressing model cell systems.

8.2 Cannabinoid-sensitivity mediated by p8

Among these down-stream signalling events critically involved in the pro-apoptotic action of cannabinoids, up-regulation of p8 can be found. p8 is a transcription factor involved in cellular stress responses and has also been termed Nuclear Protein 1 (NUPR1) or Candidate of Metastasis 1 (COM-1). Initially, p8 was associated with acute pancreatitis (106)(*Encinar, 2001),* high occurrence in pancreatic cancer (174) and was attributed with an inverse correlation to apoptosis in the same tumor type (175). Controversely, later reports indicated a pro-apoptotic role for p8, as induction of its mRNA was found to be crucial for apoptosis after THC treatment in glioma, pancreatic, and breast cancer cells (87, 107, 108). The transcription factor p8 was reported to mediate its apoptotic effect via upregulation of the endoplasmatic reticulum stress-related genes for ATF-4, CHOP, and TRB3 in glioma cells (87). A series of ER alterations such as calcium depletion, protein misfolding, and impairment of protein trafficking to the Golgi triggers the ER stress response (176), which can lead to apoptosis as is reported for several stimuli, including ischemia (177), viral infection (178), and drugs such as cisplatin (179).

Also in aRMS cells, the viability-reducing action of HU210 and other cannbinoid receptor agonists was found to be mediated through concentration-dependent up-regulation of p8 transcript levels. Further support for the importance of p8 in the pro-apoptotic cascade in Rh4 cells was derived from p8 siRNA-mediated knockdown experiments, where cells with lower p8 levels showed significantly higher viability after HU210 treatment. Interestingly, experiments in our lab also demonstrated increase of p8 levels after treatment of Rh4 cells with a broad-spectrum kinase inhibitor PKC412, indicating a general pro-apoptotic mechanism regarding different substance classes (data not shown).

Whereas experiments with cannabinoids in cancer cells lead to uniform conclusions about pro-apoptotic contribution of p8, the general cellular context remains grossly unknown and its investigation is even further complicated by the fact that mere medium change can lead to p8 up-regulation (180). Hence, p8 up-regulation, which can be very unspecifically triggered, might not serve as appropriate read-out to test the pro-apoptotic action of any cytotoxic compound but knowledge about its cellular

regulation is still helpful as there is evidence for its implication in synergistic drug effects (87). Additionally, p8 levels correlated with good prognosis in breast cancer as reported by Jiang et al, in 2005 (181), probably reflecting the necessity of p8 expression for successful pro-apoptotic cancer therapy. Nevertheless, analysis of p8 RNA levels among rather aggressive aRMS and well-treatable eRMS tumors in the microarray data published by Wachtel et al. 2004 (19) didn't reveal any conclusive pattern, with a non-significant difference between means of p8 transcript levels (in arbitrary units) in aRMS (mean = 264) and eRMS (mean = 288).

8.3 Effects of HU210 on aRMS xenograft

In the last part of our studies, a rhabdomyosarcoma xenograft experiment allowed to proof the efficacy of the anti-neoplastic cannabinoid treatment *in vivo* and furthermore gave the opportunity to validate signalling mechanisms observed *in vitro*. In detail, tumor structure, apoptosis, and proliferation were analyzed histologically. By quantitative RT-PCR, mRNA levels of p8 were addressed to study the involvement of ER-stress as well as transcript levels of the differentiation markers Myosin light chain and Troponin C2 were assessed to elucidate the effects of HU210 on differentiation in aRMS xenografts.

