Dissertation

Cannabinoids as a Potential Novel Therapeutic Approach to Metastatic Melanoma

submitted by

Dr. med. univ. Georg RICHTIG

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Prof. Dr. Akos HEINEMANN

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Statutory Declaration

"I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Guidelines of the Medical University of Graz on Good Scientific Practice".

Georg Richtig
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Disclosures

This doctoral thesis was the basis for the preparation of a manuscript, which has been published under the following title and journal:

Cannabinoids Reduce Melanoma Cell Viability and Do Not Interfere with Commonly Used Targeted Therapy in Metastatic Melanoma In Vivo and In Vitro

Georg Richtig[1,2], Melanie Kienzl[1,3], Sonja Rittchen[1], David Roula[1], Jürgen Eberle[4], Zina Sarif[4], Martin Pichler[2], Gerald Hoefler[5], Akos Heinemann[1]

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[1] Otto-Loewi Research Center for Vascular Biology, Immunology and Inflammation, Division of Pharmacology, Medical University of Graz, 8010 Graz, Austria.

[2] Division of Oncology, Department of Internal Medicine, Medical University of Graz, 8036 Graz, Austria.

[3] BioTechMed-Graz, 8010 Graz, Austria.

[4] Department of Dermatology, Venereology and Allergology, Skin Cancer Center Charité, Charité-Universitätsmedizin Berlin (University Medical Center Charité), 10117 Berlin, Germany.

[5] Diagnostic and Research Institute of Pathology, Medical University of Graz, 8036 Graz, Austria.

this doctoral thesis, which is the reason why significant parts of this doctoral thesis are similar to the manuscript. All co-authors of the manuscript agreed to the reproduction of data and images in this dissertation.

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Abbreviations and Definitions

2-AG	2-arachidonoylglycerol
5-HT_3	5-hydroxytryptamine
BRAF	B-Raf proto-oncogene, serine/threonine kinase
CBD	Cannabidiol
CBN	cannabinol
CDKN2A	cyclin dependent kinase inhibitor 2A
CML	chronic myelogenous leukemia
COSMIC	catalogue of somatic mutations in cancer
CTLA-4	cytotoxic T-lymphocyte–associated antigen 4
GBM	glioblastoma multiforme
GPR	G protein-coupled receptor
IRB	International Review Board
KIT	KIT proto-oncogene, receptor tyrosine kinase
MiS	melanoma in situ
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
mTORC1	mammalian target of rapamycin complex 1
NCI	National Cancer Institute
NF1	neurofibromin 1
NRAS	NRAS proto-oncogene, GTPase
PBMC	Peripheral Blood Mononuclear Cells
PD-1	programmed cell death-1
PD-L1	programmed cell death ligand-1
PFS	progression free survival
PI3K	phosphoinositide 3-kinase
PLCε	phospholipase C-ε
PPAR	peroxisome proliferator-activated receptors
PTEN	phosphatase and tensin homolog
QoL	Quality of Life

RCT	randomized controlled trial
RTK	receptor tyrosine kinase
SD	standard deviation
TERT	telomerase reverse transcriptase
THC	tetrahydrocannabinol
TIAM1	TIAM Rac1 associated GEF 1
TMZ	temozolomide
TP53	tumor protein p53
TRB3	tribbles homolog 3
TRPV1	transient receptor potential cation channel subfamily V member 1
UV	ultraviolet
VEGF	vascular endothelial growth factor

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Abstract in German

Hintergrund: Cannabinoide werden hauptsächlich als Freizeitdroge eingesetzt, haben aber auch ihren Einzug in die Onkologie gefunden, wo sie hauptsächlich als Appetitanreger bei Tumorkachexie verwendet werden. Nichtsdestotrotz gibt es in der Literatur Hinweise, dass Cannabinoide auch eine gewisse krebshemmende Wirkung haben könnten. Aus diesem Grund wird es auch von Patientinnen und Patienten genommen, die eine weit fortgeschrittene Krebserkrankung haben, wobei diese Art der Wirkung noch nicht ausreichend untersucht ist.

Zielsetzungen: Die Zielsetzung dieser Arbeit ist es den Mechanismus, ob und wie Cannabinoide einen pro-apoptotischen Effekt beim metastasierten Melanom haben, in-vivo und in-vitro zu untersuchen. Zusätzlich soll die Wirkung von Cannabinoiden im Vergleich zu einer zielgerichteten Therapie in-vivo untersucht werden.

Methoden: Mehrere Melanom-Zelllinien wurden mit unterschiedlichen Cannabinoid-Konzentrationen behandelt, und die krebshemmende Wirkung wurde durch Proliferations- und Apoptose-Tests untersucht. Die anschließende Analyse der Signalwege erfolgte mittels Durchflusszytometrie, Immunhistochemie, Zytokin-Array und konfokaler Mikroskopie. Die Wirksamkeit von Cannabinoiden wurde in Kombination mit dem klinisch verwendete MEK-Inhibitor Trametinib in NSG-Mäusen in-vivo untersucht.

Ergebnisse: Cannabinoide verringerten die Lebensfähigkeit von mehreren Melanom-Zelllinien in dosisabhängiger Weise. Die Wirkung wurde durch CB1-, TRPV1- und PPARα-Rezeptoren vermittelt, wobei die pharmakologische Blockade aller drei Rezeptoren vor der Cannabinoid-induzierten Apoptose schützte. Cannabinoide leiteten die Apoptose durch mitochondriale Cytochrom-c-Freisetzung mit anschließender Aktivierung verschiedener Caspasen ein. Im Wesentlichen verringerten Cannabinoide das Tumorwachstum in vivo erheblich und waren ebenso wirksam wie der klinisch verwendete MEK-Inhibitor Trametinib.

Conclusio: Cannabinoide zeigten pro-apoptotische Effekte in-vitro und in-vivo.

Abstract in English

Background: In the private sphere, cannabis is mainly used for recreational purpose, whereas in the clinical field it is used as an appetite stimulant in cachectic cancer patients. Patients usually take cannabis on the belief that it has an anti-tumor effect, which is also claimed in the literature.

Objectives: The purpose of this study was to elucidate if and how cannabinoids mediate pro-apoptotic effects in melanoma in-vitro and in-vivo and its value in comparison with conventional targeted therapy in-vivo.

Methods: Different melanoma cell lines were treated with different concentrations of cannabinoids. Anti-cancer efficacy was assessed by apoptosis and proliferation assays. Pathway analysis was performed using proliferation, apoptosis, immunohistochemistry, flow cytometry, confocal microscopy and cytokine array data. In-vivo efficacy of cannabinoids in combination with trametinib was studied using NSG mice.

Results: In multiple melanoma cell lines cannabinoids reduced cell viability in a dose-dependent manner. CB1, TRPV1 and PPARα receptors played a central role in mediating this effect. Cannabinoid-induced apoptosis was reduced by simultaneous pharmacological blockade of all three receptors. Apoptosis was mediated by activation of different caspases and mitochondrial cytochrome c release. In-vivo, cannabinoids significantly decreased tumour growth and were as potent as the clinically used MEK inhibitor trametinib.

Conclusions: These data demonstrate that cannabinoids reduce cell viability in several different melanoma cell lines. This is achieved by initiating apoptosis via the intrinsic apoptotic pathway with further activation of caspase and mitochondrial cytochrome c release. Importantly, it does not interfere with the commonly used MEK inhibitor trametinib.

Introduction

Melanocytes are pigment-producing cells in the skin, which are derived from neural crest cells.[1] They are located at the basal layer of the epidermis but can also be found in the hair bulb, eyes, ears, mucosal tissue, and meninges. During embryonic development, multipotent trunk neural crest cells migrate from the neural plate to the epidermis and dermis, which is also the reason why melanoma can occur nearly everywhere in the body.[2,3]

The main function of epidermal melanocytes is the contribution to photoprotection and thermoregulation by packaging melanin pigment into melanosomes and delivering them to neighboring keratinocytes.[4]

Melanoma

Melanoma incidence is rising worldwide and, despite all efforts, metastatic disease in melanoma is still a huge problem.[5] The minority of skin cancer cases are melanoma-associated but melanoma is responsible for the majority of skin cancer-related deaths.[6] Prognosis depends on tumor thickness, ulceration and metastatic spread decreasing 10-year survival from 88% to 24%.[7] The most common mutation suitable for targeted therapy is in the B-Raf proto-oncogene, serine/threonine kinase (BRAF) gene with a frequency of approximately 40 - 60% of all melanomas (Detailed in **Figure 1**).[8,9] In up to 95% of all cases the mutation occurs at codon 600 of the *BRAF* gene where the most common exchange is a valine to glutamic acid referred to as $BRAF^{V600E}$ mutation.[10]

Mutation of *BRAF* leads - in most cases - to an over-activation in the RAF-MEK-ERK signaling pathway (MAPK pathway) resulting in enhanced cell growth and survival.[11] However, not all *BRAF* missense mutations lead to increased BRAF kinase activity and the mutations especially in the p-loop of the BRAF kinase – encoded by exon 11 of the *BRAF* gene - tend to a have a lower kinase activity then the wild-type, BRAF kinase.[12] The clinical implication of such mutations are still unclear and under investigation. Besides *BRAF* the second most common mutation found in melanoma is affecting the NRAS proto-oncogene, GTPase (NRAS) gene with a frequency of ~20%.[9,13] However, recent studies revealed more mutations involved in the growth and progression of malignant melanoma, e.g. *GNA11/GNAQ, c-KIT, TERT, NTRK, CDKN2A*, *TP53* and others.[14–16]

Oncogenic signal transduction in melanoma

Two major pathways of clinical interest are the PI3K/PTEN/Akt/mTOR signaling pathway and the Ras/Raf/MEK/ERK signal transduction cascade (MAPK signaling pathway).[17]

The classic pathway is that RAS activates RAF, which propagates down-stream signaling through MEK to ERK. There are three RAF isoforms: ARAF, BRAF and CRAF/Raf1, whereas BRAF is one of the major targets of therapy in metastatic melanoma today.[18] The most common mutation in the BRAF gene ($BRAF^{V600E}$) has a 500 fold higher kinase activity than the $BRAF^{wt}$ kinase.[12] Therefore, drugs targeting this mutation have been developed with overwhelming success. However, nearly all patients treated with BRAF inhibitors develop resistance to those. A possible mechanism discussed is a consecutive mutation in the *RAS* gene, when a BRAF inhibitor is used.[19]

The RAS gene family is the most commonly mutated oncogene in human cancers (20 - 30%), whereof the most common and investigated isoforms are *NRAS, KRAS* (pancreatic cancer, large intestine, peritoneum, biliary tract)[20–22] and *HRAS* (skin, salivary gland, urinary tract, cervix).[21,23] In addition, there are also less investigated isoforms like *RRAS* and *MRAS*.[24,25] In melanoma the most common mutated *RAS* isoform is *NRAS* with a mutation rate between 15% and 20%.[26] The most common codons affected are codon 61 and codon 12, whereas the most common alteration is a change in Q61R (Data obtained from the catalogue of somatic mutations in cancer (COSMIC) at http://cancer.sanger.ac.uk/cosmic; accessed April 2016).[27,28]

The glycine at position 61 of the NRAS protein has a prominent role, since it stabilizes the hydrolysis of GTP to GDP. Oncogenic mutations in this position lead to a reduced intrinsic GTP hydrolysis rate and, therefore, a constitutive activation of NRAS.[25] This causes actions and alterations in cell cycle impairing actin cytoskeletal organization, cell polarity, cell movement and therefore enhancing cell growth and cell survival.[29,30]

Due to the downstream signaling to the MAPK pathway, a possible therapeutic intervention in *NRAS* mutated melanoma is the inhibition of this pathway with a MEK-inhibitor.[26] It has been shown that response to MEK inhibitors is variable, suggesting that NRAS signals not solely to the MAPK pathway and there might be some escape mechanism.[31] Indeed, many pathways, other than the MAPK pathway such as phosphoinositide 3-kinase (PI3K)[32], phospholipase C-ε

(PLCε)[33], T-cell lymphoma invasion and metastasis-1 (TIAM1)[34], and others are involved, all being responsible for proliferation and growth.[35] Interestingly, the resistance to BRAF inhibitors goes along with an increase in ERK levels, suggesting that the MAPK pathway is activated by a different protein than BRAF and indeed CRAF is held responsible for this effect.[36]

Like BRAF, CRAF activates the MAPK pathway over MEK and ERK.[37] However, MEK activated by CRAF is less susceptible to MEK inhibitors than when being activated by BRAF.[38] Apart from this, CRAF also has several other functions, including an anti-apoptotic effect mediated through binding to Bcl-2 at the mitochondrial membrane.[39] It links directly to Bcl-2 and phosphorylates BAD which leads to reduced apoptosis and more robust cancer cells.[40]

Genetic evolution of melanoma

From an evolutionary perspective, mutations play an important role since they drive cancer growth and progression. From a clinical perspective, mutations (or mutational pattern) can be targeted by therapy or used as a progression and risk marker. Importantly, melanoma is – apart from other tumors with a mismatch repair deficiency - the tumor with the highest mutational burden.[41] However, melanoma can originate in different organs and tissues. The most important sub-types are uveal melanoma, mucosal melanoma and cutaneous melanoma. Importantly, all three sub-types are genetically completely different.[42] In cutaneous melanoma, ultraviolet (UV) radiation is - on a genetic basis - the most important risk factor and responsible for the mutational burden.[43] This is due to the cancerogenic effects of UV radiation and draws a distinctive mutational pattern whereas risk factors associated with cancer can be identified on a genetic basis (including tobacco smoking for various cancer types).[44] However, cutaneous melanoma located on the head, neck and upper extremities are associated with a higher mutational burden than melanomas located on the trunk or lower extremities.[43]

In line with high mutational burden, it has been a genetic challenge to identify clinically relevant mutations as compared to by-stander-mutations to provide clinical targets. In melanoma the most commonly mutated gene with the highest clinical attention has been *BRAF*. This was due to the fact that the majority of *BRAF* mutations occurred on a distinct molecular pattern where valine is exchanged against glutamic acid ($BRAF^{V600E}$).[12] Although it has been shown that this mutation occurs at high frequency in metastatic melanoma, it was unclear if *BRAF* mutations happen at an

early stage or are a late-phase mutation. This question was addressed by Wu et al. who could demonstrate that *BRAF*^{V600E} mutations are highly frequent in moles.[45] Therefore, a *BRAF*^{V600E} mutation itself is neither a marker for melanoma nor for metastatic melanoma. This has been clinically shown in patients under BRAF inhibitor therapy: The moles of a patient with stage IV melanoma under BRAF inhibitor therapy showed changes in color, size and pattern suggesting that the BRAF inhibitor also has an effect on moles.[46] Interestingly, the *BRAF*^{V600E} mutation was far more common in nevi than in melanoma itself suggesting that the loss of *BRAF*^{V600E} mutation can lead to progression or that *BRAF*^{V600E} mutation is not necessarily strong enough to promote progression and that it needs further co-driver mutations.[47,48] Biologically, ~20% of melanoma develop from pre-existing nevi and in melanoma this part is still present in the primary tumor.[49] Therefore, it was a unique opportunity – at least in this subgroup of tumors - to sequence (histologically) different parts of melanoma to get a deeper insight into melanoma development and progression. It was shown that there are some key mutations occurring in the course of developing to a metastatic phenotype. *BRAF*^{V600E} mutations were an early sign and frequently present in benign lesions. In the further course, telomerase reverse transcriptase (TERT) promoter mutations with a preference for specific hotspots[50,51] were observed and these mutations were present in melanoma and histopathological/dermatoscopic conspicuous lesions but not in nevi. In line with this were co-occurring cyclin dependent kinase inhibitor 2A (CDKN2A) mutations, mainly deletions with a preference on germline level rather than somatic level.[52,53] Together with *TERT* promotor mutations in intermediate lesions, *NRAS* mutations were commonly found whereas the frequency of *NRAS* mutations increased from intermediate to invasive melanomas. In melanoma, from melanoma in situ (MiS) to invasive melanoma, the second important pathway that is commonly mutated, is the PI3K pathway, where mutations in neurofibromin 1 (*NF1*) and mutations in the phosphoinositide 3-kinases (*PI3K*) and their negative regulatory element phosphatase and tensin homolog (*PTEN*) are commonly found. Finally, *TP53* mutations were frequently found in melanoma metastases and T4 melanoma, although the role and prognostic value of tumor protein P53 (*TP53*) mutations are highly controversial in the literature.[54–56]

Pharmacological therapeutic options

It has been suggested that, cutaneous melanoma can be classified into four different subgroups: *BRAF*^{mt}, *NRAS*^{mt}, *NF1*^{mt} or triple wild type.[57] A different classification was suggested for mucosal

melanoma: *c-kit*^{mt} and wild-type.[57] Due to this classification, patients with metastatic melanoma have three pharmacological therapeutic options: Immune checkpoint inhibitors including PD-1/PD-L1/CTLA-4/LAG-3, conventional chemotherapy or targeted therapy including BRAF- and MEK-inhibitors.[58–62]

Targeted therapy

<u>BRAF mutated melanoma</u>

In Austria three BRAF inhibitors have been approved for systemic treatment in metastatic melanoma and are currently widely used: Vemurafenib (Zelboraf®, Roche), Dabrafenib (Tafinlar®, Novartis) and Encorafenib (Braftovi®, Pierre Fabre).[63–65] In the BRIM-3 study it could be shown that patients harboring a melanoma with a *BRAF*^{V600E} mutation had a response rate to vemurafenib of 48% compared to conventional chemotherapy (dacarbazine, DTIC) with 5%.[61]

Furthermore, systemic treatment with vemurafenib led to an improved median overall survival of 13.3 months compared with 10 months for the dacarbazine group and a median progression-free survival (PFS) of 6.9 month compared with 1.6 months in the dacarbazine group proving the success of targeted therapy in metastatic melanoma.[66]

In line with this result were clinical trials using other BRAF inhibitors including dabrafenib and encorafenib.[67,68] However, one major problem of targeted therapy is that patients develop resistance against the BRAF inhibitors by the emergence of receptor tyrosine kinase (RTK) mutations.[19]

A common side effect of BRAF inhibitors is the occurrence of new *BRAF*^{wt}*/RAS*^{mt} and *BRAF*^{wt}*/NRAS*^{wt} melanoma, which is explained by a paradoxical activation of the MAPK pathway.[69,70]

Since there were some hints from *in-vitro* experiments that *NRAS*^{mt} melanoma might be sensitive to MEK inhibition, Ascierto and colleagues conducted a phase II study where they used a MEK1/2 inhibitor (MEK162) in *NRAS*^{mt} melanoma patients. They could show that MEK inhibition might be an alternative in NRAS^{mt} melanoma and in BRAF^{V600E} mutated melanoma.[71] Furthermore, Abdel-Wahab *et al.* could show in a patient suffering from *NRAS*^{G12R} chronic

myelogenous leukemia (CML) and *BRAF*^{V600K} melanoma that a dual inhibition (initially the BRAF inhibitor for the melanoma and the MEK inhibitor for the CML) nearly led to a complete response (with no evidence of any upcoming resistance) in melanoma and a mitigation of the CML.[72] Along with studies by Flaherty *et al.* and Ribas *et al.* this led to large clinical trials testing dual inhibition of the MAPK pathway in advanced melanoma.[73–75] The coBRIM trial (vemurafenib and cobimetinib vs vemurafenib and placebo) could confirm prior results showing a higher median progression-free survival (9.9 months vs 6.2 months), higher 9-month survival (81% vs 73%) and higher response rates.[76] Similar results could be achieved when another combination of BRAF (dabrafenib) and MEK inhibitors (trametinib) was used.[77] Dual MAPK pathway inhibition is now first line therapy in direct competition to checkpoint inhibitor therapy even though there are now clinical trials investigating the chances of combining targeted therapy with checkpoint inhibitor therapy.

NRAS mutated melanoma

NRAS is mutated in up to 20% of all melanoma cases where the most common form is a mutation at codon 61, followed by codons 12 and 13.[26] Since NRAS is upstream of BRAF in the MAPK pathway and since it is known that BRAF inhibitors lead to enhanced growth in *BRAF*^{wt} melanoma, MEK inhibitors are tested in clinical trials in *NRAS*^{mt} patients.[70] Response rate to MEK inhibitors was variable, suggesting that NRAS not solely acts on the MAPK pathway and there might be some escape mechanism, or that the MAPK pathway is not exclusively responsible for oncogenic growth in this subset of melanomas.[71]

c-kit mutated melanoma

The most common mutation in mucosal and acral melanoma can be found in the KIT proto-oncogene, receptor tyrosine kinase (*c-KIT*).[78] Additionally, *KIT* is also commonly mutated in melanomas with signs of chronically sun damaged skin.[78] Functionally, *KIT* acts as a *bona fide* oncogene leading to higher cell proliferation and cell migration.[79,80] This is achieved by activation of the PI3K/mTOR- and the MAPK pathway.[79,80] Imatinib – a receptor tyrosine kinase inhibitor mainly known for its role in the treatment of the *BCR-ABL* fusion gene in CML – demonstrated high response rates among patients with exon 11 or 13 *KIT* mutated melanoma.[81,82] Furthermore, imatinib shows anti-tumorous effects in *KIT* exon 9, 11, 13, and 17-mutated GIST tumors.[83]

In addition, nilotinib, a tyrosine kinase inhibitor used in imatinib-resistant CML, gave promising results in the treatment of KIT mutated metastatic melanoma just as sunitinib.[84,85] However, it has to be mentioned that this is confined to a subset of an already small subpopulation of patients.

Resistance upon target therapy

In cancer therapy, resistance upon targeted therapy is divided in two mechanisms: intrinsic (primary) resistance and acquired (secondary) resistance.[86] Intrinsic resistance is characterized by the fact that tumors harboring a $BRAF^{V600E}$ mutation – and therefore theoretically sensitive to BRAF/MEK inhibition – do not respond to BRAF/MEK inhibitor therapy. Secondary resistance occurs in patient with a positive response to therapy with consecutive relapse.[87] Due to high tumor heterogeneity and mutational burden in melanoma, it is highly likely that patients experience recurrence upon targeted therapy. So far, only the MAPK pathway can be treated by targeted therapy. This can be done by BRAF- and MEK-inhibitor therapy. Interestingly, BRAF inhibition can kill $BRAF^{V600E}$ mutated melanoma cells but surprisingly, patients under BRAF inhibitor therapy experiences *de novo* occurrences of squamous cell carcinomas, keratoacanthomas and $BRAF^{wt}$ melanomas. It was observed that melanomas under BRAF inhibitor therapy are more likely to heterodimerize with CRAF, which lead to an increase in ERK phosphorylation and consecutive MAPK pathway reactivation.[88] This problem has been solved by adding a MEK inhibitor to BRAF inhibitor therapy (dual MAPK pathway inhibition). With this strategy the occurrence of carcinogenic side effects could be significantly reduced.[65,73] Dual-MAPK pathway inhibition led to high response rates (~95%) demonstrating that there is only a minority of patients not responding to this strong MAPK pathway inhibition if a $BRAF^{V600E}$ mutation has been found.

In contrast, despite impressive response rates only 39% patients survived five years after dual MAPK pathway inhibition.[89] Major efforts were made to identify possible resistance mechanism in these patients and it has been shown that there are some newly identified mechanisms: It seems that the MAPK pathway is of major importance for melanoma growth since the majority of resistance mechanisms interfere in one way or another with this pathway. Mutations have been detected supporting cells in being resistant to dual MAPK pathway inhibition including mutations in *MAP2K1/MAP2K2, NF1, RAS* (mainly *NRAS*) and $BRAF^{non\text{-}V600}$ mutations.[90–92] A second very important pathway that is upregulated in resistant melanoma is the PI3K pathway. Traditionally,

this signaling pathway is one of the most important escape routes when the MAPK signaling pathway is therapeutically blocked, and is associated with mutations in upstream RTKs, PI3Ks and *AKT* and loss of function mutations in negative regulators including *PTEN*.[90,93,94] Several clinical studies try to combine MAPK pathway inhibition with inhibitors of the PI3K pathway in any form.[95–97]

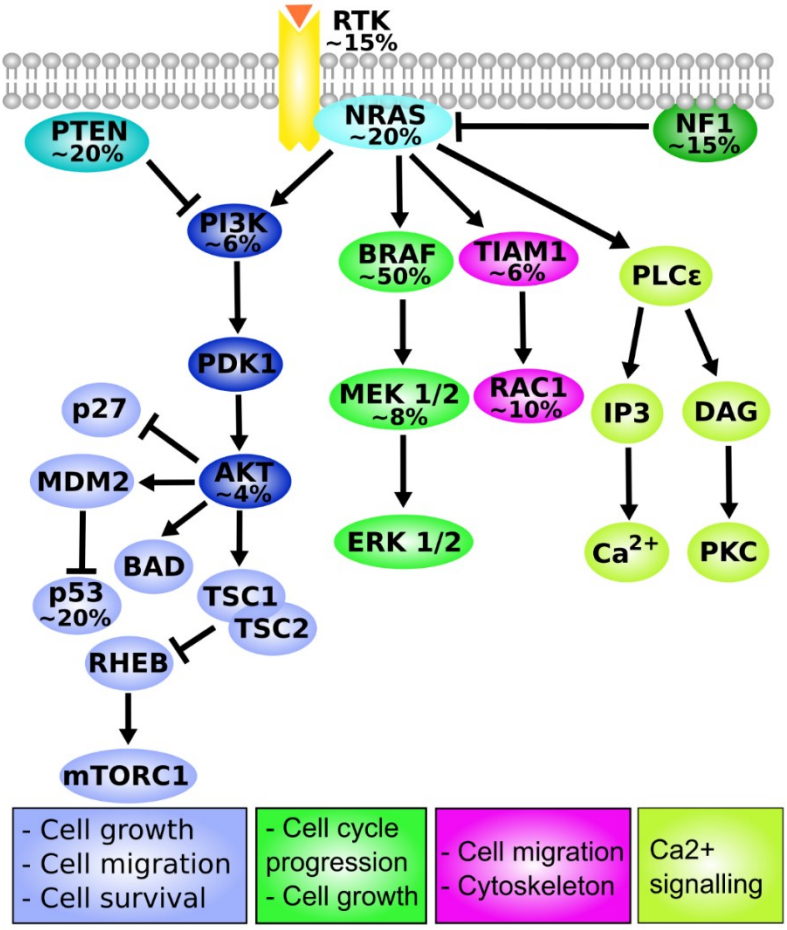

Figure 1. Important signaling pathways in melanoma including mutations rate.

The Receptor Tyrosine Kinase (RTK) is activated with down-stream activation of NRAS. NRAS activates two major pathways: The Map-Kinase pathway with BRAF – MEK – ERK and the PI3K Pathway with PDK1 and AKT. Both pathways play a crucial role in cell survival and cell cycle progression. Percent numbers indicate the proportion of somatic mutations in melanoma in the

specific gene. Reproduction of the figure with permission of Oxford University Press from Richtig et al [98]*. (Figure 1 corresponds to Figure 3 of the original publication by Richtig et al* [98]*).*

Immunotherapy

Nowadays, a second important pharmacological option for patients with metastatic melanoma is immune checkpoint inhibitor therapy. Boosting the immune system in the treatment of metastatic melanoma has a long history, but showed nearly no success and was, therefore, only suitable for a minority of patients.[99] The breakthrough came in 2011 when ipilimumab became the first immunotherapy to be approved for the treatment of metastatic melanoma by the U.S. Food and Drug Administration.[100] Ipilimumab is a fully human, IgG1 monoclonal antibody that binds to cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) resulting in increased T-cell activation and proliferation.[101] In comparison to the former used chemotherapeutic dacarbazine, ipilimumab could significantly improve overall survival in patients with advanced melanoma[100], but more importantly it could be shown that some patients demonstrated long-term survival.[102] Tremelimumab, another CTLA-4 mAb, showed similar efficacy and overall survival although has never been approved for clinical used.[103]

However, one of the major disadvantages of CTLA-4 mAb was the low response rate of ~20% due to many mechanism of immune evasion existing in cancer. One of this mechanism was the expression of B7-H1 on the surface of melanoma cells which was later described – due to its mode of action - as programmed cell death ligand-1 (PD-L1).[104] It could be demonstrated that PD-L1 was able to initiate apoptosis in activated T-cells *in vivo,* leading to a reduced immune response. When PD-L1 was blocked the immune response was restored showing a significant reduction of the tumor size.[104,105]

Therefore, soon after the development of ipilimumab, nivolumab - a mAb against programmed cell death-1 (PD-1) - was tested in clinical studies showing extraordinary results.[106] In 2012 nivolumab was tested in a large clinical trial and showed a response rate of 28% in melanoma patients.[107] The response rate could be raised up to 53% when nivolumab was combined with ipilimumab (against 10 to 20% when ipilimumab was given as a monotherapy).[108] The increase

in the rate of adverse effects was classified by the authors as manageable.[109] Lambrolizumab and pembrolizumab, other antibodies against PD-1, showed similar results.[60,110]

Apart from CTLA-4 and PD-L1 blockade Relatlimab – a LAG-3 mAB - is now another important clinically tested therapeutic option for stage-4 melanoma patients.[111]

However, immunotherapy is now a major approach to anti-cancer treatment and is evolving at high speed, with new substances targeting Tim-3, OX40, cancer vaccines[112] and CD73 are now being widely tested.[113,114]

The cannabinoid system

Cannabis is the most commonly used illicit drug worldwide in adults[115], while a number of people are consuming cannabis for medical purposes already on a legal basis.[116–118] So far cannabis formulas are used for patients suffering from nausea, vomiting and cachexia triggered by chemotherapeutics and as an additive medication for chronic pain relief.[119,120]

Cannabis (British) and Marijuana (American), also known as herbal cannabis, is obtained from the plant Cannabis sativa and from some subspecies. Around 60 cannabinoids can be found in the plant, whereby most of the research has been performed on Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN).[121] Beside these cannabinoids, the plant also contains approximately 420 other chemical components.[122] From all cannabinoids, THC is responsible for the psychotic effects of cannabis and was, therefore, the main target of research.

To date, two subtypes of cannabinoid G-coupled receptors, CB_1 (cloned in 1990) and CB_2 (cloned in 1993) could be identified and fully characterized.[123–125] Within the cell, CB_1 can signal through G_i inhibiting adenylate cyclase, G_s stimulating adenylate cyclase, G_q activating the phospholipase C, and G_o regulating Ca^{2+} and K^+ currents.[126,127] Furthermore, CB_1 can also interact with non-G protein partners like the adaptor protein FAN.

Nevertheless, cannabinoids are capable of binding multiple receptors apart from the traditionally cannabinoid-receptors. It has been shown that cannabinoids can interact with the G protein-coupled receptor (GPR) 55 and GPR35 and further with the peroxisome proliferator-activated receptors (PPAR) α and PPARγ and the transient receptor potential vannilloid-1 (TRPV1) channels..[128] Furthermore, it could be shown that cannabinoids could block 5-hydroxytryptamine

(5-HT_3) and nicotinic acetylcholine receptors.[129,130] The CB_1 receptor in the brain occurs mainly in the hippocampus, substantia nigra, prefrontal cortex and the basal ganglia. However, low to mid density of CB_1 could also be found in the thalamus, amygdala, area postrema and hypothalamus.[131,132] Using a selective CB_1-antagonist (SR141716), the psychoactive effects of THC in the brain could be completely prevented.[133] Outside the brain, the CB_1 receptor is expressed in the testis[134], hepatocytes[135100], cardiomyocytes[136], adipocytes[137] and in the adrenal cortex[138]. CB_1 receptors occur also on immune cells (monocytes, NK-cells, B-cells)[139], but at lower abundance than the CB_2 receptor and, therefore, the CB_2 receptor is often called the peripheral immunological cannabinoid receptor. The CB_2 receptor is expressed by all hematopoietic cells.[140] The order of expression levels on particular immune cell is as follows: B-cells > NK-cells > macrophages > $CD8^+$ T-cells > $CD4^+$ T-cells.[141]

Cannabinoids and cancer

In the literature there is growing evidence – especially since the 90s – that cannabinoids (including THC, endocannabinoids like 2-arachidonoylglycerol (2-AG) and anandamide) as well as various synthetic cannabinoid receptor agonists may have anti-tumor effects. Nevertheless, there are also some reports available challenging this by demonstrating tumor-promoting effect of cannabinoids.[142,143] This might be one of the reasons why cannabinoids did not enter clinical trials.

A significant amount of research so far has focused on how cannabinoids can induce apoptosis in cancer cells. It could be shown that cannabinoids mediate their pro-apoptotic effect over the up-regulation of the stress-regulated protein p8, which in turn upregulates the endoplasmic reticulum stress-regulated genes ATF-4, CHOP and tribbles homolog 3 (TRB3).[144] TRB3 acts as a negative regulator of AKT, which leads to the inhibition of the mammalian target of rapamycin complex 1 (mTORC1) and the subsequent mediation of autophagy.[145] However, there have also been reports of *in-vitro* assays showing that certain cell lines might be resistant to cannabinoid treatment, even when higher concentrations of THC were used than those that promote cell death in non-carcinogenic body cells.[144,145]

In cancer, it has been shown that cannabinoids are capable of inhibiting angiogenesis by downregulation of the vascular endothelial growth factor (VEGF) pathway.[146] Functionally,

cannabinoids therefore promote a normalized tumor vasculature, which contains fewer vessels at a smaller scale, but they are more differentiated and therefore less leaky. Furthermore, cannabinoids are able to promote the formation of distant tumor masses in both induced and spontaneous metastasis.[146] In contrast, there is *in vitro* data of breast, cervical, lung and glioma cell lines available showing that cannabinoids can inhibit migration, adhesion and invasiveness.[147–150] It seems that cannabinoids also play a significant role in the immune system and might, therefore, be of interest for the tumor microenvironment. Newton et al.[151] could show in an *in-vivo* approach that THC was able to shift the cytokine profile from Th1 to Th2 pattern and Hegde and colleagues[152] demonstrated that THC increases the number of myeloid-derived suppressor cells responsible for suppression of antitumor immunity. On the other hand, it could be shown that cannabinoids (such as the synthetic cannabinoids WIN55,212-2 or JWH-133) can enhance immune system-mediated tumor surveillance.[153] Together, the effect of cannabinoids on immune cells in the tumor microenvironment and on tumor progression is unclear.

Cannabidiol, has a low affinity for cannabinoid receptors and it has also been proposed that cannabidiol is able to mediate its apoptotic effects independently from CB_1 and CB_2 receptors.[154] The mechanism by which cannabidiol mediates its effect has not yet been completely clarified. It seems that cannabidiol relies on its ability to enhance the production of reactive oxygen species and reduces the mitochondrial membrane potential.[155]

Clinical use of cannabis in cancer

Many patients believe that cannabinoids have a beneficial impact on their disease related problems and can enhance their therapies.[156] As cannabis has been legalized in some states of the USA, a National Cancer Institute (NCI) -designated cancer center published a survey performed in 2017 conducted among cancer patients about the usage of cannabis (not only medical). They could show that the majority of patients used cannabis for physical symptoms including pain, nausea and appetite. However, one quarter of the patients stated that they specifically used cannabis to treat their cancer disease.[157]

Indeed, the clinical use of medical cannabis is – despite all conducted in-vivo and in-vitro studies – still highly controversial. Preclinical studies could demonstrate that cannabinoids have an impact on cancer apoptosis in the majority of cases.[160] In contrast to these findings, there are no

large randomized controlled trials on this important question. One important issue of translating basic science to clinical science is the fact that medical cannabis is not standardized and different cannabinoid composition may lead to different effects in cancer cells.[161,162] Therefore, in-vivo and in-vitro findings do not mirror the biological reality of cannabis consumption in cancer patients.

The majority of clinical studies focused on two natural cannabinoids - the psychoactive THC and the non-psychoactive CBD – and several industrial engineered substances (e.g. dexanabinol, nabiximol, nabilone). Importantly, the main endpoint of cannabinoid research in cancer patients is Quality of Life (QoL), reduction of pain medication, reduction in chemotherapy-induced nausea and vomiting and other cancer-related symptoms. Cannabis is claimed to be a strong 'cancer-killing' agent across the world-wide web, although cannabinoids have not been tested as mono-therapeutic agent in cancer in clinical trials.[158] So far, only tiny studies exist investigating the role of cannabis as therapeutic agents. Guzman et al. performed 2006 a pilot study where THC was given into the recurrent tumor after tumor surgery in nine patients with glioblastoma multiforme (GBM).[163] Importantly, THC was intracranially applied when patients had a relapse on radio- and/or chemotherapy. Guzman could show that patients, that received THC intracranially, had a median survival of 24 weeks (95% CI: 15 to 33). An underpowered randomized controlled trial (RCT) could recently demonstrate that patients with GBM receiving TMZ in combination with an oral-mucosal spray of CBD and THC (ratio 1:1) had a longer overall survival than patients receiving placebo and TMZ (550 days vs 369 days).[165] A recent case report could further demonstrate that CBD – one of the non-psychoactive compounds of cannabis – might also have beneficial effects in GBM treated with procarbazine, lomustine, and vincristine (PCV) chemotherapy in combination with TMZ.[166] Two cases of young patients with pilocytic astrocytoma showed spontaneous regression after continuous smoking of cannabis.[167] Another case report from a patient with an adenocarcinoma of the lung could show that self-treatment with CBD resulted in a remarkable decrease of tumor mass.[168] A juvenile patient with acute lymphoblastic leukemia has been successfully treated with cannabis oil after the family independently decided to switch therapy.[159]

Cannabinoids in melanoma

Although there is no RCT available demonstrating any benefit of cannabinoids in melanoma there are some in-vitro and in-vivo data available. THC decreased proliferation and induced Atg7

dependent autophagocytotic cell death in three different melanoma cell lines (A375, SK-Mel-28 and CHL-1) and in a CHL-1 melanoma xenograft model.[169] In addition, the same group could demonstrate that THC in combination with a MEK inhibitor (trametinib) significantly decreased cell viability in MEK inhibitor resistant A375 cell lines.[170] Glodde et al. demonstrated in a CB1/CB2-receptor deficient murine model (CB1/2$^{-/-}$) that the anti-tumor effect of THC on HCmel12 xenografts was dependent on cannabinoid receptors.[171] Interestingly, THC did not show any direct apoptotic effect in-vitro on HCmel12 cells, therefore, the authors could further demonstrate that the effect is mainly mediated by modulation of the immune system by accumulation of CD45^{+} cells in the tumor.[171] Another study focusing on non-psychoactive CBD demonstrated that mice harboring murine B16F10 melanoma cells survived significantly longer when treated with CBD as compared to vehicle.[172]

Aim of this thesis

It is hypothesized that cannabinoids might have a potential anti-cancerous effect in many cancer types and therefore I set out to address the following research aims within this thesis:

- To determine if cannabinoids have an impact on cell viability in several metastatic and non-metastatic melanoma cell lines irrespectively of their genotype.
- To gain insight in the mechanism by which cannabinoids mediate their apoptotic effect(s)
- To elucidate the biological efficacy cannabinoids in targeted therapy-resistant *BRAF*^{mt} melanoma cell lines and their interaction with cannabinoids.
- To compare the efficacy of cannabinoids as single therapeutic agent and in combination with targeted therapy in an immunodeficient mouse model.

Material and Methods

Since parts of this thesis have been already published as an original article[183], the material and methods sections were partly adapted from this original article. Therefore, similarities in wording and content do not occur unexpectedly.

Chemicals and antibodies

All drugs were purchased from Tocris (Abingdon, UK) except Z-VAD-FMK (Selleckchem, Houston, TX), THC (Gatt-koller, Absam, Austria) and UO126 and LY294002 (both Cell Signaling Technology Europe, Frankfurt am Main, Germany). All antibodies have been ordered from Cell Signaling Technology Europe (Frankfurt am Main, Germany) except Goat anti-Mouse-IgG, Alexa Fluor 488 (Thermo Scientific, Vienna, Austria) and Anti-Cannabinoid Receptor I (Abcam, Cambridge, UK). All Primers for RT-qPCR have been purchased from Bio-Rad Laboratories (Vienna, Austria).

A375, A2058, SK-Mel-28 and MRC-5 were purchased from LGC Standards GmbH (Wesel, Germany). Malme-3M, A375R, UACC-62 and Colo-800 were a generous gift from Anna Obenauf (Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Vienna, Austria). SK-Mel-30 was a generous gift from Prof Martin Pichler (Division of Clinical Oncology, Department of Internal Medicine, Comprehensive Cancer Center Graz, Medical University of Graz, Graz, Austria) and SBcl2 was a generous gift from Prof Beate Rinner (Department for Biomedical Research, Medical University of Graz, Graz, Austria). A2058, A375 and SK-Mel-28 cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in DMEM (Thermo Scientific, Vienna, Austria) supplemented with 10% fetal calf serum (FCS) (Thermo Scientific, Vienna, Austria) and 1% (v/v) penicillin/streptomycin (P/S). Malme-3M, UACC-62, SK-Mel-30 and Colo-800 cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI1640 (Thermo Scientific, Vienna, Austria) supplemented with 10% FCS (Thermo Scientific, Vienna, Austria) and 1% (v/v) P/S. SBCl2 cells were maintained in a similar way except 2% FCS instead of 10% was used. For all experimental assays, FCS was reduced from 10% to 2% and from 2% to 0.5%.

Cell viability assay

Cells were seeded into 96-well plates at a density of 5000 cells/100 µL/well and grown for 24 h at 37 °C under a humidified atmosphere containing 5% CO_2 followed by the desired treatment duration. Cell growth was determined using a commercial kit (MTS Assay Kit; G5421; Promega Corp., Madison, WI, USA), according to the manufacturer's instructions. For measurement of cell proliferation, 10 µL of MTS reagent were added into each well, and cells were incubated light protected at 37°C for 1 h, then the absorbance was read at 490 nm with a microplate spectrophotometer (xMark™ Microplate Absorbance Spectrophotometer; Bio-Rad Laboratories GmbH; Vienna; Austria). All experiments were repeated at least three times in duplicates.

Cell Cycle

After cells were detached by trypsination, cells were washed twice, permeabilized with ice cold 70% EtOH and incubated overnight at 4°C. Afterwards cells incubated with propidium iodide (Sigma-Aldrich) and Ribonuclease I (Sigma-Aldrich), followed by incubation at 37°C for 1 hours. Immediately afterwards, stained cells were analysed by flow cytometry on a BD FACScan Flow-Cytometer (Becton Dickenson Heidelberg, Germany). Percentage of cells in each cell cycle phase was analyzed with the analyzing software FlowJo® (FlowJo LLC, Ashland, OR).

JC-1 staining

Cells ($5x10^4$) were kept in DMEM supplemented with 2% FBS and 1% PenStrep and incubated with antagonists 20 minutes prior to the indicate treatment with cannabinoids (CBD, THC or CT) for the indicated time points and concentration or for 24 hours if not otherwise mentioned at 37°C. The mitochondrial uncoupler FCCP (1 µM) has been used as positive control. After cell detachment, cells were washed twice in PBS and then incubated in JC-1 dye (2 µg/mL, Thermo Fisher Scientific) for 20 min at 37°C. Fluorescence-activated cell sorting (FACS) was performed using a BD FACScan Flow-Cytometer (Becton Dickenson Heidelberg, Germany) for the detection of mitochondrial depolarization. Intact mitochondrial membrane is reflected by JC-1 monomeres that were detected in the FL-1 channel. Depolarization of the mitochondrial membrane led to the formation of orange J-aggregates that were measured in the FL-2 channel.[184]

Annexin V/PI-co-staining

A2058 cells were seeded in 24 well plates (50.000/well) and kept in DMEM containing 2% FCS and 1% PenStrep. Cells were treated – if indicated – with an antagonist (AM251, 9 µM;

AMG9810, 50 µM; GW6471, 15 µM; BafA1, 100 nM; Z-VAD, 100 µM, Trametinib, 30nM or Vemurafenib, 1 µM) 20 minutes prior to treatment with cannabinoids (CT 6 µM; CBD 6 µM; CBD 10 µM; THC 6 µM; THC 10 µM or THC 15 µM) and CQ (50 µM) – if not otherwise specified - for 24 hours at 37°C. After trypzination, cells were washed, collected via centrifugation and stained with annexin V and PI according to the manufacturer's protocol for 15 min light protected at room temperature (Annexin V-FITC Apoptosis Detection Kit I, BD Pharmingen). Analyzation was performed using a BD FACScan Flow-Cytometer (Becton Dickenson Heidelberg, Germany) and software FlowJo® (FlowJo LLC, Ashland, OR).

Cancer Genomics and RNA expression data

Data from The Cancer Genome Atlas (TCGA) and the Cancer Cell Line Encyclopedia were accessed through the www.cbioportal.org website on 19 November 2018. The database was searched for mutations in *CNR1, PPARα* and *TRPV1* for the skin cutaneous melanoma (SKCM) cohort (366 patients) and the Cancer Cell Line Encyclopedia (1020 cell lines). Mutational frequencies—depending on data availability—were summarized and plotted using the output provided by the online portal. RNA Expression data for *CNR1* and *CNR2* among different tissue types were retrieved from gtexportal.org website (Accessed 8.10.2019; 11:20).

Caspase 3/7 – glo assay

A2058 cells were seeded into 96-well plates (0.5 x 10^5/ per well) and cultured for 24 hours in DMEM containing 2 % FCS and 1% PenStrep supplemented with the indicated treatment. In some experiments cells were pre-treated with antagonists 20 minutes prior to the treatment with cannabinoids (CBD, THC or CT). Caspase 3/7 activation was determined using a commercial kit (Caspase-Glo 3/7 Assay; G8093; Promega Corp., Madison, WI, USA), according to the manufacturer's instructions. For measurement of caspase activation, 50 µL of caspase reagent were added into each well, and cells were incubated light protected at 37°C for 1 h, then the absorbance was read at 490 nm with a microplate spectrophotometer (PerkinElmer Topcount NXT; Waltham, MA).

PGE_2

For detection of immunoreactive PGE_2 a radioimmunoassay was used as described previously[185]. Briefly, samples and standards (Sigma, Vienna, Austria) were incubated with PGE_2 antiserum and [5,6,8,11,12,14,15(N)-3H]-PGE_2 (PerkinElmer, Waltham, MA) overnight at 4°C. Unbound PGE_2 was removed by adding activated charcoal the next day followed by centrifugation at 4000 rpm for 15 min at 4°C. Subsequently, supernatants were poured into scintillation tubes and scintillation cocktail (PerkinElmer) was added. Counts per minute were measured in a beta counter (HIDEX, Turku, Finland) and values were calculated in Sigma plot by employing a 4-parameter curve fit algorithm. IC_{50} of PGE_2 was 106.2 ± 10.6 pg/ml (n = 10) and detection limit, defined as 10% inhibition of binding was at 11.2 ± 1.2 pg/ml (n = 10).