Daily tumor measurement revealed a significantly reduced tumor growth in HU210-treated NOG-mice bearing Rh4 xenografts. Analysis of signalling mechanisms such as inhibition of AKT and elevation of p8-transcript levels, which were usually assessed at rather short time-points after drug treatment in vitro, didn't yield any significant differences between control and HU210-treated animals. However, on sections of HU210-treated tumors, a high occurrence of cell free areas was observed, as already described above in the manuscript. These areas filled with connective tissue are probably remainings of previously apoptotic or necrotic cells potentially reflecting successful treatment of cancer cells, which died during the first days of the treatment regimen. Although tumor size was markedly smaller in cannabinoid-treated animals, only a moderate increase of cells positively staining for cleaved caspase-3, which was variable across tumors, was detected in cannabinoid-treated animals. On the other hand, no difference in the staining pattern for Ki67

between treatment modalities was seen and thus no changes in proliferation and cell cycle could further account for reduced tumor growth.

Interestingly, upon dissection of xenograft bearing mice, HU210-treated tumors seemed rather pale with lower vascularization on the tumor surface than the vehicle-treated controls (see Fig. 6 of the Paper section), suggesting that HU210 influences the vascularization of the tumors. Histological analysis of CD31, which is a marker for vascularization, didn't reveal a difference in the inner sections of the differently treated tumors (data not shown) reflecting that the anti-angiogenic impact of HU210 might be restricted to the tumor surface of aRMS xenografts.

Earlier *in vivo* studies in our lab have demonstrated a higher pro-apoptotic action of the substance PKC412, a broad spectrum kinase inhibitor, on aRMS xenograft tumors than observed after cannabinoid-treatment with HU210. Application of this kinase inhibitor led to tumor growth inhibition by a significant increase of apoptotic cells as well as decrease of Ki67 positive tumor cells (51). Hence, the relatively moderate increase of apoptotic cells upon HU210-treatment demands yet additional mechanisms contributing to lower xenograft growth.

Cancer cells are known to be in a rather undifferentiated state than their healthy tissue of origin causing them to divide constantly and grow aggressively. Therefore, cues stimulating their differentiation can help limiting uncontrolled cell divisions. In a number of publications, the endocannabinoid system was claimed to play a role in cell differentiation in the hematopoietic system (72, 182), in neural cells (183), in adipose tissue (184), or in skeletal remodelling (185). In regard to this project, the most relevant finding was reported by Aguado et al. in 2007 on the cannabinoid-induced differentiation of glioma stem cells (149). JWH133 or HU210-treatment of glioma-stem like cells deriving from tumor biopsies or cell lines promoted glial differentiation and decreased the efficiency of in vivo glioma development as well as neurosphere formation.

To test whether a similar process could be involved in the reduced tumor growth of the HU210-treated aRMS xenografts, mRNA levels of several differentiation markers were evaluated. The origin of rhabdomyosarcoma hasn't been definitively proven yet,

but due to its resemblance to skeletal muscle cells as well as expression of specific muscle markers such as myogenin or myosin light chain, it is believed to originate from this tissue. Therefore, it is not surprising that RMS tumor cells can be driven into differentiation by exogenous stimulation with eg. phorbol esters (186) or retinoid acid (187). Similarly, experiments in our lab showed that aRMS cells can be guided into differentiation by PAX3/FKHR silencing leading to a significant increase of myosin light chain 1, (Myl1) and troponin C2 (TNNC2)(50). Based on this knowledge, expression levels of Myl1 and TNNC2 were addressed on tumors excised 14 days after HU210-treatment start. As shown in the section of additional results, a slight increase of both markers could be observed. However, the values obtained didn't differ significantly between treatment and control suggesting that differentiation doesn't account for the observed reduction in aRMS tumor growth.

Nevertheless, it should be mentioned that the time-point of analysis is very critical for such approaches. In fact, observable and measurable growth reduction of tumors is only the result of a process happening earlier on. For instance, apoptotic cells are reabsorbed by the tumoral tissue resulting in a smaller tumor volume. Similarly, AKT inhibition is a signalling mechanism occurring instantly after drug incubation *in vitro*, possibly recovering to normal levels after 13 daily cycles of treatment. Furthermore, peritumoral injections are placed next to the tumor, what could even in the case of vehicle (DMSO)-application have disturbing effects on tumor environment and hence would also explain general slow Rh4-tumor growth in this experimental setting.