Immunoflurescence

1x 10^5 cells were seeded in each of an eight well chamber slide (Nunc™ Lab-Tek™ II Chamber Slide™ System, Thermo Scientific, Austria) in full growing medium (DMEM, 10% FBS, 1% PenStrep) and rested for 2 days. After two washing steps cells were pre-treated for 30 min with PPARα antagonist (GW6471; Tocris, Abingdon, UK) followed by a 6 hours treatment with CT (CBD 6µM + THC 6µM) in starving medium (DMEM, 2% FBS, 1% PenStrep). After 6 hours medium was sucked off and washed two times carefully with pre-warmed PBS. Cells were then fixed with a solution of 4% Paraformaldehyd for 10 minutes at room temperature. Afterwards cells were washed once with PBS with 0.1% Triton-X100 (Triton™ X-100, Merck KGaA, Darmstadt, Germany) followed by incubation with the same substance for 5 minutes at room temperature. Afterwards cells have been blocked for 60 minutes in 2% Bovine Serum Albumin (BSA; Merck KGaA, Darmstadt, Germany) at room temperature followed by overnight incubation of cytochrome c anti-body (#12963, clone: 6H2.B4; Cell Signaling Technology Europe, Frankfurt am Main, Germany) at 4°C. Cells were then washed 2x times with PBS with supplementation of 0.1% Tween 20 (Merck KGaA, Darmstadt, Germany). After incubation with 2nd anti-body (Goat anti-Mouse IgG, Alexa Fluor 488, #A-11001; Thermo Scientific, Vienna, Austria) for 1 hours light-protected at room-temperature, cells were washed 3x times with PBS with supplementation of 0.1% Tween 20, covered with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI)(#D8417; Merck KGaA, Darmstadt, Germany) and then sealed.

Real-time PCR

Cells were pelleted via centrifugation (400 x g, 7 min) and carefully resuspended in 500 µL of TRI Reagent® (Sigma, Vienna, Austria) and incubated on ice for 10 min. 100 µL of chloroform was added followed by shaking and incubation (2-3 min at RT) in order to accomplish phase separation. The mixture was centrifuged (12 000 rpm for 15 min at 4°C) and the aqueous phase was transferred to a new tube. Total RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Afterwards, cDNA was synthesized using the iScript cDNA Synthesis Kit (Biorad, Vienna, Austria) according to the manufacturer's instruction. Real-time PCR was performed using SsoAdvanced™ Universal SYBR® Green Supermix with PrimePCR™ SYBR® Green Assay primers for *CNR1, CNR2, TRPV1, TRPV2, PPARA, PPARG, GPR55* and *GAPDH*, which was used as reference gene (all from Biorad). Samples were measured in duplicates and mRNA expression was quantified due to their respective Ct values. Data are shown as absolute Ct values of each gene.

Crystal Violet Assay

For the quantitative determination of cells adhering to the plate after the 24 h treatment with different concentrations of CBD, THC and PPARα, the violet crystal assay was used. Cells were carefully washed after treatment and stained with crystal violet-solution (0.05% crystal violet, 1% formaldehyde, 1% methanol in PBS) for 20 minutes. After careful washing, plates were dried overnight, methanol added and absorbance was determined with a spectrometer (VICTOR Multilabel Plate Reader, PerkinElmer, Waltham, MA) at 560 nm.

Human phospho-kinase antibody array

Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems, Minneapolis, MN, USA) was used following the manufacturer's instructions. Briefly, cells were treated with a combination of THC (6 µM) and CBD (6 µM) or the MEK inhibitor Trametinib (30 nM) or a combination of both for 2 hours in DMEM supplied with 2% FCS and 1% P/S. After 2 hours cells have been lysed in array buffer. BCA assay (Thermo Scientific, Waltham, MA) was performed to normalize protein amount among treatment groups. The arrays were blocked with blocking buffer and incubated with 120 µg of cell lysate overnight at 4 °C. The arrays were washed, incubated with a horseradish peroxidase (HRP)-conjugated phospho-kinase antibody, treated with Clarity Western ECL Substrate (Bio-Rad, Hercules, CA) and visualised by

ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA). Experiment was done in duplicates.

Human cytokine array

100.000 A2058 cells per mL were seeded and treated with CT, trametinib or a combination of both for 24 hours in DMEM supplied with 2% FCS and 1% P/S at 37°C. Supernatant was collected and analyzed using the Proteome Profiler Human Cytokine Array Kit (R&D Systems, Minneapolis, MN, USA; #ARY005B) according to the manufacturer's instructions. The average pixel density was analyzed in duplicate spots of each cytokine, each group was normalized to the respective reference spot and compared between the four different groups.

In vivo Experiments

Ten- to 26-week-old male NOD Scid gamma (NSG) mice were a generous gift of Dr. Andreas Reinisch (Medical University of Graz, Graz, Austria) and originally obtained from Charles River Laboratories (Wilmington, MA, USA). A2058 cells ($1x10^6$ cells/mouse) were injected subcutaneously (s.c.) in the area of the right flank.

The mice were bred and hold under pathogen-free conditions in the animal facility of the Medical University of Graz (Graz, Austria). All experimental procedures were done according to Europeans Guidelines and our governmental ethics commission regulations (BMBWF-66.010/0139-V/3b/2019). After 7 days from the injections, when tumors were palpable in all mice, they were randomized that were no significant difference between age and weight among all four groups (8 mice per group). Treatment assignment to any group was done in a blind fashion. CT (CBD 10 mg/kg BW and THC 10 mg/kg BW) and Trametinib (Selleckchem, Houston, TX)(0.75 mg/kg BW) were dissolved in ethanol and kolliphor (#C5135-500G, Merck, Darmstadt, Germany) one to one. For immediate subcutaneously application drugs have been further diluted in a 1 to 10 ratio with PBS and all treatments were given every day (weight adjusted; ~150 µl/mouse) for 21 days. Control mice were injected with empty kolliphor/ethanol dissolved in PBS. Treatment-related toxicity was determined by monitoring mouse weight and appearance every day. The tumor size was measured with a caliper and tumor volume was calculated according to the following equation: $Volume = 0.5*(length*width^2)$.[186] Mice were

sacrificed 21 days after treatment initiation and tumor samples were collected afterwards for further analysis.

Immunohistochemistry

Immunohistochemistry was performed on 4µm thick FFPE sections of each single mouse. Staining was done using the Dako Omnis platform (Agilent, Santa Clara, CA).

Ki67 (Agilent, Santa Clara, CA; #GA626), PD-L1 (Agilent, Santa Clara, CA; #M3653; Clone: 22C3; 1:30) and S100 (Agilent, Santa Clara, CA; #GA504) staining has been performed according to the manufacturers' protocol. In summary, pre-treatment was performed using the DAKO OMNIS EnVision FLEX Target Retrieval Solution Low pH solution (Agilent, Santa Clara, CA; #GV805) for 50 minutes at 97°C (PD-L1) or for 30 minutes at 97°C (Ki67 and S100), followed by blocking using peroxidase from the EnVision FLEX Mini Kit, High pH (Agilent, Santa Clara, CA; #GV82311-2) for 3 minutes. Incubation time for the PD-L1 Ab was 40 min at 25°C, for S100 12 min at 25°C and for Ki67 20 min at 25°C. As Detectionsystem the ENV FLEX HRP from the EnV FLEX, High pH (Dako Omnis)(Agilent, Santa Clara, CA; #GV80011-2) for 20 minutes at 25°C with mouse linker for 10 minutes at 25°C (PD-L1) or without any linker (S100 and Ki67) was used. Afterwards, samples have been incubated for 5 minutes at 25°C with DAB+Substrate Chromogen (Agilent, Santa Clara, CA; #GV80011-2). Counter staining was performed using Hematoxylin (Merck, Darmstadt, Germany; #H3136) for 1 minute at room temperature. Between each step, each sample has been washed with wash buffer 20x (Agilent, Santa Clara, CA, #GC807).

Following immunohistochemical staining, tumor sections were scanned using Pannoramic 1000 (3D Histech, Budapest, Hungary), whereby 5 non-overlapping regions were randomly selected (35x magnification) and extracted for quantification of PD-L1 or nuclear Ki67 staining with QuPath Software [187]. Additionally, S100 expression was evaluated in one randomly chosen image. One image resulted in approximately 2000 cells for quantification. Expression analysis was performed in batch mode, whereby nuclei were detected according to hematoxylin counterstain with following standard settings: background radius 15px, Sigma 3 px, minimum area 10 px^2, maximum area 1000 px^2, intensity threshold 0.1. Cell expansion was set at 5 px. For

characterization of $Ki67^+$ cells, only the nuclear score compartment considering DAB OD mean was chosen, whereby threshold 1 was 0.2, threshold 2 was 0.5 and threshold 3 was 1. PD-L1 expression was determined using the whole cell as score compartment (DAB OD mean) with a threshold of 0.1. S100 staining was evaluated using the whole cell as score compartment (DAB OD mean) with thresholds 0.1, 0.2 and 0.6. The thresholds were set by a blinded person after evaluation of 10 images chosen randomly from the data set.

Statistical Analysis

GraphPad Prism version 6 (GraphPad Software Inc., San Diego, CA, USA) was used to perform statistical tests. Bar plots and graphs were created with Graphpad or ggplot2. All data are shown as mean + SD for n observations. For three or more groups, one-way or two-way ANOVA followed by Dunnett's, Tukey or Bonferroni multiple comparison test were used or for two groups, Mann–Whitney-U test. Survival curves were estimated using the Kaplan–Meier product-limit method and compared using a log-rank test. Two-sided p value of < 0.05 was considered statistically significant and indicated as * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$ and **** = $p<0.0001$. ns = not significant.

Results

Melanoma progresses from benign lesions to melanoma in-situ to metastatic disease by accumulation of somatic mutations including several key mutations. Melanoma evolution starts with acquiring the therapy relevant *BRAF*^{V600E} mutation at a very early stage since this mutation can also be found in nevi.[16] Progression to metastatic disease includes the acquisition of *TP53, PTEN* and mutations in other genes.[16] Although there have been more than 60 types of cannabinoids described in the literature[188], research is mainly focusing on the psychoactive THC and the non-psychoactive CBD. Apart from using both substances as single therapeutic approach, the combination of CBD and THC (ratio 1:1) has been approved for clinical use in patients with chronic pain, chemotherapy induced nausea and vomiting and spasticity in multiple sclerosis.[1] Due to its clinical value and availability, we included the combination of THC and CBD in a 1:1 ratio in all our experiments.

Effect of cannabinoids on different melanoma cell lines and non-cancerogenous cells

Firstly, we were interested if a commonly used *BRAF*^{V600E} mutated non-metastatic melanoma cell line is responsive to cannabinoids since this cell line is widely used and also serves as resistance model for commonly used targeted therapy.

Indeed, cannabinoids were capable of reducing the cell viability in a concentration-dependent manner in A375 cells (**Figure 2**). All three combinations were capable of reducing cell viability whereas the 1:1 combination of THC and CBD (CT) showed the most pronounced effect followed by CBD alone and THC alone. This result is in line with previous reports.[169]

[1] https://www.parlament.gv.at/PAKT/VHG/XXVI/III/III_00233/imfname_728963.pdf, Accessed 26.11.2019; 11:20

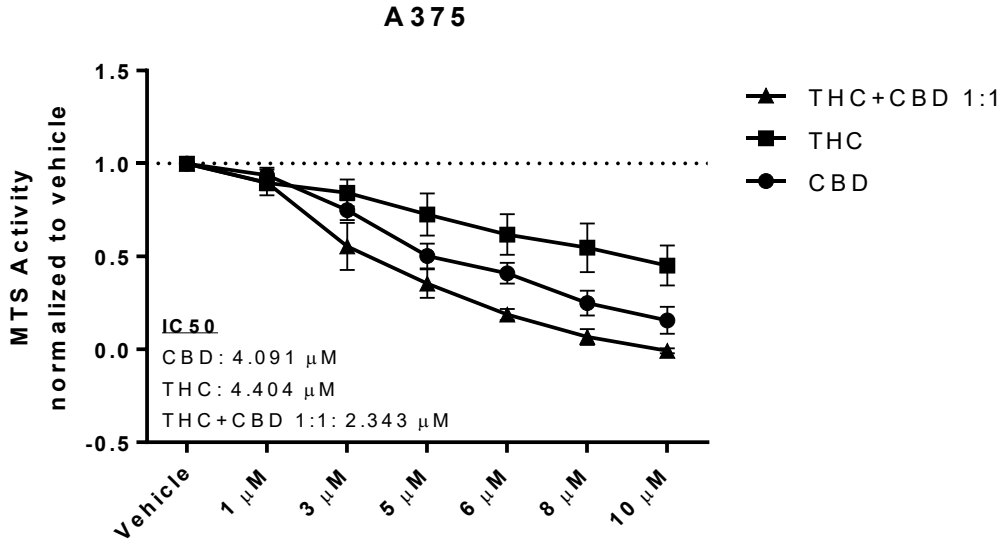

Figure 2. Cannabinoids can reduce the cell viability of A375 cells in a concentration-dependent way.

A375 cells were treated with different concentrations (1, 3, 5, 6, 8 and 10 µM) of CBD, THC or a 1:1 combination of THC and CBD for 24 hours. Cell viability was assessed by using the Promega MTS assay. Data are presented as mean ± SD. IC50 = Half maximal inhibitory concentration. Experiment was performed four times.
(Figure 2 corresponds to Figure 1A of the original publication by Richtig et al [183])

The *BRAF*^{V600E} mutation is the most common one found in melanoma with a prevalence of 40 to 60%, *NRAS* mutation comes second with a prevalence of 12 to 20%.[92] Therefore, we were interested if the apoptotic effect of cannabinoids depends on *BRAF* mutation and might also be seen in cell lines harboring a *NRAS* mutation.

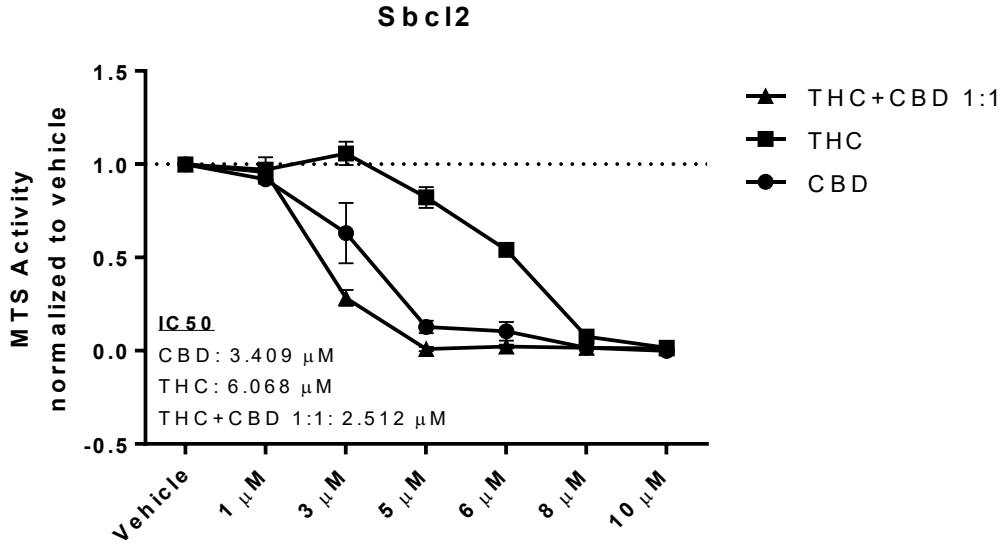

Figure 3. Cannabinoids can reduce the cell viability of SBcl2 cells in a concentration-dependent way.

SBcl2 cells were treated with different concentrations (1, 3, 5, 6, 8 and 10 µM) of CBD, THC or a 1:1 combination of THC and CBD for 24 hours. Cell viability was assessed by using the Promega MTS assay. IC50 = Half maximal inhibitory concentration. Data are presented as mean ± SD. Experiment was performed five times.
(Figure 3 corresponds to Figure 1B of the original publication by Richtig et al [183])

Similar to the A375 cell line tested, the *NRAS*^{Q61L} mutated cell line SBcl2 (**Figure 3**) showed similar patterns in response to cannabinoid treatment, although the effect of THC was not as pronounced as seen in A375 cells.[189]

The primary treatment of non-metastatic melanoma is surgical excision but it is of clinical interest if metastatic melanoma cells would also respond to cannabinoid therapy. Importantly, melanoma – especially cutaneous melanoma – is characterized by the fact that they harbor the highest mutational burden compared to any other cancer type.[41] This is of clinical relevance since it increases the likelihood of having primary resistance to any treatment. For this reason, we tested several metastatic melanoma cell lines for the efficacy of cannabinoids.

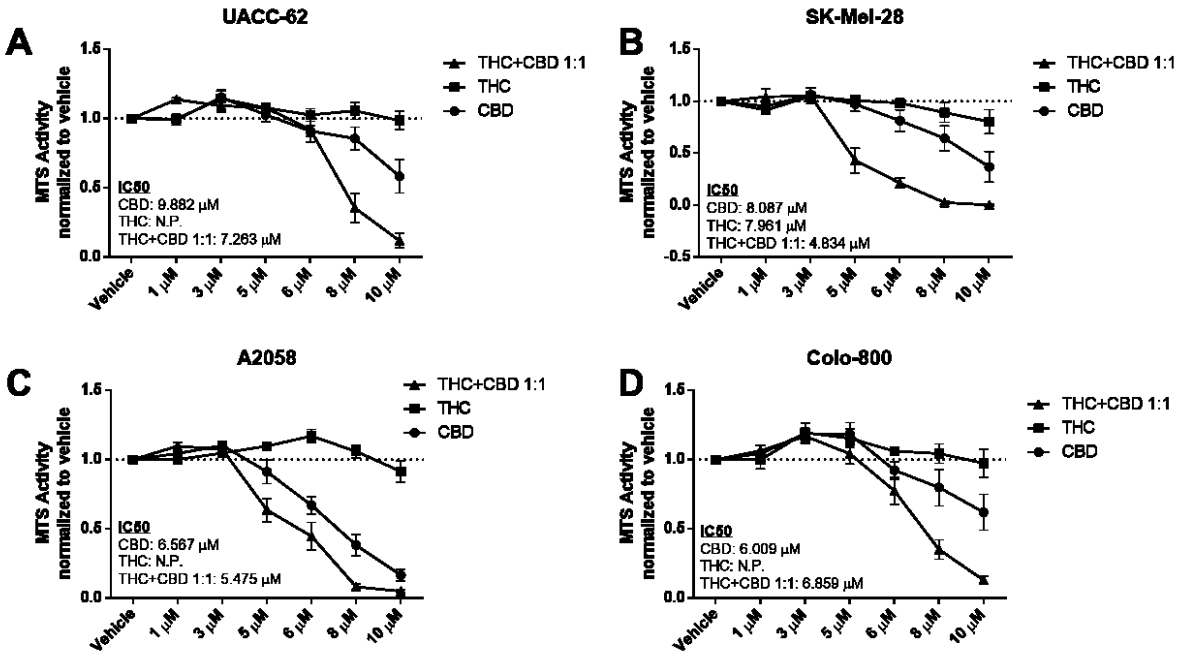

Figure 4. Four different human metastatic melanoma cell lines show different sensititivities to cannabinoid-induced toxicity.

UACC-62, SK-Mel-28, A2058 and Colo-800 cells were treated with different concentrations (1, 3, 5, 6, 8 and 10 µM) of CBD, THC or a 1:1 combination of THC and CBD for 24 hours. Cell viability was assessed by using the Promega MTS assay. Data are presented as mean ± SD. IC50 = Half maximal inhibitory concentration. N.P. = not possible. Experiments were performed at least three times.

(Figure 4 corresponds to Figure 1 and SFigure 1 of the original publication by Richtig et al [183])

When cannabinoids were tested in metastatic melanoma cell lines, we observed that THC was incapable of reducing cell viability in all four tested melanoma cell lines. CBD alone had a concentration-dependent effect on all cancer cell lines although some cell lines (A2058) (**Figure 4C**) were more sensitive to CBD than others (UACC-62 (**Figure 4A**), SK-Mel-28 (**Figure 4B**) and Colo-800 (**Figure 4D**)). Similar effects were seen when CBD and THC were combined since A2058 and SK-Mel-28 cells were more responsive to the treatment than Colo-800 and UACC-62 cells. These results demonstrate that different metastatic cell lines respond – as expected – differently to cannabinoids suggesting that cannabinoids might not be effective against all melanoma cells due to their genetic heterogeneity (see **Table 1**).

Table 1. **Key mutations of different melanoma cell lines.**

Cell line	Key mutations	COSMIC ID or source
A375	*BRAF*^{V600E}	COSS906793
SBcl2	*NRAS*^{Q61L}, *CDKN2A*^{del}	Sini et al.[189]
SK-Mel-28	*BRAF*^{V600E}, *APC*^{S130G}, *PTEN*^{T167A}, *TP53*^{L145R}	COSS905954
UACC-62	*BRAF*^{V600E}, *PTEN*^{P248fs*5}	COSS905976
A2058	*BRAF*^{V600E}, *TP53*^{V274F}, *PTEN*$^{L112Q/V175fs*3}$, *MAP2K1*^{P124S}	COSS906792
Colo-800	BRAF^{V600E}, *TP53*^{C135R}	COSS906813
SK-Mel-30	*BRAF*$^{D287H/E275K}$, *NRAS*^{Q61K}, *TP53*^{T284fs*21}, *CDKN2A*^{P114L}, *APC*$^{G1339R/Q1406*}$	COSS909726

Given the importance of the tumor microenvironment[190], it was of interest if cannabinoids could reduce cell viability of normal fibroblasts since these cells play a pivot role in the skin and are one of the first barriers metastatic melanoma has to overcome. As a surrogate for this type of cells we used the fibroblastic cell line MRC-5 as a control.

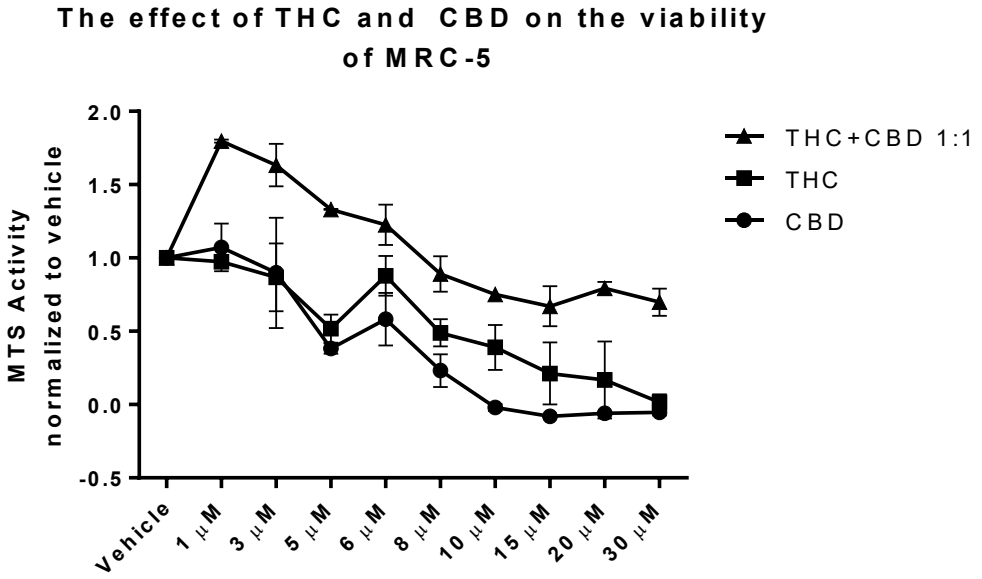

Figure 5. **Cannabinoids induce different effects on cell viability in the fibroblast cell line MRC-5 in a concentration-dependent way.**

MRC-5 cells were treated with different concentrations (1, 3, 5, 6, 8, 10, 15, 20 and 30 μM) of CBD, THC or a 1:1 combination of THC and CBD for 24 hours. Cell viability was assessed by using the Promega MTS assay. Data are presented as mean ± SD. Experiment was performed two times.

THC and CBD reduced cell viability of MRC-5 cells (**Figure 5**) in a concentration-dependent manner. Surprisingly, when CBD and THC were combined, this effect was abrogated with no effect seen even in very high concentrations. Another highly important type of immune cells when it comes to successful therapy of stage IV melanoma patients are eosinophils.[191] It has been suggested that eosinophils may play a pivotal role in patients receiving immunotherapy. Therefore, it was of interest if cannabinoids have any effects on the viability of these cells and other immune cells.

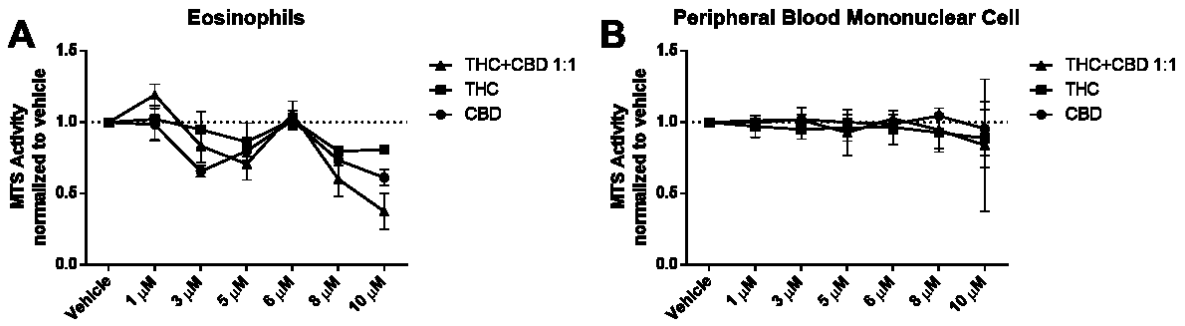

Figure 6. Cannabinoids mediate different effects on cell viability in human eosinophils in a concentration-dependent fashion and no effect on peripheral blood mononuclear cells.

Freshly isolated eosinophils and peripheral blood mononuclear cells (PBMCs) *were treated with different concentrations (1, 3, 5, 6, 8 and 10 µM) of CBD, THC or a 1:1 combination of THC and CBD for 24 hours. Cell viability was measured using the Promega MTS assay. Data are presented as mean ± SD. Experiments were performed three times.*
(Figure 6 corresponds to Figure 1H-I of the original publication by Richtig et al[183]*)*

All three combinations tested reduced cell viability in eosinophils, although in a bimodal way. Six µM of any cannabinoid did not reduce cell viability of eosinophils although reduced cell viability was observed at lower and higher concentrations (**Figure 6A**). When peripheral blood mononuclear cells (PBMC) were tested for the effect of cannabinoids, we did not observe any significant effect on cells' viability (**Figure 6B**).

Receptor-mediated action

Sativex®, a clinically used spray on the market consists of CBD and THC in a 1:1 ratio. This spray is approved for therapy in patients with chronic pain, chemotherapy induced nausea, and vomiting and spasticity in multiple sclerosis.[2] Therefore, we decided to further investigate cannabinoids at a concentration of 6 µM since it has been shown that this concentration could reduce melanoma cell viability and did not impair other cell types, such as fibroblasts and immune cells. From the metastatic melanoma cells tested, A2058 was of interest since it harbored a $BRAF^{V600E}$, a $TP53^{V274F}$ and a $PTEN^{L112Q/V175fs*3}$, suggesting a highly aggressive phenotype[16] and in combination with a $MAP2K1^{P124S}$ mutation a cell line potentially not suitable for targeted

[2] https://www.parlament.gv.at/PAKT/VHG/XXVI/III/III_00233/imfname_728963.pdf, Accessed 26.11.2019; 11:20

therapy with BRAF and/or MEK inhibitors.[192] For this reason, all subsequent experiments were performed with this specific cell line and this specific reference concentration.

To confirm the effects seen in the viability assay, we performed annexin-V/PI-apoptosis staining and cell cycle analysis. Cells were treated for 24 hours or 48 hours with a combination of CBD and THC (CT) in a 1:1 ratio at a concentration of 6 µM or with its respective single agents.
In the annexin-V/PI-apoptosis staining we observed that CT significantly induced apoptosis after 24 hours and after 48 hours (**Figure 7A**). When cells were treated for 24 hours with different concentrations of cannabinoids and then analyzed for their cell cycle, we observed that CT and CBD (10 µM) significantly induced the accumulation of cells in the subG1 phase (**Figure 7B**).

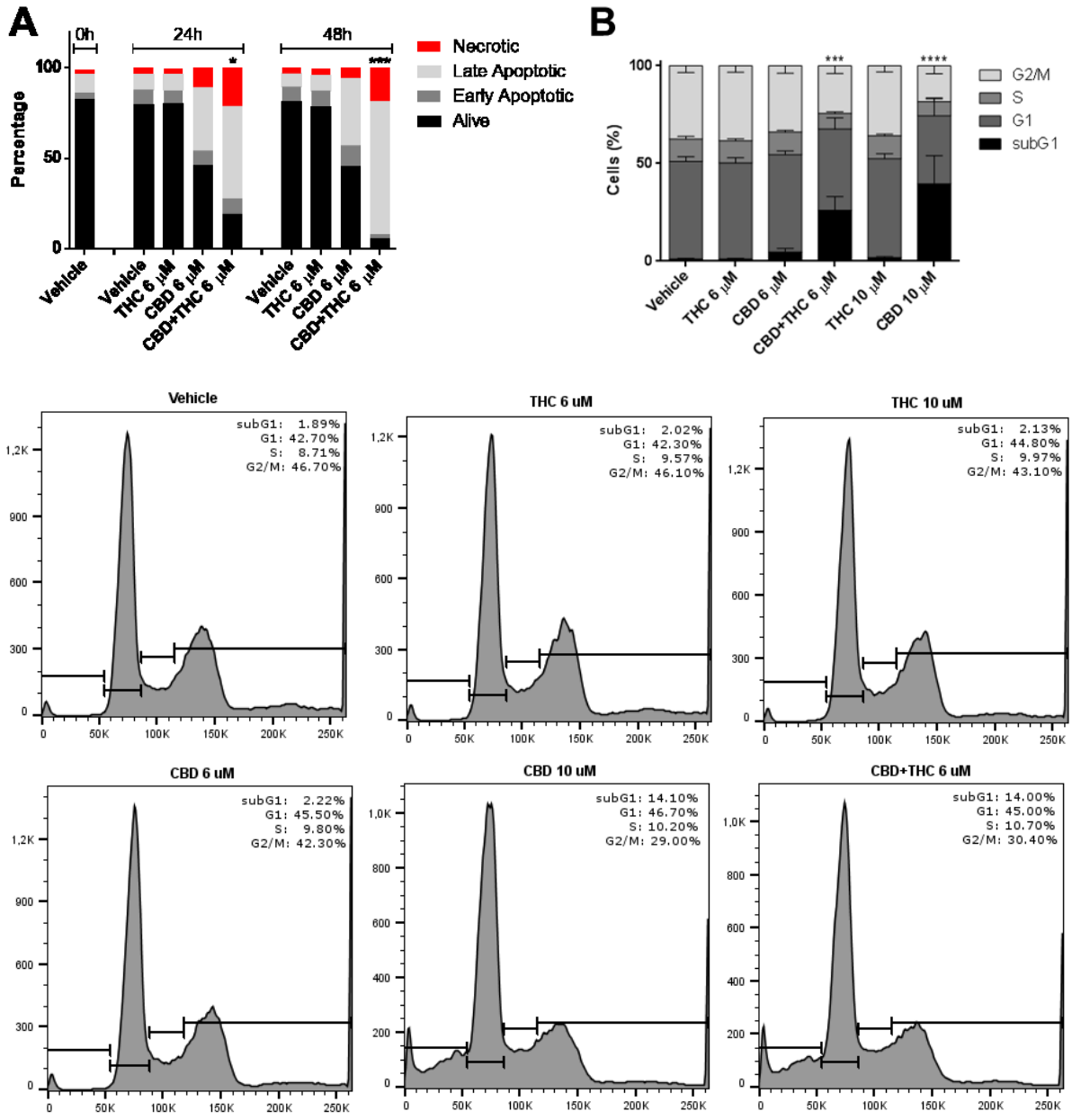

Figure 7. Combination of CBD and THC and high-concentration CBD mediate apoptosis and cell cycle alterations.

A2058 cells were treated with different concentrations of cannabinoids (CT 6 uM, THC 6uM, CBD 6uM and THC 10uM) for 24 hours and 48 hours. (A) Cell apoptosis was assessed 24 hours and 48 hours post-treatment using flow cytometry annexin-V/PI-co staining. (B & C) Cell Cycle was investigated after 24 hours post-treatment using flow cytometry PI staining. Data are

*presented as mean ± SD. Experiments were performed five times. Statistical significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*
(Figure 7 corresponds to Figure 1F-G and SFigure 1C of the original publication by Richtig et al [183])

Two main subtypes of cannabinoid G-coupled receptors have been described in the literature[123,124] whereas CB1 is mainly expressed in the brain and different other organs (**Figure 8A**). In contrast, CB2 is mainly known for its role on immune cells and immune-related organs (**Figure 8B**).

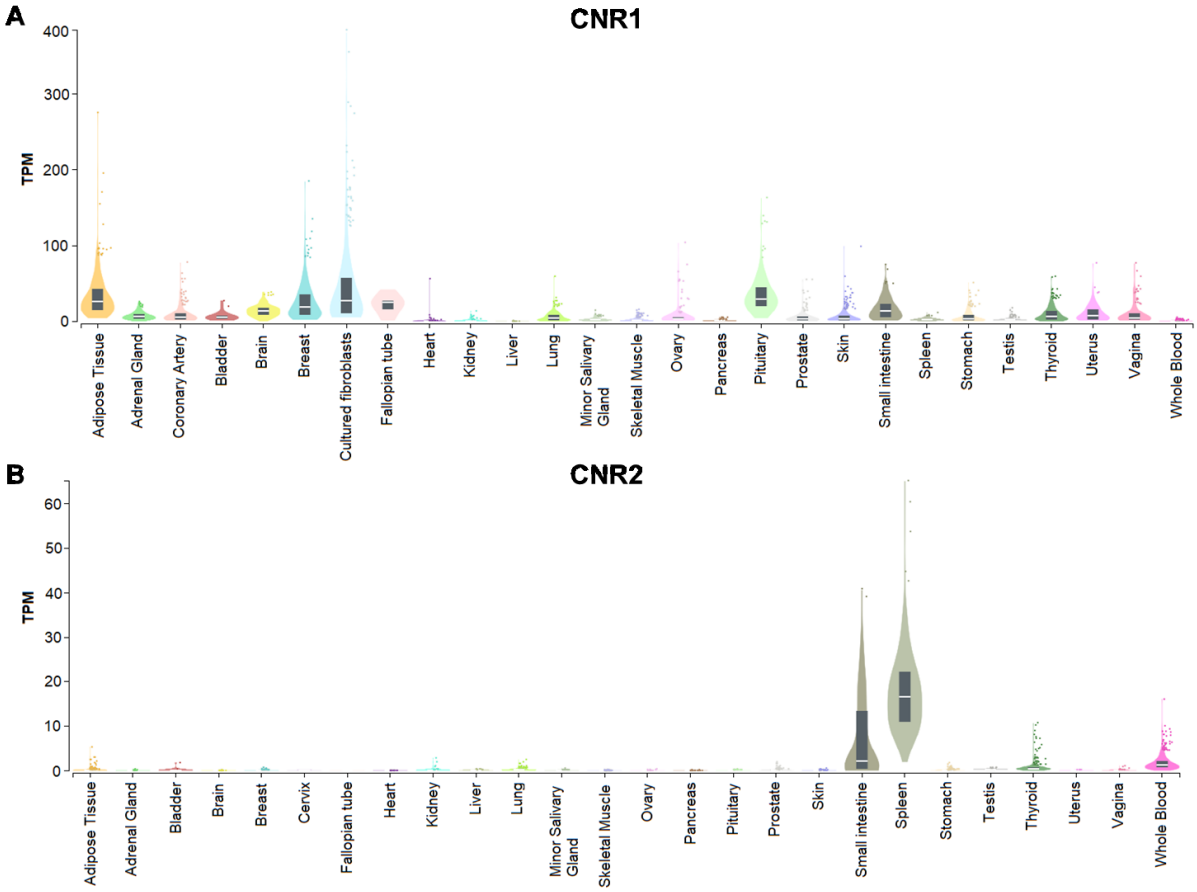

Figure 8. Cannabinoid receptor 1 and 2 are differently expressed among different tissue types.

Expression patterns of CNR1 (A) and CNR2 (B) among different tissue types were looked up by using the gtexportal.org website (Accessed 8.10.2019; 11:20).

To investigate through which cannabinoid receptor the apoptotic effect of cannabinoids is mediated in melanoma cells, we used one CB1 antagonist (AM251) and one CB2 antagonist (AM630).

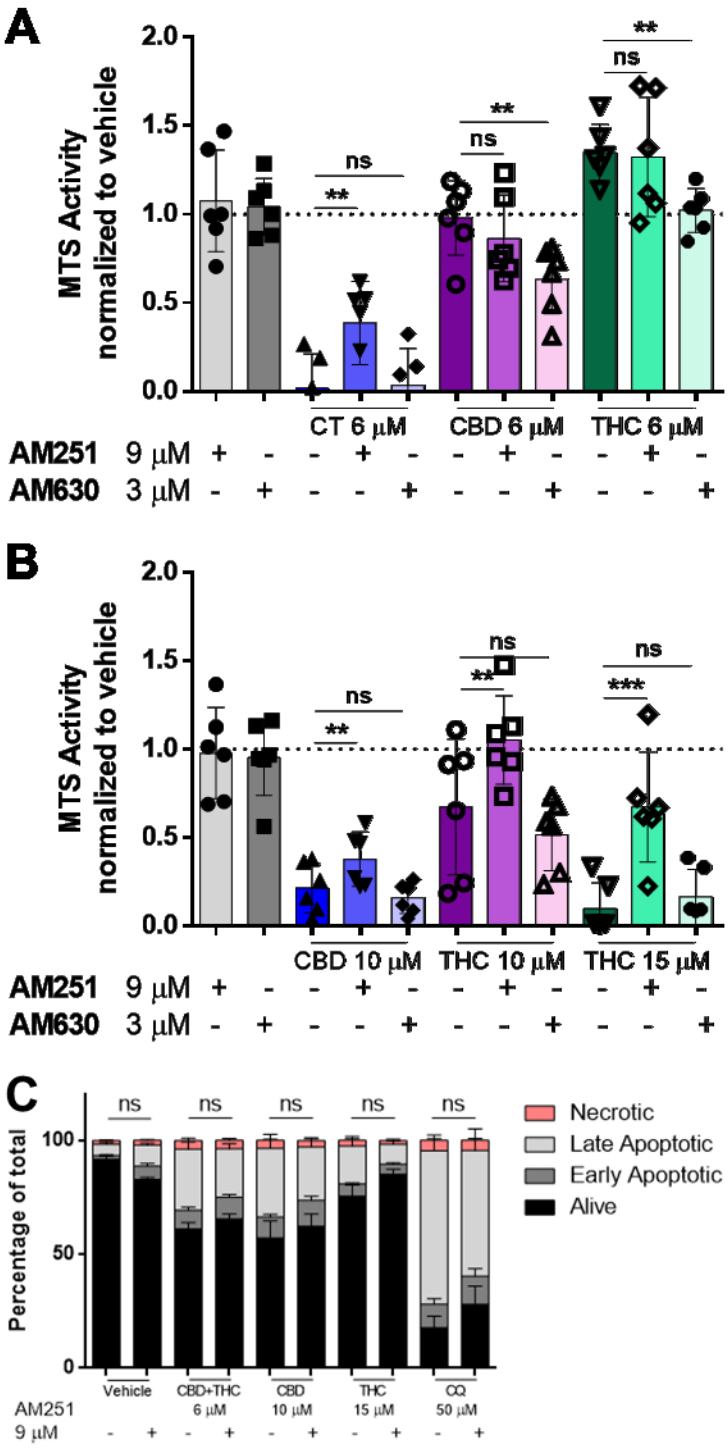

Figure 9. Effects of cannabinoids is more likely to be mediated by CB1 receptor than CB2 receptor.

*A2058 cells were pre-treated with a CB1 receptor antagonist (AM251) or a CB2 receptor antagonist (AM630) 15 minutes prior to cannabinoid or chloroquine (CQ) treatment for 24 hours. (A & B) Cell viability was measured using the Promega MTS assay. (C) Apoptosis was investigated by flow cytometry annexin-V/PI-co staining. Data are presented as mean ± SD. Experiment were performed five times. Statistical significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*
(Figure 9 corresponds to Figure 2A-B and SFigure 1D of the original publication by Richtig et al [183])

Both inhibitors did not reduce the cell viability when used at a concentration of 9 µM for AM251 and 3 µM for AM630 (**Figure 9A**). When cannabinoids were used in a concentration of 6 µM, the CB1 antagonist AM251 significantly increased cell viability treated with CT. When CBD and THC were used alone, AM251 did not restore cell viability. In contrast to the CB1 antagonist, the CB2 antagonist (AM630) did not increase cell viability in CT treated cells and in addition decreased cell viability when used in combination with CBD and THC (**Figure 9A**). When concentrations were increased to 10 µM for CBD and 10 µM and 15 µM for THC, AM251 was capable of increasing cell viability under all treatment conditions, whereas AM630 had no significant effect (**Figure 9B**). In annexin-V/PI apoptosis co staining, the findings of the MTS assay were not confirmed since AM251 had no significant effect on apoptosis when given in combination with CBD, THC or CT (**Figure 9C**). It also had no effect when given with chloroquine (CQ), a drug known to have inhibitory properties on autophagy.[193]

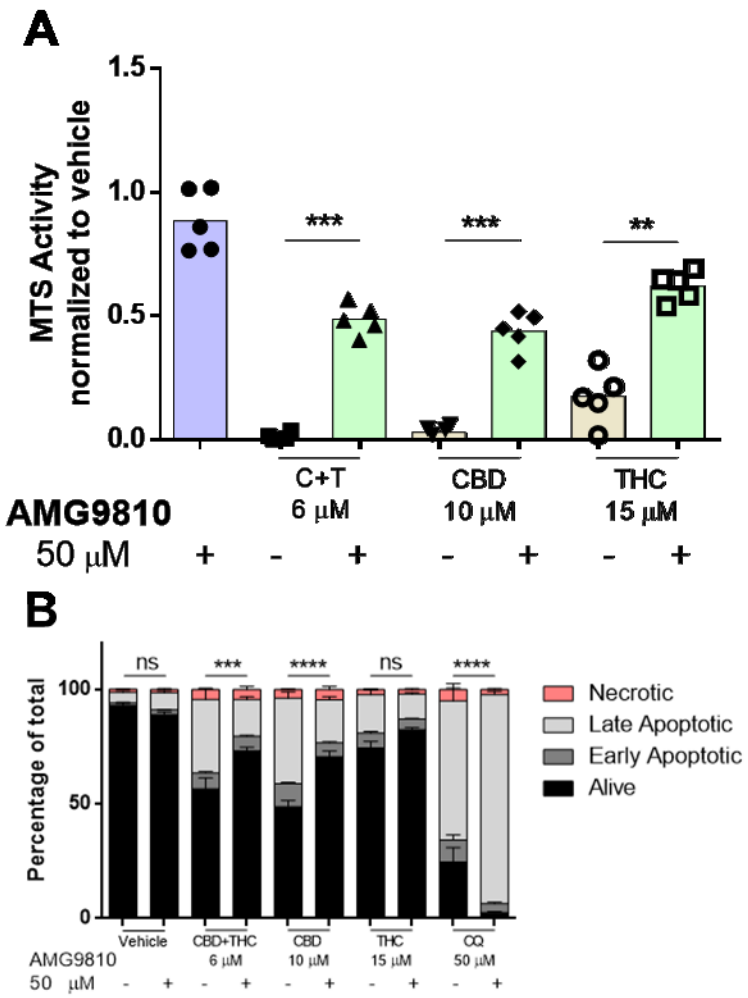

Figure 10. Cannabinoid-induced apoptosis involves TRPV1 signaling.

*A2058 cells were pre-treated with a TRPV1 receptor antagonist (AMG9810) 15 minutes prior to cannabinoid or chloroquine (CQ) treatment for 24 hours. (A) Cell viability was assessed by using the Promega MTS assay. (B) Apoptosis was investigated by flow cytometry annexin-V/PI-co staining. Data are presented as mean ± SD. Experiment were performed five times. Statistical significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*

(Figure 10 corresponds to Figure 2C and SFigure 1E of the original publication by Richtig et al [183]*)*

Another receptor thought to be involved in cannabinoid signaling is the TRPV1 receptor.[194] It was shown, that this receptor can be activated by CBD in different cancer cell lines.[194] Therefore, we used the TRPV1 specific antagonist AMG9810 to test this hypothesis. AMG9810 increased viability when cells were treated with 6 µM CT, 10 µM CBD and 15 µM THC (**Figure 10A**). In contrast to AM251, this effect could be confirmed at least in cells treated with CBD and CT in the annexinV-/PI co staining. For cells treated with 15 µM THC, there was no significant increase in living cells when AMG9810 was given prior to treatment. In cells treated with AMG9810 and 50 µM CQ, AMG9810 significantly reduced the number of viable cells (**Figure 10B**).