8.4 Psychoactivity and other side effects

Peritumoral injections of the substance HU210 were chosen based on former publications in order to circumvent problems with the lipohilicity of cannabinoids and even more importantly to avoid psychoactive effects. Several years ago, it was discovered that i.v. injection of cannabinoids in mice produced sedation, anti-nociception, catalepsy, and hypothermia (188). These are such typical effects of cannabinoids that they were even used to classifiy substances as cannabinoids in the early days of cannbinoid research although current understanding relates these actions mainly to CB1 stimulation. For medical use of cannabis-based substances,

some of these side effects are of course not bearable and solutions to circumvent this problem are warranted.

One approach is the local administration of drugs as is it was performed in our xenograft mouse model or as it is also used in the clinical trial phase I with late-stage glioma patients, where patients receive intra-cranial THC-injections (167). Because many groups conducting research on the anti-neoplastic actions of cannabinoids found not only CB1 but also CB2 to be implicated in the viability-reducing effects on cancer cells, current studies often focus on use of agonists with higher CB2 affinity such as WIN55,212-2 or JWH133. With this strategy, i.p. WIN55,212-2-treatment was successfully performed in melanoma or pancreatic tumor models (100, 107). Still, CB2 receptor expression on cancer cells is a prerequisite for this approach and thus the issue of CB1 targeting hasn't been resolved. Towards this aim, efforts are being undertaken to develop CB1 ligands which lack the capability to cross the blood-brain-barrier and are overt of any psychoactive effects.

Although there apparently are several strategies to prevent psychoactive effects, longterm-use of these substances could have physiological consequences which have so far not been possible to determine. Cannabinoind receptor agonists could potentially cause a shift in the regulation of immune responses through CB2 (53), whereas many other side effects can not be ruled out being mediated either through CB1 or CB2, among them influences on bone and adipose tissue formation as these systems are subject to regulation by cannabinoids as described earlier on. None of the possible mentioned implications is acutely life threatening and cannabinoid-induced side effects might be even better tolerated than those of standard chemotherapies taking into consideration the already established use of THC-analogous compounds Nabilone or Sativex as palliative agents during chemotherapy (152). However, treatment of children is in general more critical due to ongoing development and thus especially cannabinoids should be considered carefully as they could, even despite low affinity to CB1, affect cells in the brain, which express functional CB2 (189), and hence cause neuronal developmental disturbances.

Regarding this major drawback of cannabinoids as well as limited pro-apoptotic potential on aRMS tumors in comparison to other novel treatment strategies e.g. the

kinase inhibitor PKC412, targeting of CB1 in aRMS might have to endure various optimizations and should rather be envisaged in combinatorial therapeutic strategies than as a single compound approach.

8.5 Old and novel treatment options in aRMS

Current RMS treatment consists of chemotherapy, surgery, and radiation not really discriminating between the histological RMS subgroups. So far, these categories rather serve as prognostic markers, while treatment is based on the primary tumor location and disease stage (*C.Arndt, Book: Nelson Pediatrics*). RMS tumors are considered as highly chemo- and radiotherapy sensitive. Therefore, primary surgery is only indicated when the tumor can be resected without any microscopic residual tumor mass (*Buch: Krebs bei Kindern und Jugendlichen*). Cytostatic drugs used for RMS comprise Vincristine, Actinomycin D, Cyclophosphamide, Ifosfamide, Adriamycin, Epirubicin, Carboplatin, VP-16, Topotecan, and Irinotecan, of which also several combinations can be applied. Thanks to adjuvant medication allowing dose increases of all these unspecific agents, RMS survival rates could be enhanced over the last 30 years, but now side effects are limiting and novel therapeutic approaches give hope for further treatment improvement better tolerability.