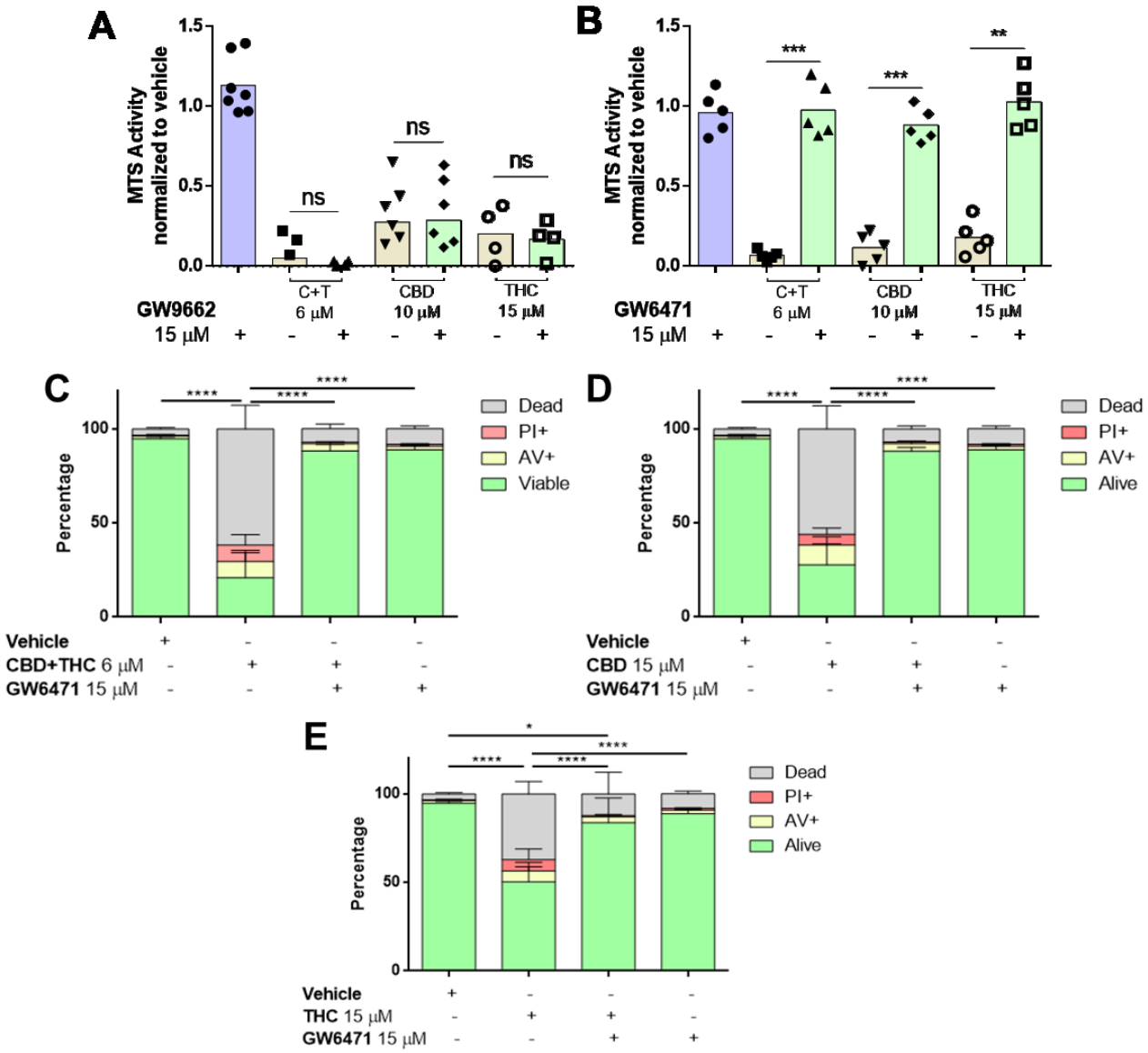

Figure 11. Effects of cannabinoids are mediated by PPARα signaling rather than PPARγ signaling.

A2058 cells were pre-treated with a PPARγ (GW9662) receptor antagonist or a PPARα (GW6471) receptor antagonist 15 minutes prior to cannabinoid treatment for 24 hours. Cell viability effectsof cannabinoids in combination with PPARγ receptor antagonist (A) or in combination with PPARα receptor antagonist (B) were assessed by using the Promega MTS assay. Apoptosis was investigated by annexin-V/PI flow cytometry staining in cells treated with PPARα receptor antagonist and CT (C), high-concentration CBD (D) and high-concentration

*THC (E). Data are presented as mean ± SD. Experiments were performed five times. Statistical significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*
(Figure 11 corresponds to Figure 2D-G and SFigure 2A of the original publication by Richtig et al [183])

A common pathway of cannabinoid signaling and that of TRPV1 is the peroxisome proliferator-activated receptor (PPAR) signaling pathway.[195] Several studies have demonstrated that both PPARα and PPARγ are involved in cancer apoptosis mediation.[195] To elucidate whether PPARα or PPARγ were in fact involved, we used two specific antagonists (PPARα: GW6471 and PPARγ: GW9662). When the PPARγ antagonist (GW9662) was tested, we did not observe any effect on cell viability (**Figure 11A**). However, the PPARα antagonist (GW6471) could effectively rescue cell viability in all treated conditions (**Figure 11B**). Importantly, this could be confirmed by annexinV/-PI staining in cells treated with CT (**Figure 11C**), high-concentration CBD (**Figure 11D**) and high-concentration THC (**Figure 11E**). However, in cells treated with the PPARα antagonist and THC there still remained a significant reduction in viable cells compared to vehicle (**Figure 11E**).

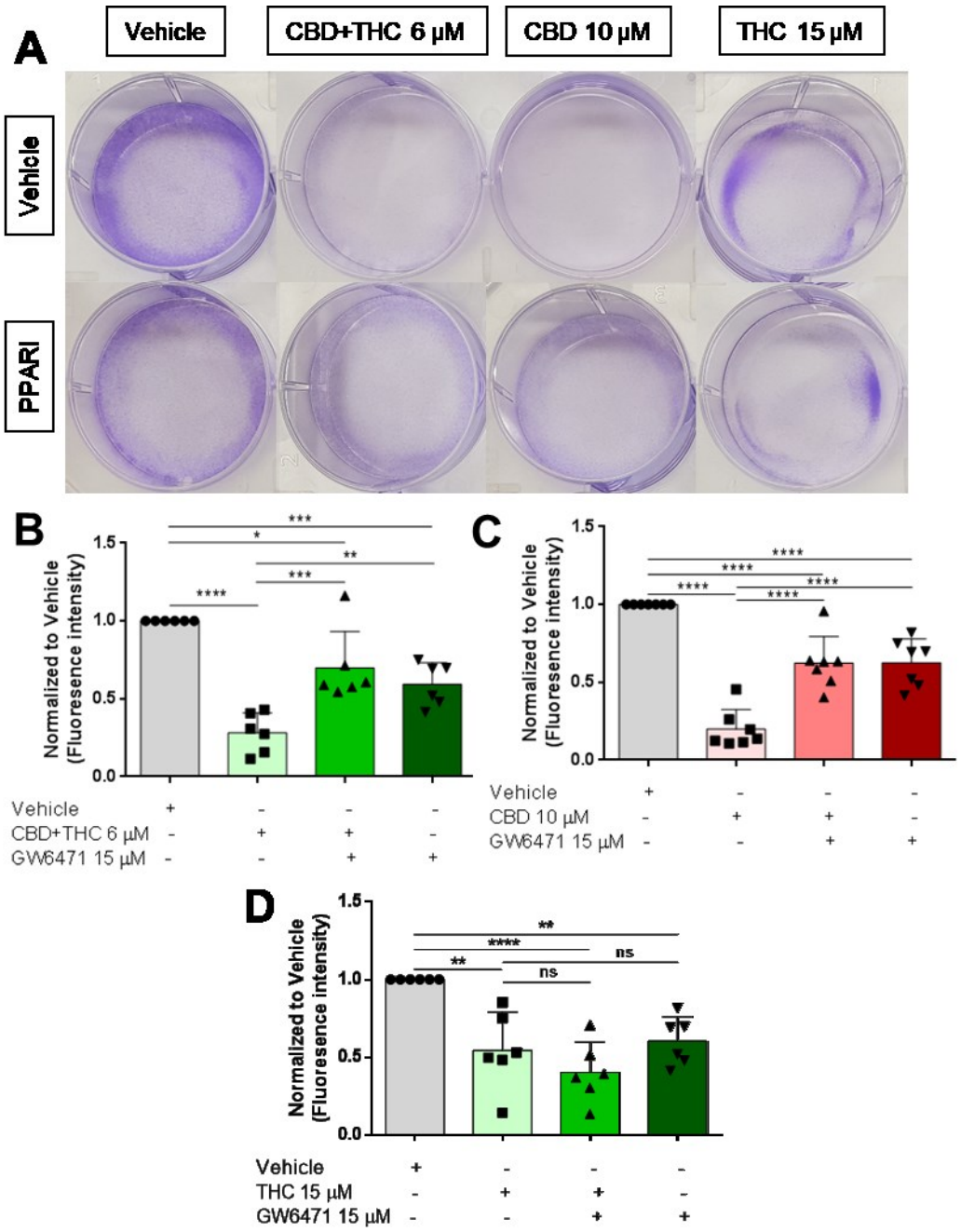

Figure 12. Inhibition of PPARα signaling is capable of blocking CBD- rather than THC-mediated effects.

A2058 cells were pre-treated with a PPARα (GW6471) receptor antagonist 15 minutes prior to cannabinoid treatment for 24 hours. (A) Cristal violet staining was performed to visualize viability of cells treated with GW6471 and different concentrations of CBD and THC.

*Quantification was measured by a spectrophotometer at 570 nm. Data are presented as mean ± SD. Experiment was performed six times. Statistical significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*
(Figure 12 corresponds to SFigure 2B-E of the original publication by Richtig et al [183])

When cells were stained with crystal violet and analyzed by absorbance, we observed that CT significantly reduced absorbance compared to vehicle (**Figure 12A & B**). In combination with GW6471, there was a significant increase in fluorescence intensity although not back to vehicle. We could also show that GW6471 significantly reduced absorbance (**Figure 12B**). Similar findings were made when high-concentration CBD (10 µM) was used instead of CT 6 µM (**Figure 12A & C**). High-concentration THC (15 µM) significantly reduced fluorescence intensity similar to GW6471, but GW6471 was not able to correct the decrease of absorbance back to baseline (**Figure 12D**).

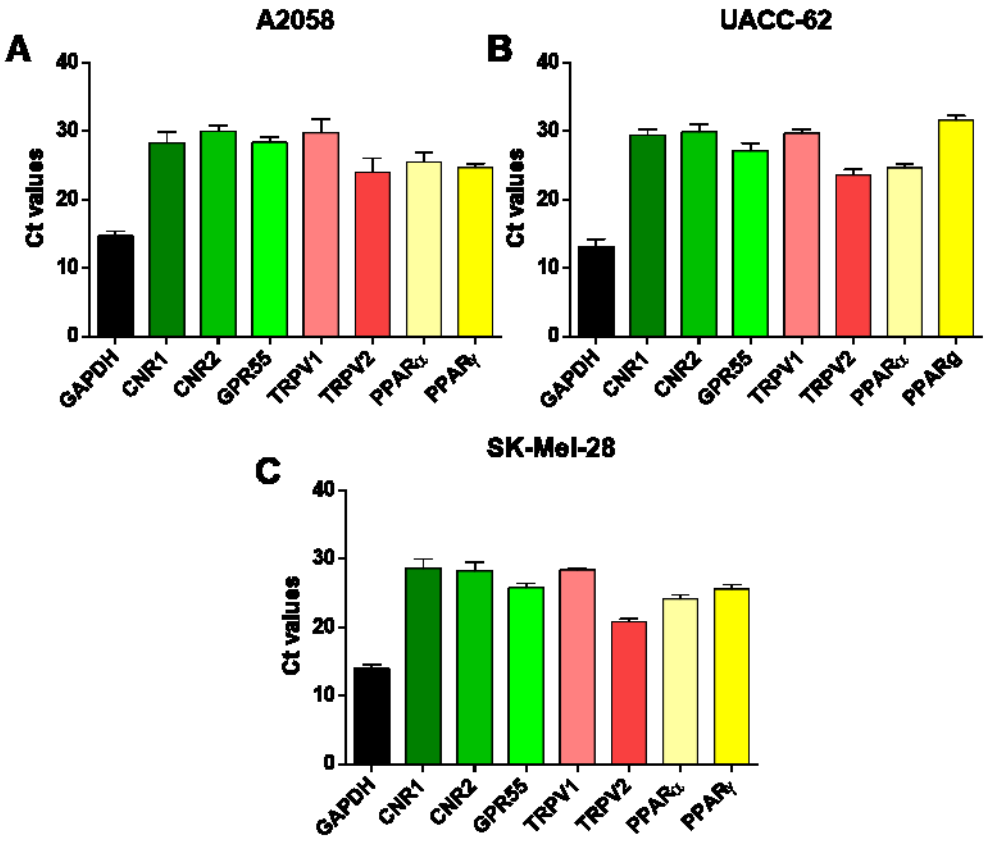

Figure 13. Expression of receptors known to be targeted by cannabinoids on different melanoma cell lines.

Total RNA from A2058, UACC-62 and SK-Mel-28 melanoma cells was isolated and cDNA synthesis was performed. All receptors were expressed on transcriptional levels with higher amounts of TRPV2, PPARα and GPR55 in A2058 (A), UACC-62 (B) and SK-Mel28 (C) cells. Data is shown as absolute Ct values. Experiments were performed at least five times in each cell line. Data are presented as mean ± SD.

Real-time PCR revealed that in A2058 cells *CNR1, CNR2, GPR55* and *TRPV1* showed lower mRNA levels than *TRPV2, PPARα and PPARγ*, respectively (**Figure 13A**). Similar findings were made in UACC-62 (**Figure 13B**) and SK-Mel-28 (**Figure 13C**) cells although in UACC-62 cells there were also lower levels of PPARγ mRNA.

Figure 14. Genes involved in cannabinoid signaling are rarely mutated in melanoma.

Mutations in CNR1, PPARA and TRPV1 have been looked up by using cbioportal.org (Accessed 19.11.2018). Query has been performed in the Cancer Cell Line Encyclopedia from Broad Institute and Novartis (A)(n=1020) and melanoma patient samples from The Cancer Genome Atlas (B)(n=366).
(Figure 14 corresponds to SFigure 2G-F of the original publication by Richtig et al [183])

Of clinical and scientific importance was the question, how common mutations in cannabinoid signaling pathways are, since these might contribute to primary resistance. Unfortunately, there was no information in the Cancer Cell Line Encyclopedia stored on *CNR1* and *TRPV1* mutations in 1020 cancer cell lines. *PPARA* alterations occurred only in 1.7% of all cases (**Figure 14A**). In samples, from melanoma patients the most common mutation was found in *CNR1* (3% of all cases) followed by *PPARA* (2.7%) and *TRPV1* (1.1%)(**Figure 14B**). Overall, all genes are rarely mutated in melanoma and therefore highly suitable as drug targets.

Caspase related activation of apoptosis

Apoptosis can be regulated by two different ways: the intrinsic pathway by activation of caspase 8 and by the extrinsic pathway by damaging mitochondrial integrity and further caspase 9/3 activation.[196] Cancer is known to interfere with this pathways to gain an advantage against normal cells.[197] This leads to longer survival and the accumulation of cell damage including mutations, which in turn increases invasiveness, tumor progression, neo-angiogenesis and deregulated cell proliferation.[198] A key element of the intrinsic apoptotic pathway activation is the involvement of the mitochondrial machinery, whereas intrinsic pathway activation leads to a disturbance of the mitochondrial membrane.[199]

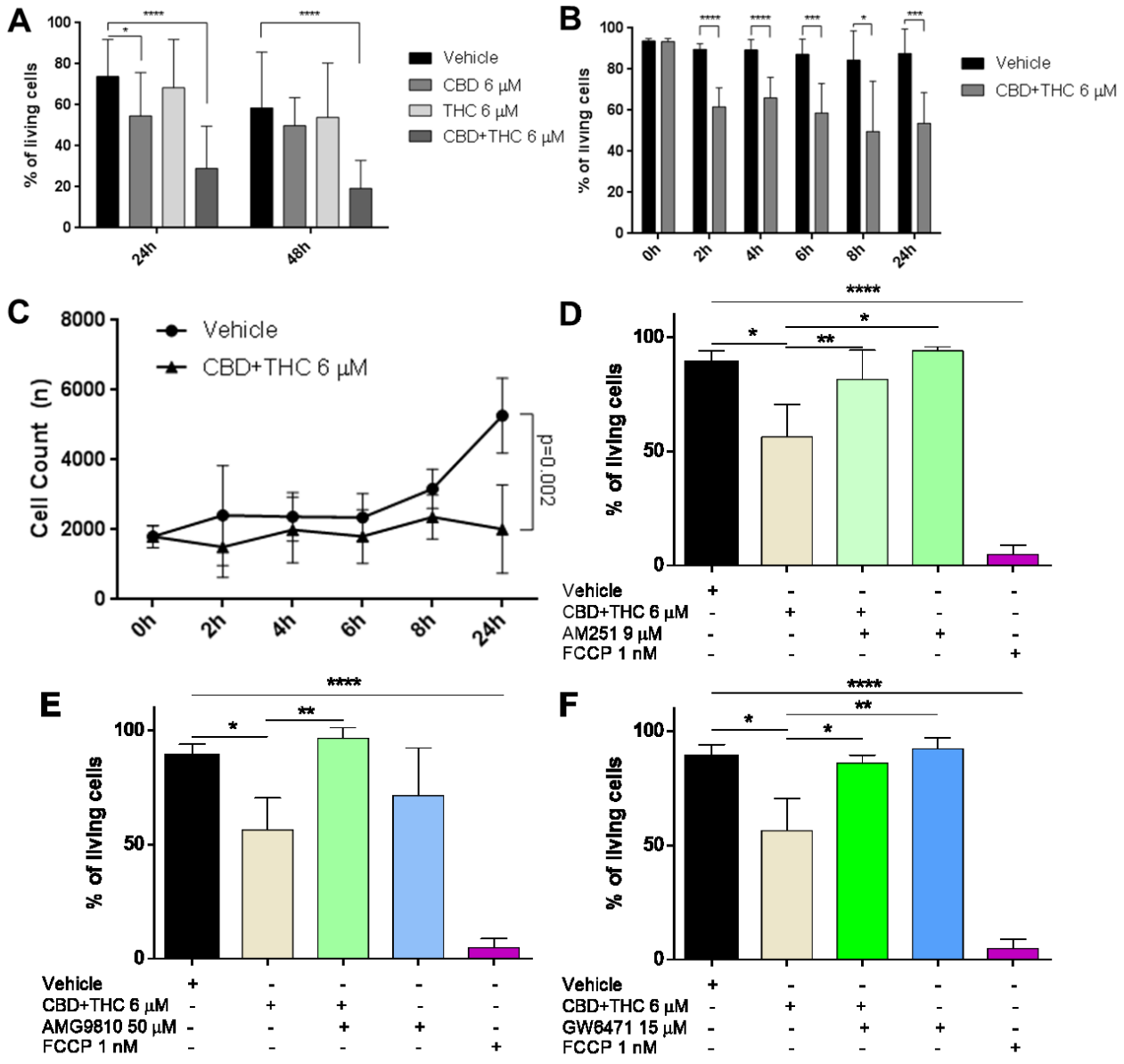

Figure 15. Cannabinoid treatment impacts mitochondrial depolarization in a time-dependent way.

A2058 cells were treated with cannabinoids (CT (6 µM), CBD (6 µM) and THC (6 µM)) for 0, 2, 4, 6, 8, 24 and 48 hours with and without inhibitors for CB1 (AM251), TRPV1 (AMG9810) and PPARα (GW6471) receptor. Mitochondrial depolarization was investigated by flow cytometry. Treatment with CBD and CT could increase mitochondrial depolarization (A) whereas mitochondrial depolarization is an early event (B). Cell count was reduced after 24 hours (C).

*Pretreatment with a CB1 (D), TRPV1 (E) or PPARα (F) receptor antagonist could significantly prevent mitochondria from depolarization. FCCP was used as positive control. Data are presented as mean ± SD. Experiments were performed at least five times. Statistically significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant. (Figure 15 corresponds to Figure 3A and SFigure 3A-E of the original publication by Richtig et al [183])*

Since all previous assays were performed within a timeframe of 24 hours, we assessed mitochondrial integrity by JC-1 FACS staining with time points of 24 and 48 hours. We could demonstrate that mitochondrial integrity was significantly impaired after 24 hours in the CBD (6 µM) and CT (6 µM) group and after 48 hours in the CT group (**Figure 15A**). Considering that mitochondrial impairment is a relatively early event in extrinsic pathway-mediated apoptosis, we performed JC-1 staining over a time course of 2, 4, 6, 8 and 24 hours. Mitochondrial impairment started as early as 2 hours after the treatment and remained significantly impaired throughout the time course investigated (**Figure 15B**). Although mitochondrial impairment started to be significant at 2 hours, there was no significant difference in cell counts by FACS until the 24-hour time point (**Figure 15C**). Investigating the receptors involved in cannabinoid signaling, we observed that AM251 (specific CB1 antagonist) significantly increased mitochondrial integrity when given prior to treatment (FCCP served as positive control) (**Figure 15D**). In addition, pre-treatment with a TRPV1 antagonist (AMG9810) (**Figure 15E**) or a PPARα-antagonist (GW6471) showed similar effects (**Figure 15F**).

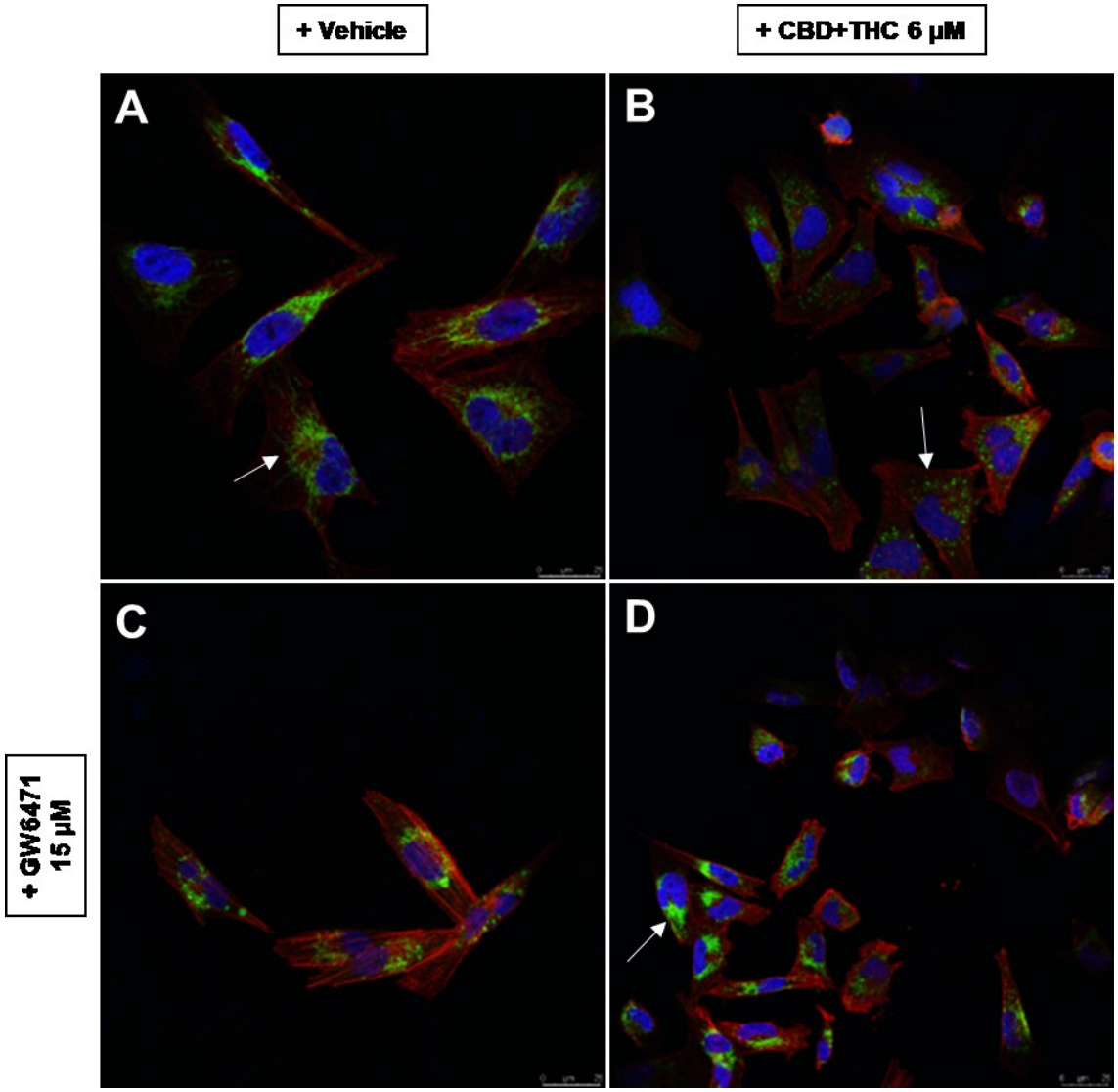

Figure 16. Treatment with CT leads to mitochondrial cytochrome c release.

Cells were treated with CT for 2 hours with or without prior treatment of PPARα antagonist (GW6471). After treatment, cells were stained for their nucleoli (DAPI staining, blue), cytochrome c (green) and their actin skeleton (phalloidin, red) and representative images were taken. In normal cells, cytochrome c is stored within the mitochondria that build a spider web like network within the cell (A, arrow). Under CT treatment cytochrome c is released into the cytosol resulting in fading fluorescence and loss of spider web structure (B, arrow). PPARα antagonist had little effect on cytochrome c release from mitochondria by its own (C) but could

reverse the effect of CT treatment (D, arrow).
(Figure 16 corresponds to Figure 3C of the original publication by Richtig et al [183])

Along with the loss of mitochondrial integrity goes the release of cytochrome c from the mitochondria into the cytosol. Under normal circumstances, cytochrome c is stored within the mitochondria insinuated by sharply confined long structures aggregated in confluent networks.[199] In line with literature, we could observe that untreated cells had this mitochondrial network within the cancer cells (**Figure 16A**, arrow). When CT was added, these networks were disrupted and cytochrome c was found at lower concentrations, aggregated in dots distributed along the cells (**Figure 16B**, arrow). Although PPARα antagonist (GW6471) seemed to have also an effect on cytochrome c release (**Figure 16C**), in combination with CT the mitochondrial networks could – at least partially – be restored (**Figure 16D**, arrow).

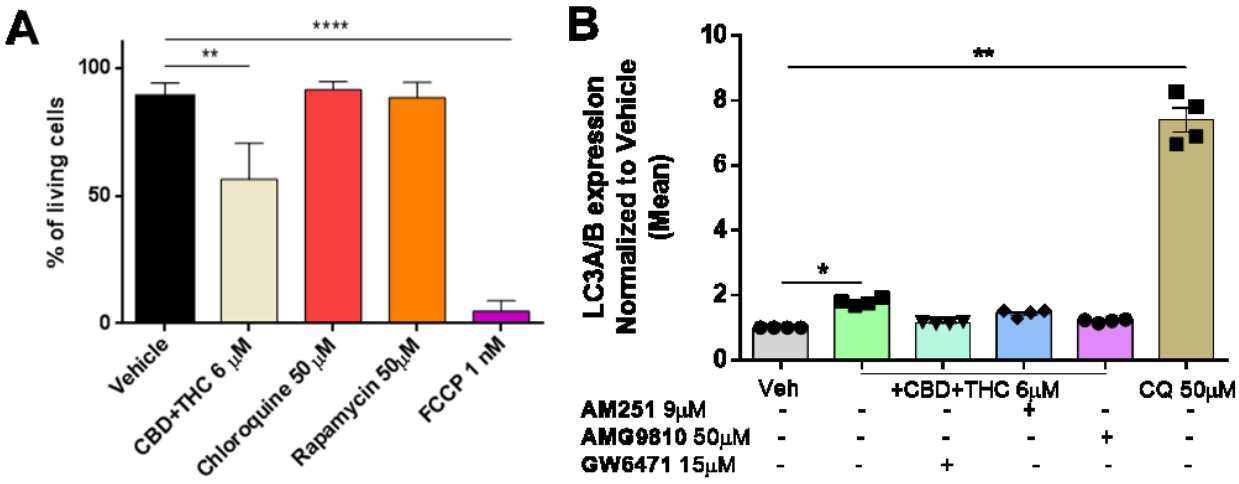

Figure 17. Cannabinoid treatment has little impact on the building of autophagosomes.

A2058 cells were treated with CT, rapamycin – an autophagy inducer – and chloroquine – an autophagy inhibitor - for 24 hours and mitochondrial depolarization was assessed by flow cytometry (A). Only CT significantly reduced mitochondrial depolarization, whereas rapamycin and chloroquine had no impact (A). Cells were treated with CT and CB1, TRPV1 and PPARα receptor antagonists and LC3A/B formation was assessed by flow cytometry (B). Chloroquine served as positive control for the accumulation of autophagosome formation. CT significantly induced autophagosome formation but all antagonists failed to reverse it back to baseline (B).

*Data are presented as mean ± SD. Experiments were performed at least three times. Statistically significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*
(Figure 17 corresponds to Figure 3D-E of the original publication by Richtig et al [183])

In the literature, it has been suggested that autophagy might be responsible for apoptosis in melanoma cells treated with cannabinoids.[169] Two substances associated with autophagy were rapamycin and chloroquine whereas the first is a known inducer of autophagy[200] and the latter an inhibitor of autophagy.[201] Since disturbance of mitochondrial integrity was a key element in cannabinoid-mediated apoptosis, we were first interested if two autophagy associated agents would have an impact on mitochondrial integrity. Neither chloroquine, nor rapamycin had a significant impact on mitochondrial membrane integrity in comparison to CT (**Figure 17A**). Autophagy is known to be mediated by forming autophagosomes that are consistent of LC3A/B bodies.[202] When investigating this by FACS staining, we could see that CT significantly induced autophagic body formation, although to a lesser extend compared to chloroquine (2x fold against 9x fold change) (**Figure 17B**). Importantly, TRPV1-, CB1- and PPARα-antagonists did not significantly reverse autophagosome formation in cells treated with CT.

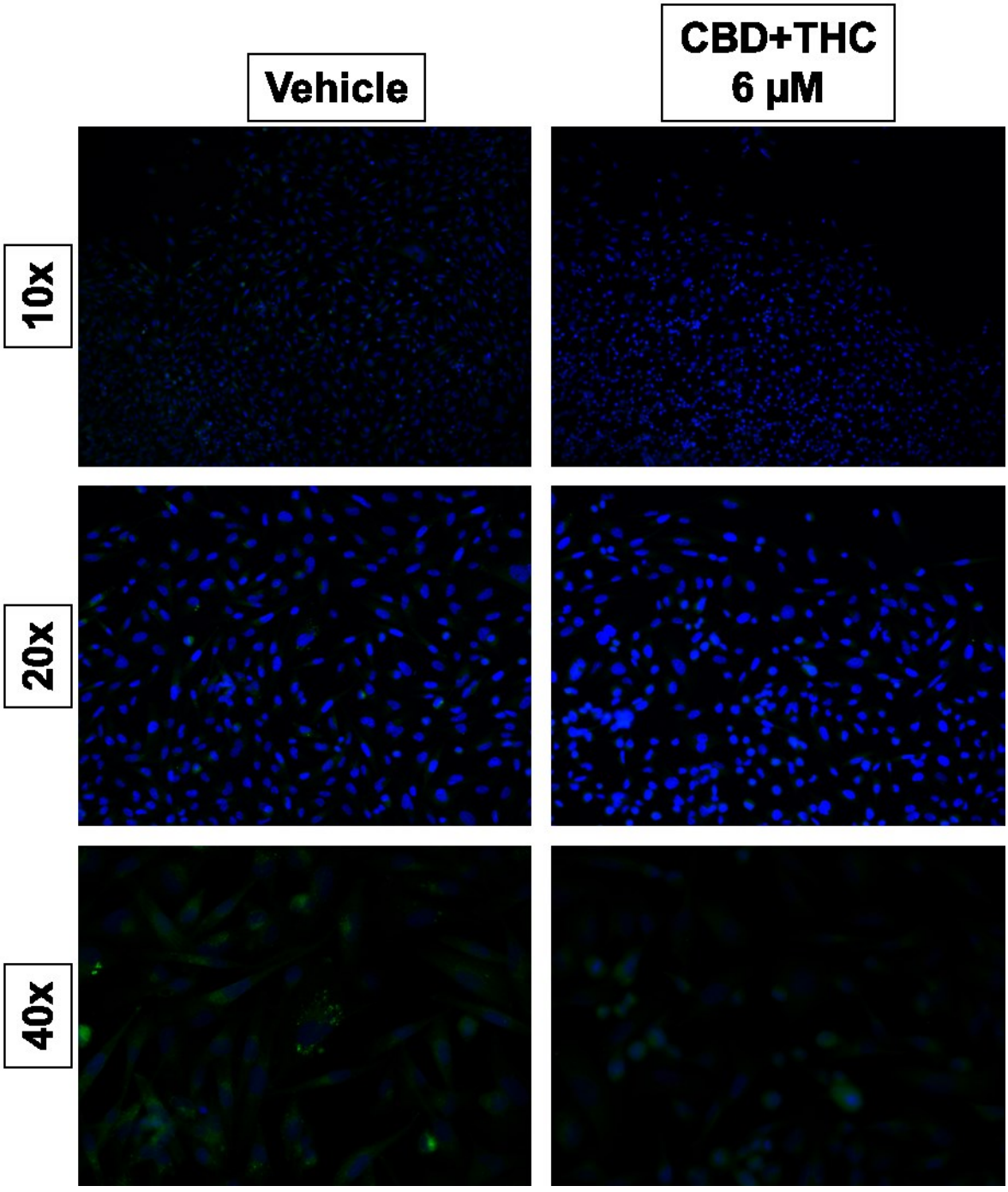

Figure 18. Cannabinoids have little impact on autophagosome formation.

Cells were treated with CT for 4 hours and immunofluorescence against DAPI (blue) and LC3A/B (green) was performed. Representative images are shown. There was no increase in autophagosome formation in CT treated cells.

Visualization by immune fluorescence microscopy demonstrated, that there was only a visual moderate to no increase in LC3A/B body formation in CT treated melanoma cells (**Figure 18**).

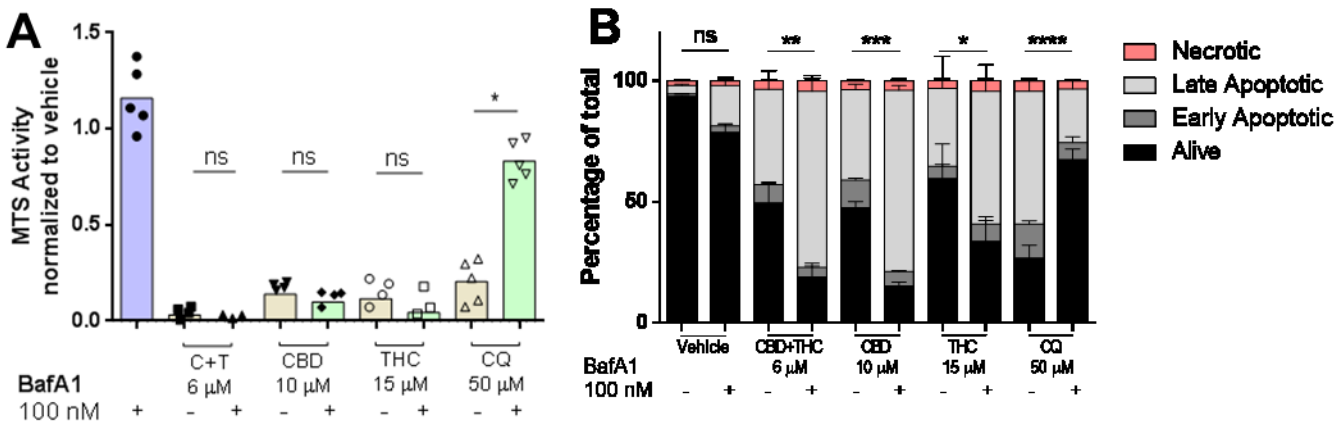

Figure 19. Inhibition of autophagosome formation could enhance the pro-apoptotic effect of cannabinoids.

*Cell were pre-treated with an autophagosome inhibitor (Bafilomycin A1; BafA1) and then with CT, CBD, THC or chloroquine for 24 hours followed by assessment of cell viability (A) and apoptosis (B). BafA1 was unable to increase cell viability in cells treated with cannabinoids (A) but could increase the degree of apoptosis (B). Data are presented as mean ± SD. Experiments were performed at least five times. Statistically significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*
(Figure 19 corresponds to Figure 3F-G of the original publication by Richtig et al [183])

Bafilomycin A1 (BafA1) is an ATPase inhibitor that blocks the autophagic flux acutely by inhibiting autolysosome acidification and autophagosome–lysosome fusion and has been widely used to pharmaceutically block autophagy formation.[203–205] It has been shown that BafA1 could resensitize cancer cells to anti-tumorous therapies suggesting that autophagy is of importance for cancer cells under cellular stress.[206–208] For melanoma and cannabinoids, it has been proposed that autophagy might be necessary for successful apoptosis in melanoma cells under THC treatment.[169] Chloroquine (50 µM) successfully induced apoptosis in melanoma cells after 24 hours treatment period and this effect was significantly reduced by the autophagy inhibitor BafA1 (**Figure 19A & B**). However, BafA1 did not restore cell viability when given in combination

with CT (6 µM), CBD (10 µM) or THC (15 µM) (**Figure 19A**). To see if BafA1 could have any effects that induce apoptosis in cannabinoid-treated cells, we performed an annexin-V/PI co-staining and saw that BafA1 significantly increased the amount of non-viable cannabinoid treated cells whereas BafA1 itself had no significant impact on cell viability (**Figure 19B**).

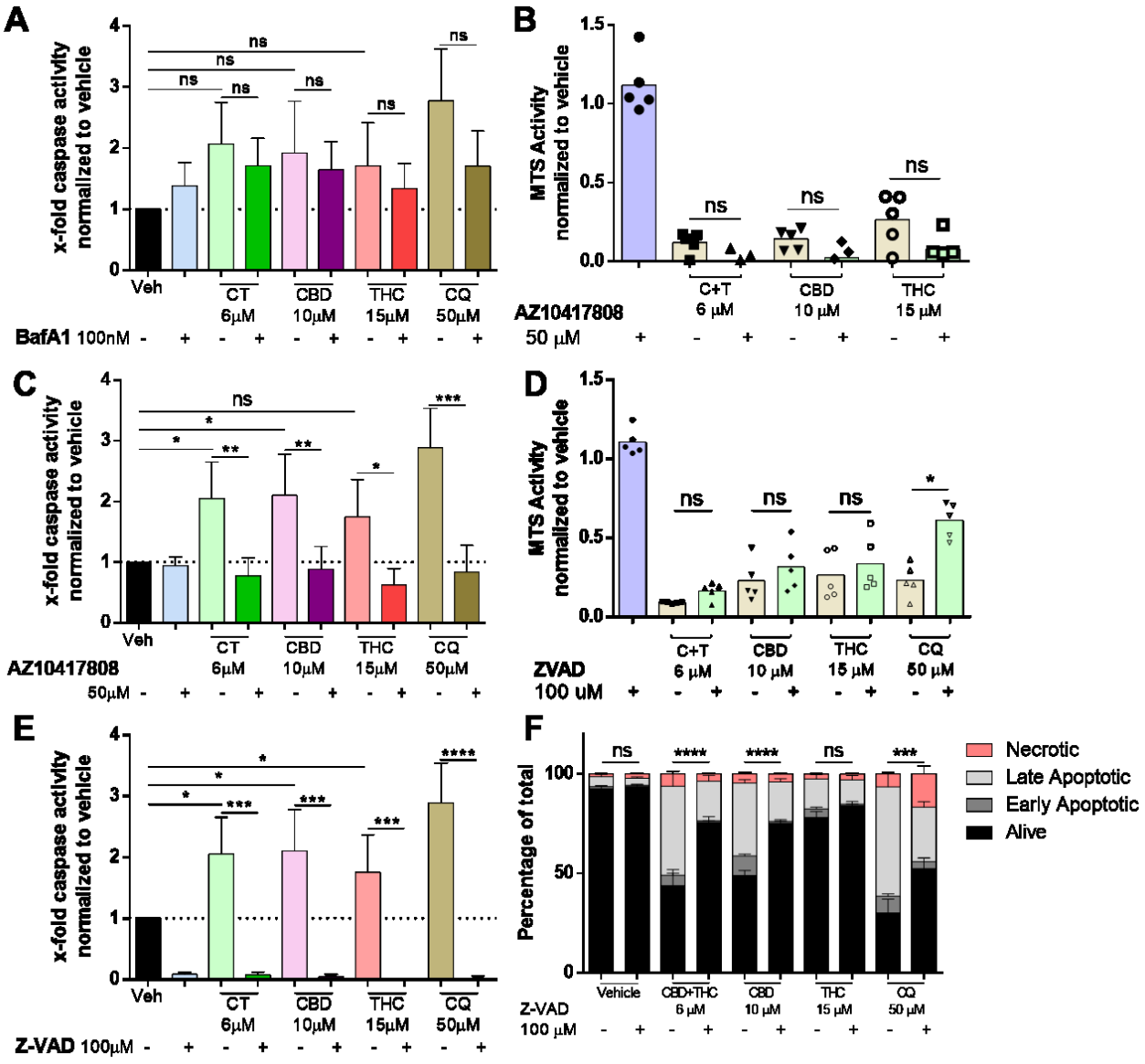

Figure 20. Cannabinoids mediate their apoptotic effect in a caspase-dependent fashion.

A2058 cells were pre-treated with antagonists against autophagosome formation (BafA1)(A), caspase-3 (AZ10417808) or pan-caspase (Z-VAD) followed by cannabinoid and chloroquine

*treatment. Caspase activation was assessed by caspase Glo-assay (A, C, E), cell viability by MTS-assay (B, D) and apoptosis by annexin-V/PI-co-staining (F) after 24 hours. Pre-treatment with BafA1 was unable to significantly decrease caspase-3/7 expression in cannabinoids and chloroquine treated cells (A). Caspase-3 specific inhibitor AZ10417808 had no significant impact on cell viability (B) but could significantly reduce caspase-3/7 expression (C). Similar effects on cell viability (D), caspase-3/7 expression (E) and apoptosis (F) were seen when the pan-caspase inhibitor Z-VAD was used. Data are presented as mean ± SD. Experiments were performed at least five times. Statistically significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*
(Figure 20 (A,C,E-F) corresponds to Figure 4 of the original publication by Richtig et al [183])

Caspases are widely known as the key modulators of apoptotic cell death.[209] Common caspases activated by extrinsic and intrinsic apoptotic pathway are caspases-3, -6 and -7.[210] Whereas the extrinsic pathway is mainly activated by caspase-8 and by death ligands, the intrinsic pathway is regulated by cellular stress including disturbance of mitochondrial integrity.[210] A recent report suggested that BafA1-mediated cell death is independent from caspase activation in hepatocellular carcinoma cells.[211] BafA1 was not capable of significantly reducing caspase 3/7 activity in A2058 cells treated for 24 hours with CT (6 µM), CBD (10 µM), THC (15 µM) or CQ (50 µM)(**Figure 20A**). AZ10417808 - a selective caspase-3 inhibitor[212] - was used to further investigate, if caspase-3 plays a crucial role in cannabinoid induced cell death as cannabinoids increase caspase 3/7 activity (**Figure 20A**). In MTS assay AZ10417808 did not significantly increase cell viability, on the contrary, it reduced cells' viability (**Figure 20B**). In the caspase 3/7 Glo assay, AZ10417808 reduced caspase 3/7 activity back to baseline in all cannabinoid treated cells as well as in CQ treated cells (**Figure 20C**). To broaden the approach of caspase-mediated cell death, we used the pan-caspase inhibitor Z-VAD-MFK (ZVAD). Interestingly, in the MTS assay ZVAD was only capable of increasing cell viability in CQ-treated cells but not in cannabinoid-treated cells (**Figure 20D**). However, ZVAD successfully reduced caspase activity to zero in CT (6 µM), CBD (10 µM), THC (15 µM) and CQ (50 µM) treated cells (**Figure 20E**). To finally assess whether ZVAD can prevent cannabinoid apoptosis, annexin-V/PI staining was conducted. Except in THC (15 µM) treated cells, ZVAD significantly increased the fraction of viable cells (**Figure 20F**).

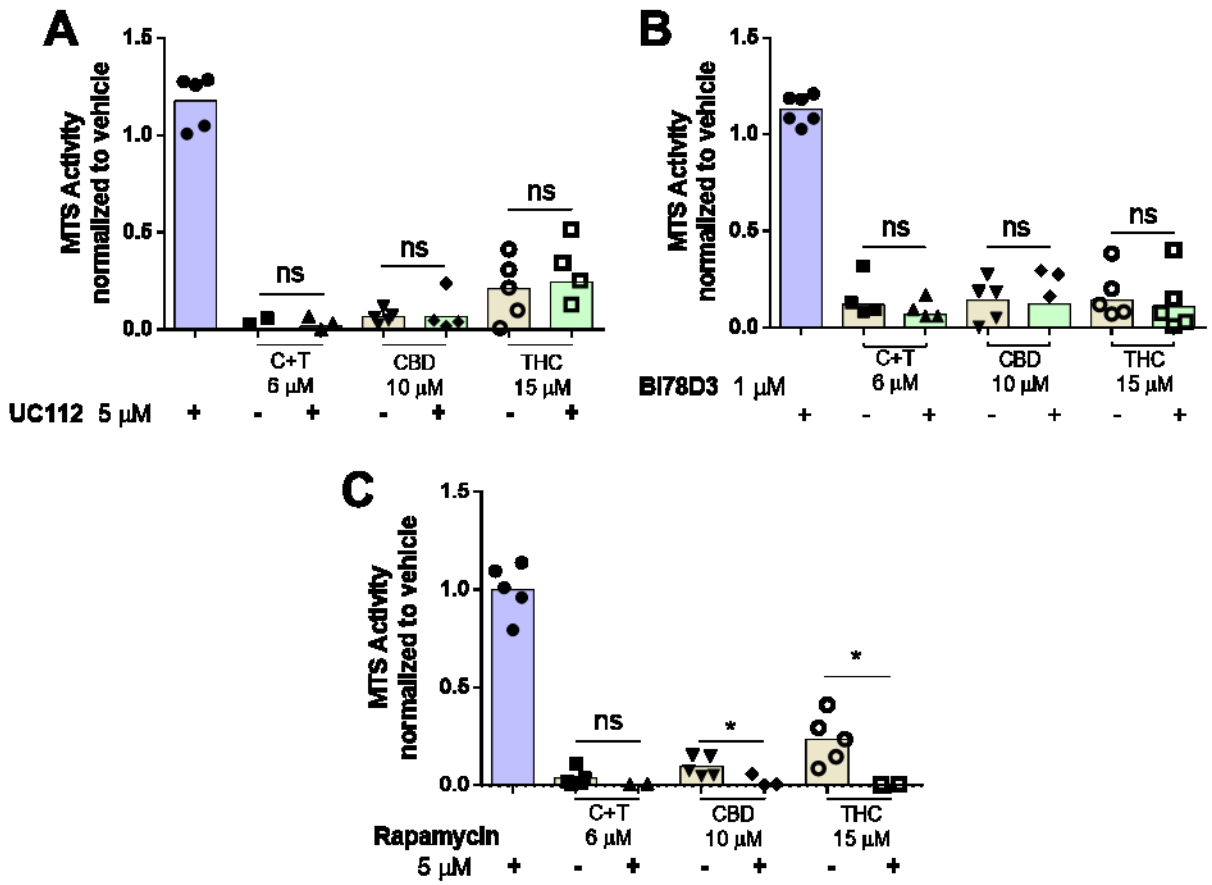

Figure 21. Inhibitors of IAP and XIAP, JNK and mTOR could not restore cannabinoid-compromised cell viability.