Most of the currently investigated compounds or antibodies rely on interfering with molecules needed for carcinogenesis and tumor growth, among them receptors and kinases, rather than simply interfering with fast dividing cells – a concept named targeted therapy.

Also targeting of CB1 in translocation positive aRMS cancer cells follows this concept as the CB1 is up-regulated on these cancer cells and moreover cannabinoid receptor agonists only interfere with cell viability of cancer cells sparing healthy cells. Noteworthy is the fact that cancer cells go into cell cycle arrest or apoptosis after activation of cannabinoid receptors, which is in gross contrast to other novel therapeutic approaches which interfere with cancer cells by inhibiting signalling molecules. What impact this difference has in regard to development of drug resistance isn't clear yet. Nevertheless, former drug testing experiments in our lab on

Rh4 xenografts in immune-deificient mice have shown that the broad kinase-inhibitor PKC412 has a higher apopotosis-inducing capability than HU210 (51), indicating that cannabinoids might not be the most efficient compound towards novel treatment strategies in aRMS. Moreover, many other novel targeted approaches have been investigated to find better treatment options for RMS cases, as is summarized in the following table (Fig. 7;(16, 25, 39, 51, 165, 187, 190-218)).

Such approaches include interference with RMS cells by small molecules, antibodies, and silencing of certain genes. Although tested cell lines and read-outs for assessment of drug efficiency may differ between studies, these experiments indicate the existence of a whole array of promising new therapeutic strategies for RMS, while their clinical impact remains to be determined.

Until now, remarkable therapy improvements were obtained with Imatinib (BCR/ABL) against chronic myeloid leukaemia (CML) or Trastuzumab (ErbB2 antibody) for certain cases of breast cancer. On the other hand, rather disillusioning was the case of the EGFR inhibitor Gefitinib (also called Iressa), whose treatment success in non-small-cell-lung-cancer patients correlated with the presence of certain somatic EGFR kinase domain mutations in tumors (219). Furthermore, anti-angiogenic compounds also turned out to be only successful in combination with other chemotherapeutics.

The current information shows that solely targeting one mechanism of cancer cells might not always be efficient and depends on sometimes unknown molecular features such as mutations. Targeting malignant tumors at various levels by combining anti-neoplastic agents can help reducing dosis and thus side effects of involved compounds and furthermore decreases the development of drug resistant cells. Optimally, combination of drugs leads to synergism, as was also shown for co-treatment of colon cancer cells with 5-Fluorouracil and HU210 by Gustaffson, 2008 (150). Therefore, combining the targeted approach of cannabinoids with currently used chemotherapeutics, among them Vincristine or Actinomycin D, or also with novel approaches such as PKC412 could represent a promising treatment combination for translocation-positive RMS displaying high CB1 expression.