*A2058 cells were pre-treated with inhibitors of inhibitors of apoptosis proteins (IAP)(UC112 (5 µM)), c-Jun N-terminal kinases (JNK)(BI78D3 (1 µM)) or mammalian target of rapamycin (mTOR)(rapamycin (5 µM)) followed by treatment with cannabinoids (CT (6 µM), CBD (10 µM) and THC (15 µM)) for 24 hours. Inhibitors against IAP (A), JNK (B) or mTOR (C) could not significantly increase cell viability. Data are presented as mean ± SD. Experiments were performed at least five times. Statistically significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*

(Figure 21 corresponds to SFigure 3F-H of the original publication by Richtig et al[183]*)*

Apoptosis is a highly complex process and often dysregulated in cancer. Therefore, several drugs are now under clinical evaluation that interact somewhere within this signal cascade to promote cancer cell death.[213] Inhibitors of apoptosis proteins (IAPs) include proteins that modulate many pathways including the MAPK pathway and pathways involved in mitochondrial integrity.[214] IAPs have been suggested to have potential anti-cancer effects, since they form a complex with survivin, which is commonly upregulated in cancer and inhibits caspase-3/-6/-7 mediated apoptosis.[215] However, UC112 - an IAP and XIAP inhibitor - could not restore cell viability in cannabinoid-treated A2058 cells (**Figure 21A**). Another important family of kinases associated with programmed cell death are the Jun N-terminal kinases (JNKs). JNKs are key players involved in apoptosis, ferroptosis, pyroptosis, autophagy and necroptosis.[216] However, it was interesting to note that BI78D3 – a competitive JNK inhibitor – could not restore cell viability in cannabinoid treated A2058 cells (**Figure 21B**). Rapamycin is an inhibitor of 'mechanistic target of rapamycin' kinase (mTOR) and some mTOR inhibitors have already been approved for cancer therapies.[217] MTOR itself regulates many pathways in cancer including the important PI3K pathway, that is commonly upregulated in melanoma with primary resistance to BRAFi/MEKi therapy.[92,218] Indeed, it seems that cannabinoids stimulate the PI3K pathway, since rapamycin significantly reduced cell viability when given in combination with CBD (10 μM) and THC (15 μM)(**Figure 21C**).

Cannabinoids can help to re-sensitize melanoma cells with primary resistance to targeted therapy

When it comes to primary resistance to targeted therapy in melanoma, mutations that prevent efficacy of BRAFi/MEKi, can occur upstream or downstream of their respective molecular targets. On one hand some *BRAF*$^{non\text{-}V600}$ mutations with an increased kinase activity might be targetable by BRAFi/MEKi inhibitors, but on the other hand *BRAF*$^{non\text{-}V600}$ mutations with a decreased or no kinase activity are resistant to BRAFi/MEKi therapy.[92] Another mechanism to prevent successful targeted therapy are mutations in downstream targets including *MAP2K1* and upstream targets including *NRAS*.[90,91] In the former case, downstream ERK activation is warranted by autonomous activation of MAP2K1 and in the latter case, *NRAS* mutations lead to activation of the PI3K and other escape pathways. Therefore, melanoma cells do not solely rely on the MAPK pathway.[219] With this in mind, it would be of major clinical interest if cannabinoids

might be effective in such tumors, re-sensitize cells back to targeted therapy or at least have no pro-carcinogenic effects. In A2058 cells, which are harboring a *BRAF*^{V600E} mutation in combination with a *MAP2K1*^{P124S} mutation (detailed in **Table 1**), we could see that CBD and CT were capable of reducing cell viability in a concentration-dependent way (**Figure 4C**). THC had no effect on the viability of these cells in the tested concentration range.

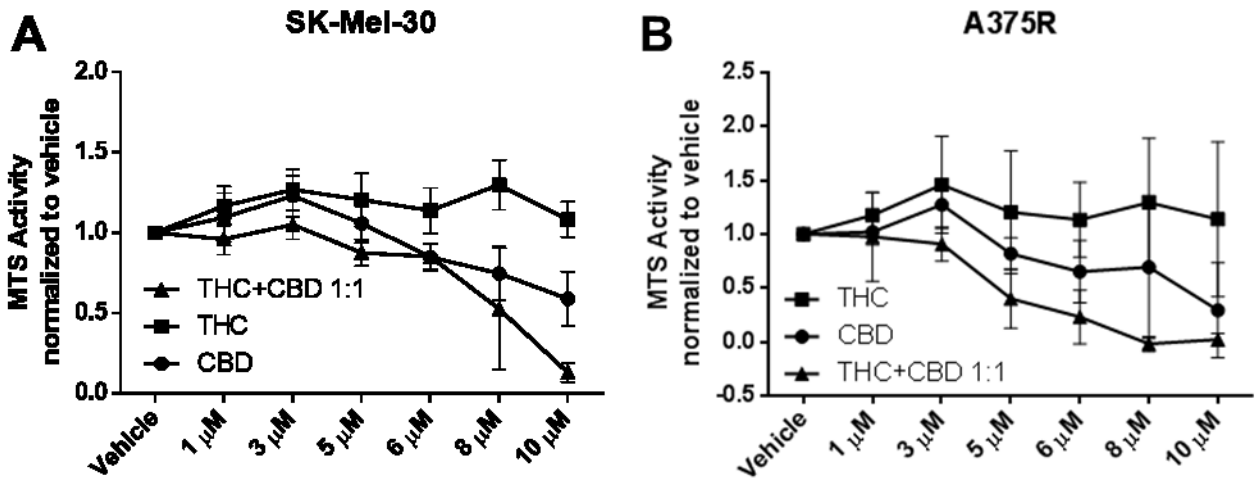

Figure 22. Cannabinoids mediate different effects on cell viability in two melanoma cell lines resistant to BRAF inhibitor therapy in a concentration-dependent way.

SK-Mel-30 and A375R melanoma cells were treated with different concentrations (1, 3, 5, 6, 8 and 10 µM) of CBD, THC or a 1:1 combination of THC and CBD for 24 hours. Cell viability was measured by MTS assay. Data are presented as mean ± SD. Experiments were performed at least three times.

SK-Mel-30, a cell line harboring a *BRAF*$^{D287H/E275K}$ (*BRAF*$^{non\text{-}V600}$) mutation in combination with a *NRAS*^{Q61K} (**Table 1**) mutation showed similar sensitivity against cannabinoids as A2058. CT and CBD reduced cell viability in a concentration dependent matter, whereas THC had no effect (**Figure 22A & Figure 4C**). A375R, a clone of the widely used A375 cell line that was made resistant to BRAF inhibitor therapy[220], confirmed the results gathered from the two previously used cell lines. CT and CBD reduced cell viability in a concentration-dependent way and THC had no effect (**Figure 22B**).

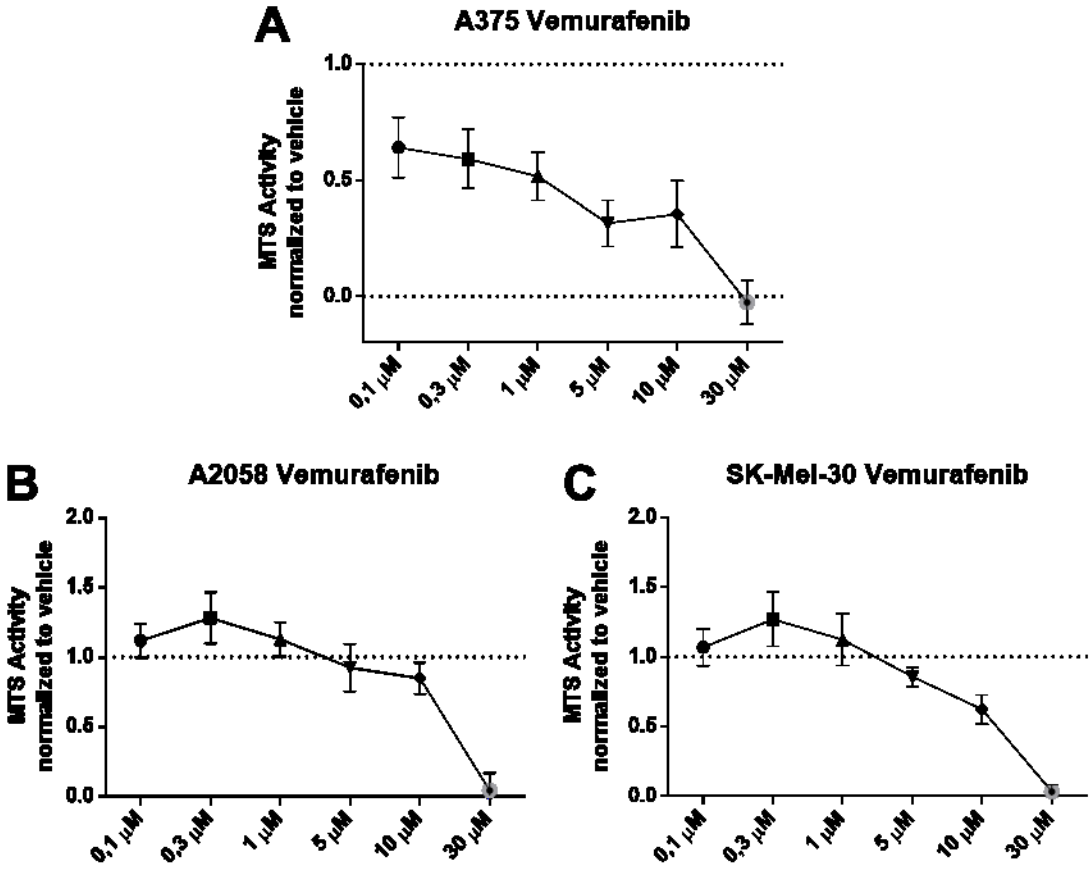

Figure 23. Metastatic melanoma cell lines with *BRAF*$^{non\text{-}V600}$ mutation show resistance to BRAF inhibitor treatment in a concentration-dependent fashion.

SK-Mel-30 and A375R melanoma cells were treated with different concentrations (0.1, 0.3, 1, 5, 10 and 30 µM) of the BRAF inhibitor Vemurafenib. Cell viability was assessed by MTS assay 24 hours later. Non-metastatic A375 melanoma cells showed a concentration-dependent reduction of cell viability even in the low concentration range (A). The BRAF$^{non\text{-}V600}$ *metastatic melanoma cell lines A2058 (B) and SK-Mel-30 (C) showed reduced cell viability when vemurafenib was given in high-concentrations. Data are presented as mean ± SD. Experiments were performed at least three times.*

(Figure 23A corresponds to Figure 5A of the original publication by Richtig et al [183]*)*

One of the BRAF inhibitors on the market, approved for the treatment of *BRAF*^{V600E} mutated metastatic melanoma and widely used clinically, is vemurafenib (formerly known as PLX4032).

To test if our cells are sensitive to this inhibitor, we performed a concentration-dependent cell viability assay. The *BRAF*^{V600E} mutated melanoma cell line A375 showed a concentration-dependent reduction of cell viability (**Figure 23A**) which is in line with the literature.[221,222] For A2058 cells, there was no effect on cell viability up to 30 µM (**Figure 23B**) and in SK-Mel-30 cells up to 10 µM (**Figure 23C**). These results confirmed that the normal *BRAF*^{V600E} mutated cell line A375 was sensitive to BRAF inhibitor therapy, whereas the latter two cell lines harboring mutations known for primary resistance to targeted therapy were resistant to BRAF inhibitor therapy.

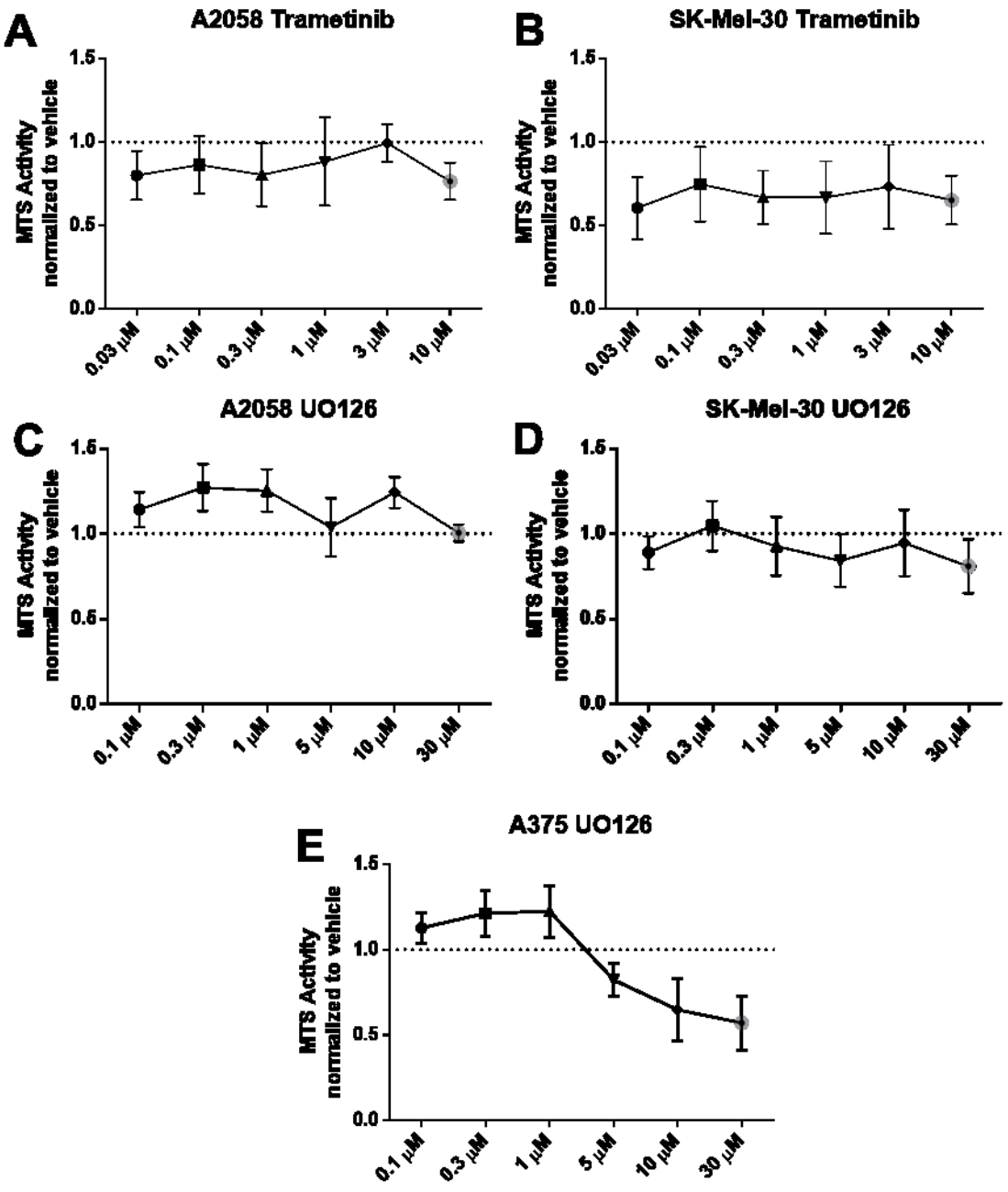

Figure 24. Metastatic melanoma cell lines with *BRAF*$^{non\text{-}V600}$ mutation show different resistance pattern to MEK and ERK inhibitor therapy.

A2058, SK-Mel-30 and A375 melanoma cells were treated with different concentrations (0.03, 0.1, 0.3, 1, 3 and 10 μM) of the MEK inhibitor trametinib or with different concentrations (0.1, 0.3, 1, 5, 10 and 30 μM) of the ERK inhibitor UO126. Cell viability was assessed by MTS assay

24 hours later. A2058 cells showed no reduction in cell viability at all concentrations of trametinib tested (A), whereas SK-Mel-30 cells showed reduced viability upon trametinib treatment (B). ERK inhibitor treatment did not reduce cell viability in A2058 (C) and SK-Mel-30 (D) cells. ERK inhibitor treatment did reduce cell viability in a concentration-dependent way in non-metastatic A375 cells (E). Data are presented as mean ± SD. Experiments were performed at least three times.
(Figure 24A corresponds to SFigure 4A of the original publication by Richtig et al [183])

The downstream target of BRAF is MEK and BRAF inhibitors are nowadays used in patients in combination with MEK inhibitors due to the occurrence of de novo tumors under BRAF inhibitor mono-therapy.[89] Therefore, we were interested if cells used in this study were intrinsically resistant to a clinically approved MEK inhibitor (trametinib, formerly known as GSK1120212). Trametinib had only little effect on the viability of A2058 cells (**Figure 24A**) whereas it showed some activity in SK-Mel-30 cells (**Figure 24B**). Downstream of MEK is ERK, its activation leads to cell survival and cell proliferation. Therefore, blocking ERK is of major clinical interest.[223] Pharmacological ERK inhibition by UO126 did not reduce cell viability in A2058 (**Figure 24C**) or SK-Mel-30 (**Figure 24D**) cells whereas in the BRAF/MEK inhibitor-sensitive $BRAF^{V600E}$ mutated A375 cells UO126 reduced cell viability in a concentration-dependent way (**Figure 24E**).

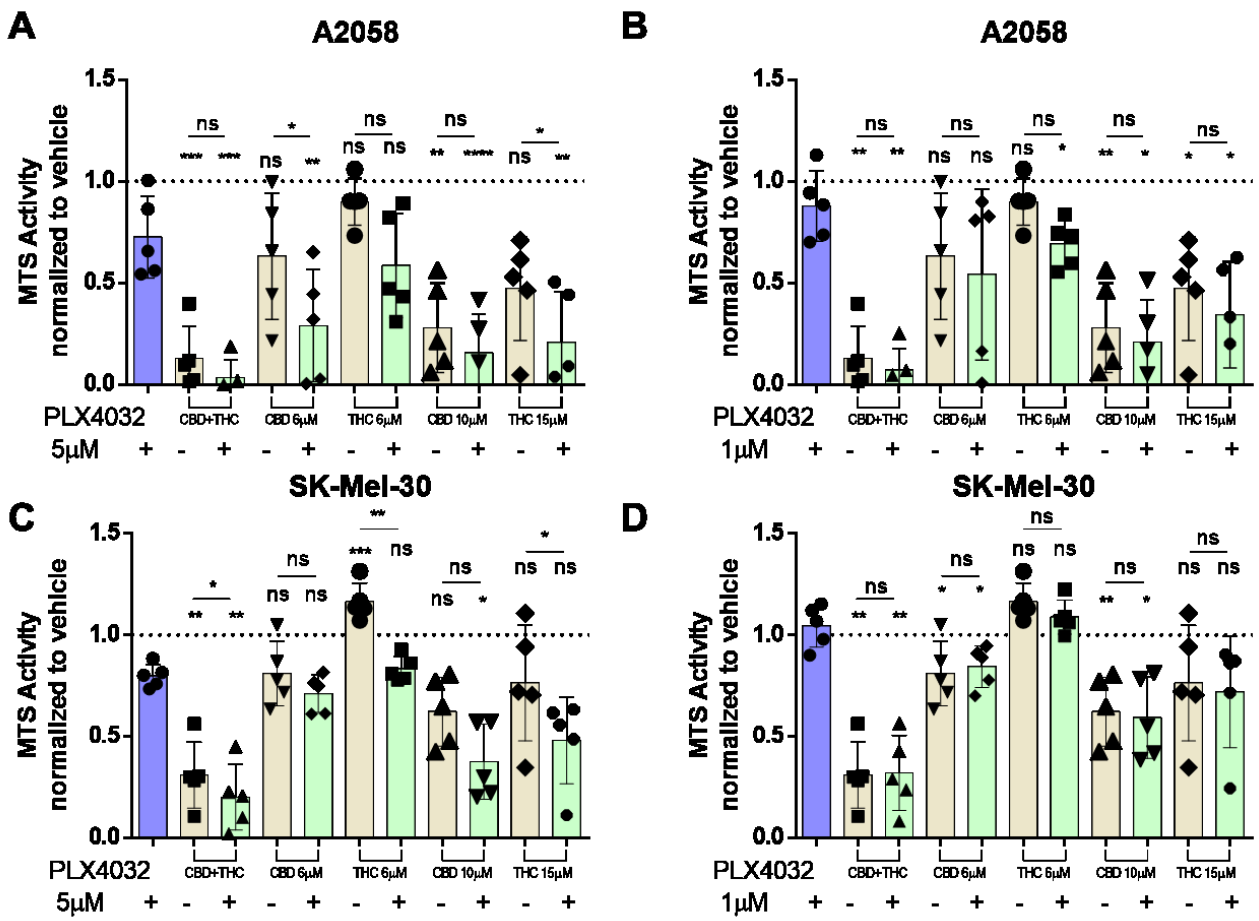

Figure 25. BRAF inhibitor therapy has no antagonistic effect on cannabinoid treatment in metastatic *BRAF*$^{non\text{-}V600}$ melanoma cell lines.

*A2058 and SK-Mel-30 melanoma cell lines were pre-treated with PLX4032 (vemurafenib) and afterwards incubated with different concentrations of cannabinoids (CT (6 µM), CBD (6 µM), CBD (10 µM), THC (6 µM), THC (15 µM)). After 24 hours cell viability was assessed by MTS assay. High-concentration (A) and low-concentration (B) PLX4032 did not restore cell viability in A2058 cells. Similar findings were made in SK-Mel-30 cells when high-concentration (C) and low-concentration (D) PLX4032 was given. Data are presented as mean ± SD. Experiments were performed five times. Statistically significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*

(Figure 25A-B corresponds to Figure 5D-E of the original publication by Richtig et al [183]*)*

Due to the legalization of cannabis and the fact that medical cannabis can be used for several conditions, it is highly likely that cannabis will be taken by patients under anti-cancer therapies.[157] In addition, there are several promises being made in the internet that cannabinoids can help in stage IV cancer, increasing the chance that patients are going to take such drugs after unsuccessful primary therapy.[156] When high-concentration (5 μM) of PLX4032 (vemurafenib) was given in combination with cannabinoids in A2058 cells, we observed that PLX4032 was capable of significantly reducing cell viability in cells receiving it in combination with CBD (6 μM) and THC (15 μM), respectively (**Figure 25A**). Although, there was a trend towards reduced cell viability, when PLX4032 was given in all other tested combinations (+CBD (10 μM), +CT (6 μM) or +THC (6 μM)), results did not reach statistical significance. When PLX4032 concentration was reduced to 1 μM, it failed to enhance any cannabinoid treatment effects (**Figure 25B**). Conversely and in both used concentrations of PLX4032, it did not increase cell viability in any tested cannabinoid concentration (**Figure 25A & B**). When high-concentration PLX4032 was used in SK-Mel-30 cells, it significantly augmented the efficacy of CT (6 μM), THC (6 μM) and THC (15 μM) (**Figure 25C**). However, this effect vanished when PLX4032 concentration was reduced to 1 μM (**Figure 25D**). Similar to A2058 cells, PLX4032 could not increase SK-Mel-30s cell viability, when given in combination with any cannabinoid (**Figure 25C & D**).