Target	Subtype specific	Compound / inhibition	Cell lines / model	Assay	Finding	Author / year
PDGFR-A	no	RNAi, imatinib, antibody	aRMS cell lines (hRh5, hRH30, primary cells)	v, cf, *in vivo* aRMS mouse model	viability ↓	Taniguchi E 2008
Midkine (MK) cytokine	no	RNAi	RD, Rh30	v	viability ↓	Jin Z 2008
Proteasome	no	Bortezomib	RD, Rh4 & Rh30	cf, a, x	viability ↓	Bersani F 2008
Proteasome	no	Bortezomib	Rh10, Rh30, Rh41, Rh28, Rh18, Rh36	v, x	viability ↓, tumor growth ↓	Houghton P 2008
MET	no	shRNA	RD, Rh30	v, cf, x	viability ↓, invasiveness ↓	Taulli R 2006
PDK-1	no	OSU-03012	RD, SMS-CTR, Rh3, Rh30, CW9019	v, a	viability ↓	Cen L 2007
mTOR/Hif-1a/VEGF	no	CCI-779 (rapamycin analogue)	RD, Rh30	x	anti-angiogenic	Wan X 2006
mTOR	no	Curcumin	Rh1, Rh30	v, cc, ac, m, signalling	growth arrest, apoptosis, motility ↓	Beevers CS 2006
mTOR	no	Rapamycin	Rh10, Rh28, Rh30, Rh41, Rh18	v, x	resistant *in vitro*, tumor growth↓, anti-angiogenic	Houghton P 2008
mTOR	no	CCI-779	Rh18	x	tumor growth↓	Dudkin L 2001
IGF-R1	no	Monoclonal antibody EM164	RD	v	IGF1-induced viability ↓	Maloney EK 2003
IGF-R1	no	Antibody	RD, Rh1, Rh4, Rh18, Rh28, Rh30, Rh36, RMS13, CTR	v, cf, x	viability↓, tumor growth↓	Cao L 2008
IGF-R1	no	IGF-R1 antibody	RD	v	tumor growth ↓	Maloney EK 2003
IGF-R1	no	SCH717454 monoclonal antibody	Rh18, Rh28, Rh30, Rh41	v, x	minor effect *in vitro*, tumor growth↓	Kolb E 2008
EGFR	eRMS	NVP-AEW541	Rh4, Rh30, RC2, RD/18, CCA	v, cc, a	cell cycle inhibition, apoptosis	Scotlandi k 2005
MYC-N	aRMS	Immunotoxin (saporin coupled to AB)	RD, Rh4, Rh30	v, protein synthesis, a	viability ↓, apoptosis	Ricci C 2002
PAX3/FKHR & EWS/FLI1, 2	aRMS	Mad/Myc inh. construct, ERK inhibitor U0162	RD	v, cf, cc	viability ↓, cell cycle inhibition	Marampon F 2006
PAX3/FKHR	aRMS	Immunotherapy	clinical study	disease progression, survival	Median 5year-OS ↑	Mackall C 2008
PAX3/FKHR	aRMS	PKC412 (broad spectrum kinase inhibitor)	Rh4, Rh30	v, a, x	viability ↓, apoptosis, tumor growth ↓	Amstutz R 2008
PAX3/FKHR	aRMS	Antisense oligos	Rh1, RD, Rh30	v	viability ↓, apoptosis	Bernasconi M 1996
Mirk/Dyrk1b	no	RNAi	RD, Rh30	a, cf	colony formation ↓, apoptosis	Mercer SE 2006
CTGF	no	RNAi	RD, CCA, RC2, Rh4, Rh30	v, a, d	apoptosis, myogenic differentiation ↓	Croci S 2004
HMG-CoA reductase	no	Lovastatin	SJRH,RD, A204, HS729T	v, cc	viability ↓	Dimitoulakos J 2001
Src	no	Dasatinib	Rh10, Rh28, Rh30, Rh41, Rh18	v, x	minor effect *in vitro* and *in vivo*	Kolb E 2008
Src	no	Dasatinib	RD, Rh18	m	migration ↓	Shor AC 2007
MEK/ERK	no	U0126	RD, Rh30	cc, d	viability ↓, differentiation in RD	Ciccarelli C 2005
VEGFR2/3, KIT, PDGFR, FLT3	no	Sunitinib	Rh10, Rh18, Rh28, Rh30, Rh41	v, x	minor effect *in vitro*, tumor growth↓	Maris J 2008
VEGFR	no	AZD2171	Rh18, Rh28, Rh30, Rh41	x, v	resistant *in vitro*, tumor growth ↓, anti-angiogenic	Maris J 2007
VEGFR2/EGFR	no	ZD6474	TE 617	x	delayed tumor growth	Rich J 2005
Survivin	no	shRNA	Rh30	x	tumor growth ↓	Caldas H 2006
Estrogen receptor β	no	Tamoxifen (4OHT)	Rh4, Rh18, Rh28, Rh30, RD	x, cf, a	viability ↓, apoptosis	Greenberg J 2008
Ornithine decarboxylase	no	Antisense oligonucleotides	RD	v, x	viability ↓, tumor growth ↓	Nakazawa K 2007
TGFβ/Smad4	no	Smad4 shRNA	RD	v, cc, a	viability ↓, growth arrest, apoptosis	Ye L 2006
Retinoic acid receptor	no	Retinoic acid (ATRA, CRA)	Rh3, Rh4, Rh5, Rh6, Rh7, RD, Rh18, Rh28, Rh30	v, cc, d	growth arrest, differentiation	Barlow J, 2006
Cdk4/6	no	PD0332991	Rh18, Rh28, Rh30, Rh41, RD, JR1	cc, d, x	cell cycle arrest, differentiation, tumor growth↓	Saab E 2006
Hsp90	no	Alvespimycin	RD, Rh18, Rh30, Rh41, Rh28	v, x	viability↓, tumor growth↓	Smith M, 2008
BH3	no	ABT-263 (BCL-inhibitor)	Rh18, Rh28, Rh30, Rh41	v, x	viability↓, xenograft growth of Rh30 and Rh41 ↓	Lock R 2008

Figure 10: Novel therapeutic approaches for RMS. (Abbreviations used as follows: a: apoptosis, cc: cell cycle, cf: colony formation, d: differentiation, m: migration, v: viability, x: xenograft)

8.6 CBs in cancer: Proto-oncogenes or tumor suppressors?

Despite all the efforts undertaken to elucidate the anti-tumoral actions of cannabinoids, an emerging question remains: Why do cancer cells display receptors such as CB1 or CB2, which upon activation reduce their survival? The physiological role of cannabinoid receptors and the endocannababinoid system in cancer remains still to be explained. Certain reports suggest loss of CB1 (60) as well as reduction of the endocannabinoid tone to be involved in the development of colon cancer (61), pointing at a tumor-suppressive role of CB1 and its activating ligands. Controversly, others have claimed that hematopoietic precursor cells with high levels of CB2 possess increased susceptibility for leukaemia development (123) indicating an oncogenic potential of CB2. In translocation-positive RMS cells the case might yet be different as CB1 probably is upregulated as a bystander effect of the oncogenic PAX3/FKHR, which has been shown to activate the promoter region of CB1 (49). Anyway, to answer this complex question, further attempts will have to be undertaken by studying cancer cells with altered cannabinoid receptor levels. Lack of any publication using the before mentioned molecular biological tools reflects probably the complexity of the addressed question. Additionally, studies on biopsy samples of different tumor stages as well as analysis of pre-malignant samples could be more relevant in regard to elucidation of the role of cannabinoid receptors and the endocannabionid system in the formation and maintenance of certain cancer types.

However, shortage of aRMS patients and samples as well as no appropriate translocation-positive mouse model might limit such studies in the case of aRMS. Findings concerning the role of CB1 obtained from other cancer types will probably have to be validated on histological sections or cell lines deriving from translocation-positive aRMS. Probably, future knowledge demands also other read-outs than viability or apoptosis assays to verify effects of cannabinoids on aRMS and other cancer cells, which could potentially occur at much lower concentration levels than the ones used to induce apoptosis.

8.7 Perspectives of cannabinoids as therapeutic approach for aRMS

In summary, the project of this thesis could show the potential of different cannabinoid receptor agonists to induce apoptosis in translocation-positive RMS cells through the depicted signalling mechanism (Fig. 9). The tumor growth-inhibiting action of the compound HU210 was verified *in vivo* in a xenograft mouse model and thus cannabinoids should be further pursued as novel therapeutic approach. However, better understanding of the underlying anti-tumoral mechanisms as well as refined knowledge about their side effects, which can be gained during the first ongoing clinical trial in glioma patients, are needed before any further attempts to induce cannabinoids in the clinics of aRMS therapy can be envisaged.

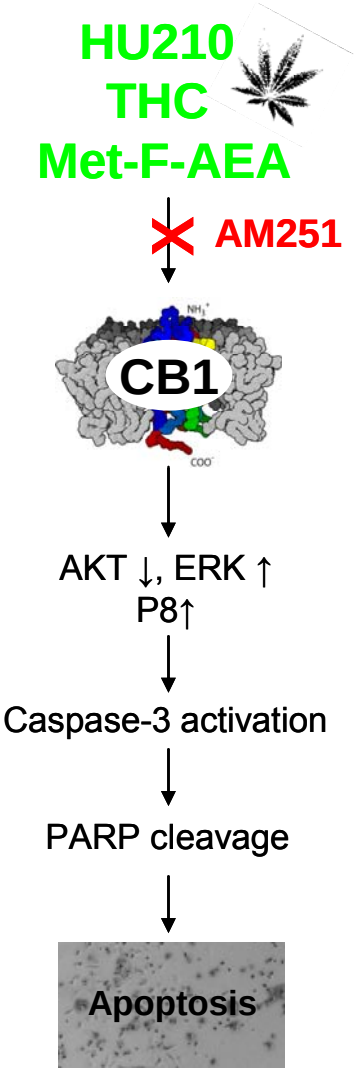

Figure 11: Proposed pro-apoptotic signalling in aRMS cells after cannabinoid challenge

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10 Curriculum Vitae

Susanne Oesch

Date of birth:	21.4.1980
Nationality:	Swiss
Postal address:	Magnusstr. 5
	8004 Zürich
Telefone number:	078/876 65 85
e-mail address:	S.Oesch@gmx.net

Education

03/2005 – 01/2009	ETH Zürich PhD studies in Biology External dissertation at the University Children's Hospital
10/2000 – 10/2004	ETH Zürich Studies in biology with the following subjects: Neurobiology, pharmacology, cell biology, immunology
09/2003 – 06/2004	University Zürich, division of psychiatric research Diploma thesis about Alzheimer's Disease
09/2002 – 03/2003	Imperial College London, ERASMUS-exchange semester
09/1993 – 06/1999	Gymnasium Einsiedeln, humanistic type B

Publications and Presentations

Susanne Oesch, Dagmar Walter, Marco Wachtel, Kathya Prêtre, Maria Salazar, Manuel Guzmán, Guillermo Velasco, Beat W. Schäfer:
CB1 is a potential drug target for treatment of translocation-positive rhabdomyosarcoma (Submitted to Molecular Cancer Therapeutics)

Susanne Oesch, Jürg Gertsch:
Cannabinoid Receptor Ligands as Potential Anti-Cancer Agents – High Hopes for New Therapies? (Review)

Susanne Oesch, Beat W. Schäfer:
Investigation of CB1 as a potential drug target for alveolar rhabdomyosarcoma
Poster presentation at the ICRS meeting 2008 in Scotland

Susanne Oesch, Beat W. Schäfer:
Investigation of CB1 as a potential drug target for alveolar rhabdomyosarcoma
Poster presentation at the D-BIOL symposium 2008 in Davos

Susanne Oesch, Beat W. Schäfer:
Investigation of CB1 as a potential drug target for alveolar rhabdomyosarcoma
Poster presentation at the Cancer Network Retreat 2008 in Fiesch

Susanne Oesch, Beat W. Schäfer:
Investigation of CB1 as a potential drug target for alveolar rhabdomyosarcoma
Poster presentation at the CNIO meeting 2007 in Madrid, Spain

Susanne Oesch, Beat W. Schäfer:
Investigation of CB1 as a potential drug target for alveolar rhabdomyosarcoma
Poster presentation at the Cancer Network Retreat 2006 in Ascona

Susanne Oesch, Beat W. Schäfer:
Investigation of CB1 as a potential drug target for alveolar rhabdomyosarcoma
Poster presentation at the ICRS meeting 2006 in Hungary

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