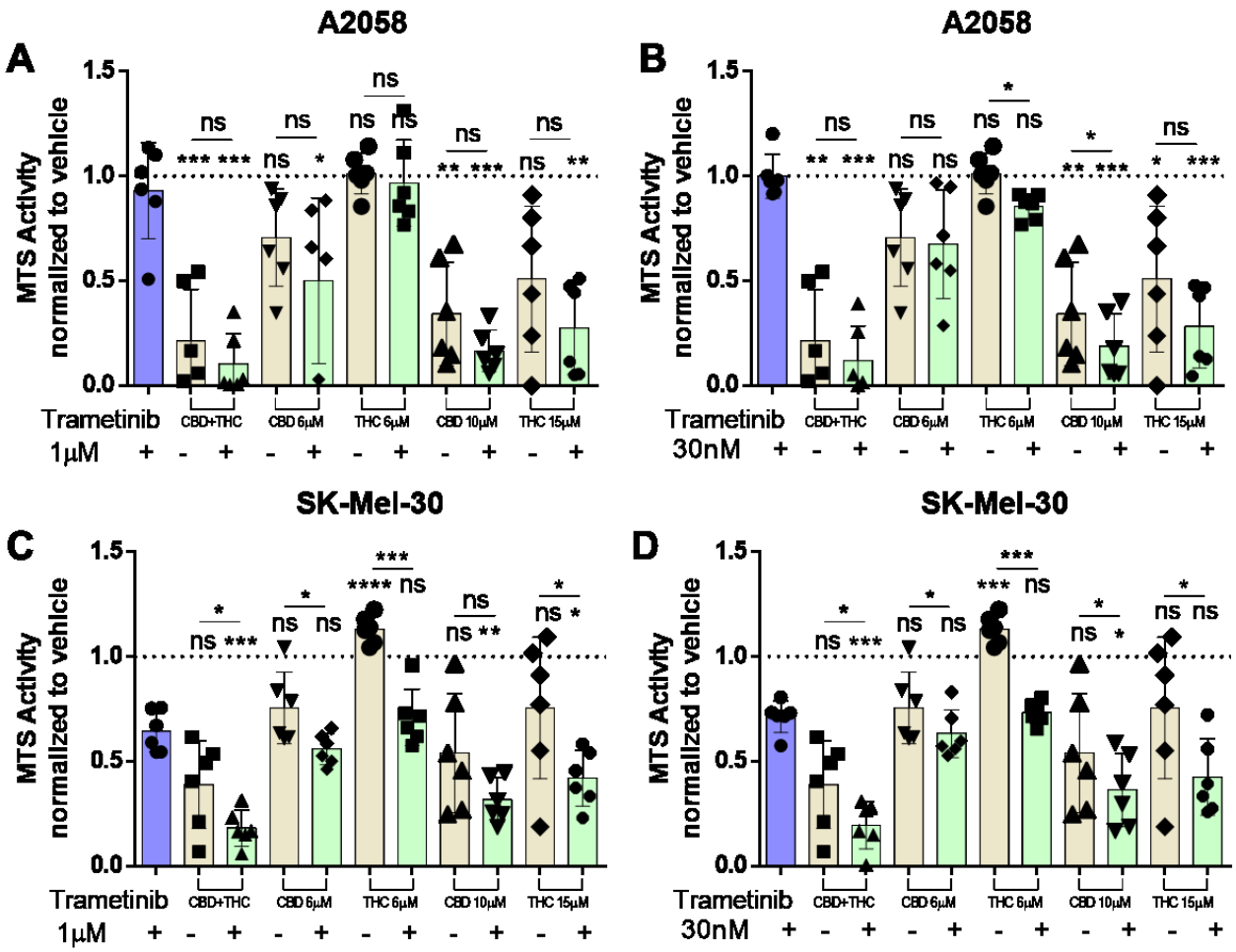

Figure 26. MEK inhibitor treatment has no antagonistic effect on cannabinoid treatment in metastatic *BRAF*$^{non\text{-}V600}$ melanoma cell lines.

*A2058 and SK-Mel-30 melanoma cell lines were pre-treated with trametinib and afterwards with different concentrations of cannabinoids (CT (6 µM), CBD (6 µM), CBD (10 µM), THC (6 µM), THC (15 µM)). After 24 hours, cell viability was assessed by MTS assay. High-concentration (A) and low-concentration (B) trametinib did not restore viability of A2058 cells. In contrast, high-concentration (C) and low-concentration (D) trametinib pre-treatment significantly reduced cell viability, when given in combination with cannabinoids in SK-Mel-30 cells. Data are presented as mean ± SD. Experiments were performed five times. Statistically significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*
(Figure 26 corresponds to Figure 5B-C of the original publication by Richtig et al[183]*)*

When high-concentration (1µM) trametinib in combination with cannabinoids was used in A2058 cells, we observed no significant increase of efficacy in all combinations tested (**Figure 26A**). Reduction of the concentration to 30 nM increased the efficacy of THC (6 µM) and CBD (10 µM), respectively, in combination with trametinib (**Figure 26B**). In SK-Mel-30 cells, trametinib had a more distinct effect, when combined with cannabinoids. Increased efficacy was observed, when high-concentration trametinib was given in combination with CT (6 µM), CBD (6 µM), THC (6 µM) and THC (15 µM), respectively (**Figure 26C**). Even when the trametinib concentration was reduced to 30 nM, the combinatory effect of Trametinib with cannabinoids remained (**Figure 26D**). Similar to PLX4032, trametinib did not increase cell viability in all cannabinoid combinations tested (**Figure 26C & D**).

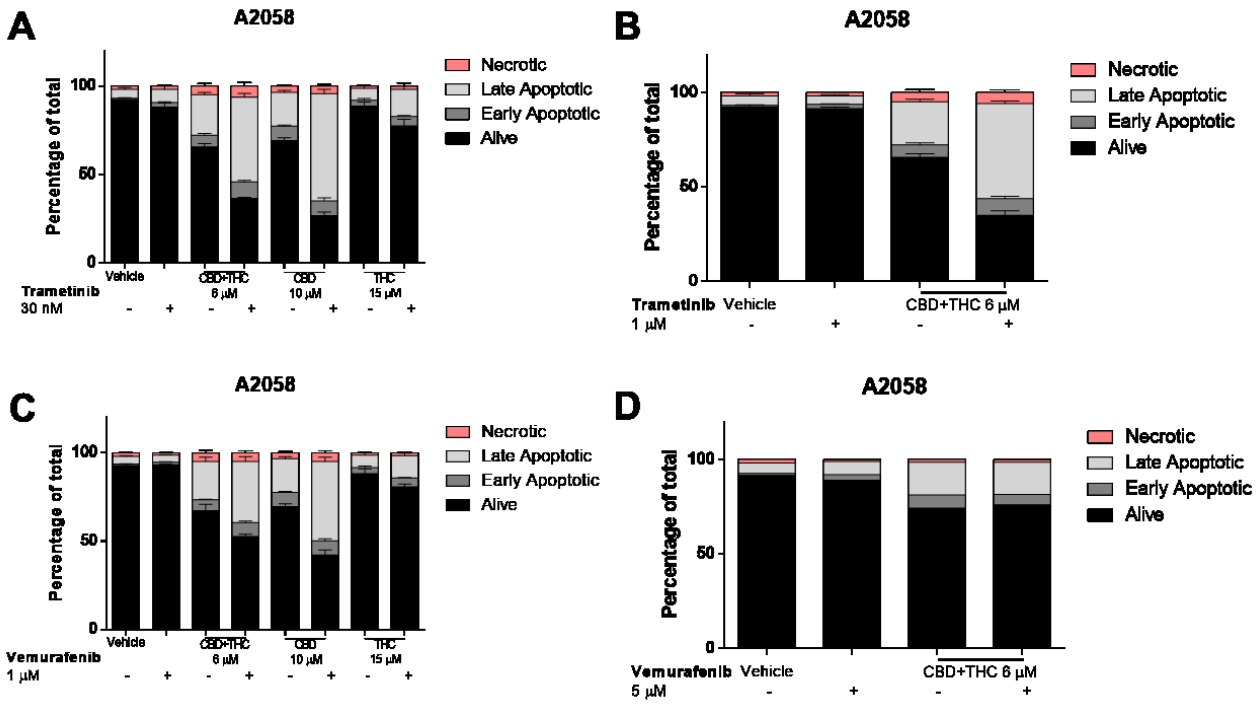

Figure 27. MEK and BRAF inhibitor treatment can have synergistic effects in cells treated with cannabinoids.

A2058 cells were pre-treated with the MEK inhibitor trametinib or the BRAF inhibitor vemurafenib in high- or low-dose followed by differential cannabinoid treatment. After 24 hours, apoptosis was investigated by flow cytometric annexin-V/PI-co staining. Trametinib enhanced cannabinoid effect at low (A) as well as high concentrations (B). In vemurafenib-treated cells,

only the 1 µM concentration (C) enhanced cannabinoids apoptotic effects in contrast to 5 µM (D). Data are presented as mean ± SD. Experiments were performed five times.

Since we could observe, that cannabinoids might have a synergistic effect on apoptosis in cells pre-treated with clinically used targeted therapy, we were further interested if this effect is directly related to increased apoptosis. Therefore, we performed annexin-V/PI-co-staining. Trametinib itself had no significant effect on apoptosis in low- (**Figure 27A**) or high-dosage (**Figure 27B**) but increased apoptosis in low- (**Figure 27A**) and high-dose (**Figure 27B**) when given in combination with CT (6 µM). In addition, low-concentration trametinib was also capable of increasing the degree of apoptosis in high-concentration (10 µM) CBD treated cells. No significant increase was observed in high-concentration THC (15 µM) treated cells (**Figure 27A**). When the BRAF inhibitor vemurafenib was used, there was only a significant increase in apoptosis, if low-concentration vemurafenib was given in combination with high-concentration CBD (**Figure 27C**). Interestingly, this effect was not observed, when high-concentration THC or CT (6 µM) was used. Even when high-concentration (5 µM) vemurafenib was given in combination with CT, there was no significant increase in apoptosis (**Figure 27D**).

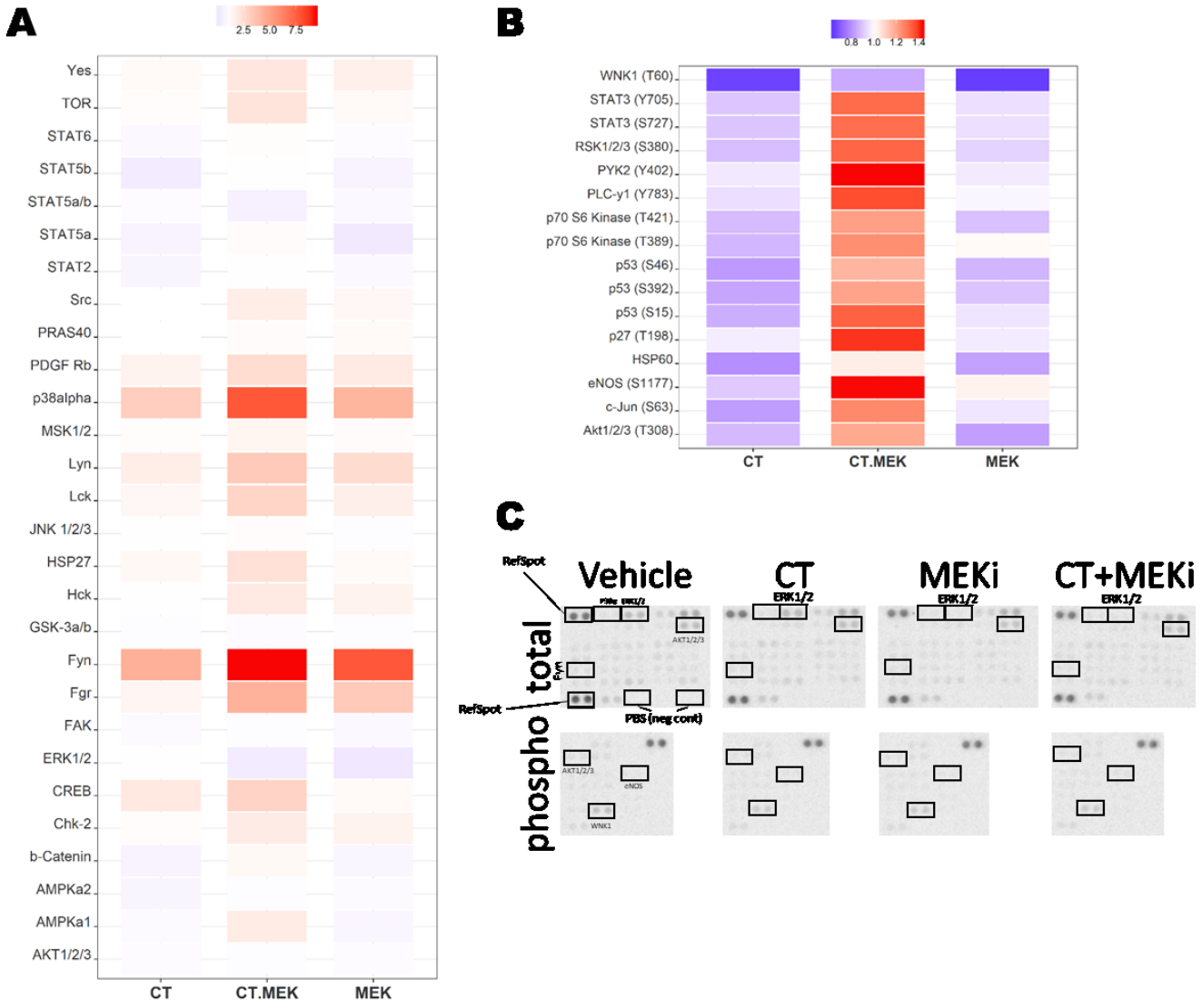

Figure 28. Cannabinoid and MEK inhibitor treatment alters protein signaling.

Human A2058 melanoma cells were treated with a combination of CBD and THC (6 µM, each) with or without MEK inhibitor (30 nM) for 2 hours. Human Phospho-Kinase Array was used to assess different protein expression levels among treatment groups. Results were normalized to vehicle. Cannabinoids and MEK inhibitor treatment were able to alter total protein expression levels (A) and phospho-protein expression levels (B) of various proteins. (C) Images of the original phospho-Kinase array are shown with labeling of the most significant/relevant spots.

Cannabinoids are known to elicit a variety of effects including strong immunomodulation. Therefore, we were interested, how cannabinoids and a clinically used MEK inhibitor

(trametinib) could influence melanoma cell signaling. For this reason, we performed a phosphor-kinase array. When total protein levels were assessed, ERK levels were reduced, when treated with a MEK inhibitor with and without CT (**Figure 28A**). Furthermore, Fyn and p38alpha expression were increased in the course of any treatment. However, changes in phosphor-protein levels were moderate, where a reduction in WNK1 expression was observed in cells treated with CT and MEK inhibitor with a contrary effect when combined (**Figure 28B**). Plots are visualized in **Figure 28C**.

In vivo experiments

In-vitro data indicate that cannabinoids can have a pro-apoptotic effect in many different melanoma cell lines independent of their pheno- and genotype. Therefore, we were interested in further investigating the effect of cannabinoids in combination with a commonly used MEK-inhibitor (trametinib) *in-vivo*. Since A2058 melanoma cells are of human origin, we decided to use NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}*/SzJ* (NSG) mice. These mice are heavily immunosuppressed with dysfunctional B-, T- and NK-cells and therefore excellent to study the direct effects of these substances on melanoma cells *in-vivo*. For this purpose, $1x10^6$ tumor cells were injected in the right lower flank of the mice and treatment was initiated after a tumor was palpable (**Figure 29A**). Each mouse received vehicle, trametinib (0.75 mg/KG BW), CBD+THC (CT) (7.5mg/kg BW CBD + 7.5mg/kg BW THC) or a combination of both every day. On day 21 after a tumor was palpable and, therefore, start of the treatment period all mice were sacrificed. Since all mice came from the 3R program, they differed in age and weight and had to be distributed equally among groups. There was no significant difference among groups regarding the age of the mice (**Figure 29B**) but in the MEK inhibitor group, mice were significantly heavier than the vehicle and the cannabinoid plus MEK inhibitor groups (**Figure 29C**).

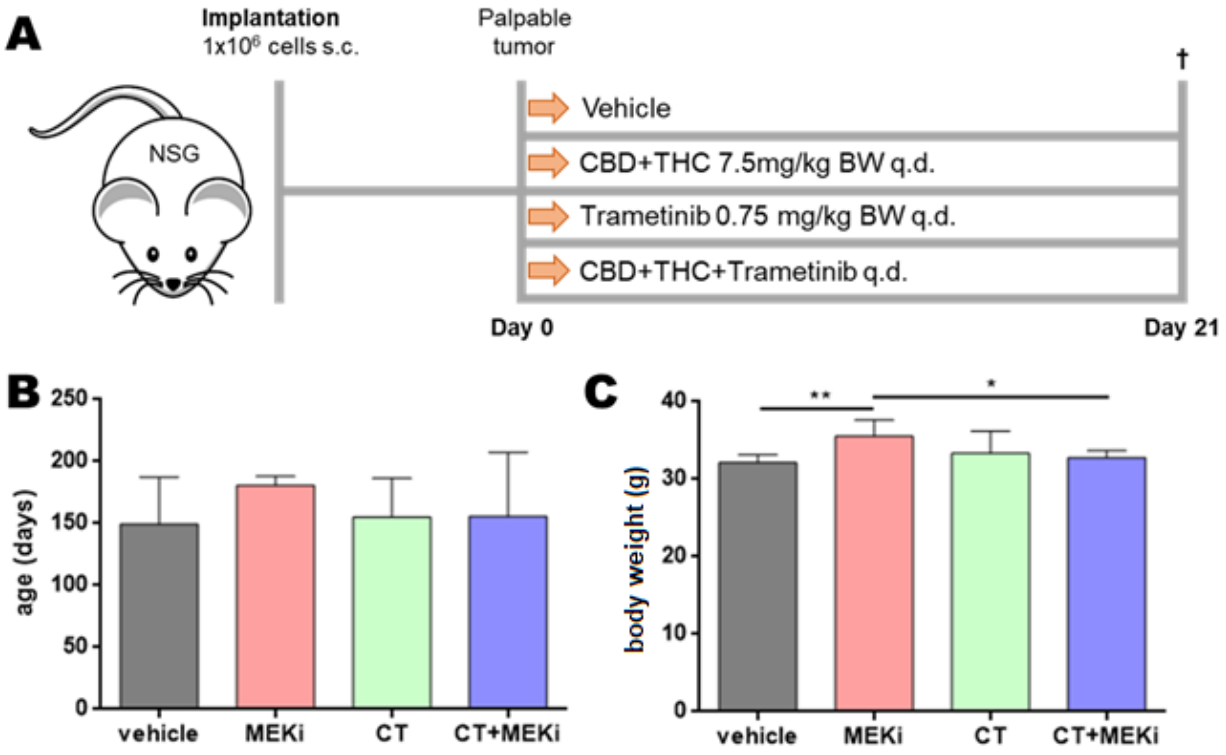

Figure 29. Schematic presentation and descriptive statistics of the *in-vivo* cancer model.

*$1x10^6$ human A2058 melanoma cells were subcutaneously injected in the lower right flank of male NSG mice (A). When a palpable tumor was detected, mice were randomly divided in their treatment groups: Vehicle, CT 10 mg/kg BW s.c. daily, trametinib (MEKi) 0.75 mg/kg BW s.c. daily, CT+MEKi s.c. daily. Mice were sacrificed on day 21. Among groups there was no significant difference in age (B) but in body weight (C). The group receiving trametinib was significantly heavier than the vehicle and the CT+MEKi group. Eight mice per treatment group. Statistically significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*

(Figure 29 corresponds to Figure 6A and SFigure 4B-C of the original publication by Richtig et al [183])

S100 is an important marker for immunohistochemical identification of melanoma and immunohistochemical tracing of melanoma progression.[224] For this reason, we were interested if cannabinoid and/or trametinib treatment had an effect on S100 expression in melanoma. There

was no statistically significant difference in S100 expression among all treatment groups (**Figure 30**) although there were some samples that had a very low S100 expression.

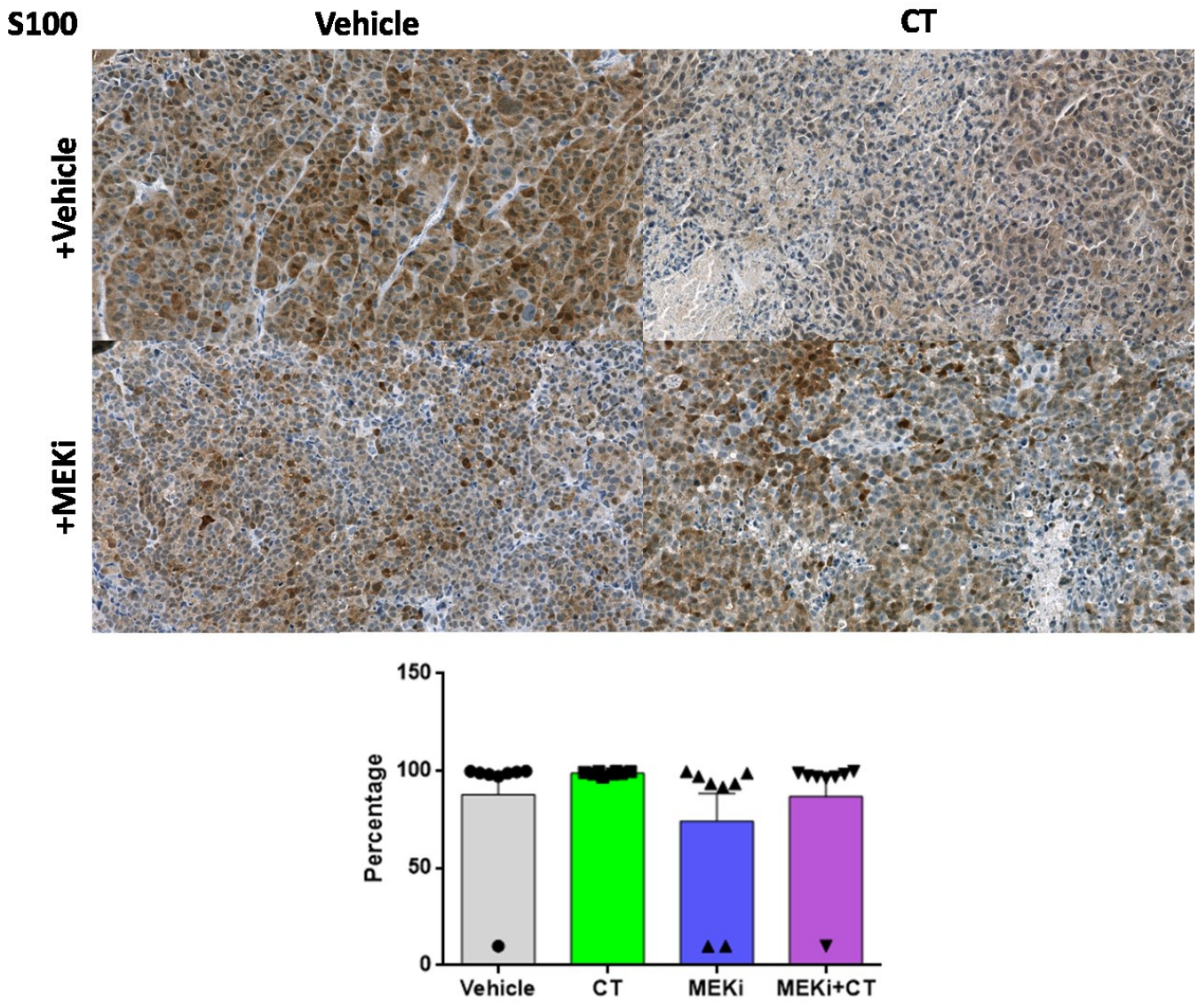

Figure 30. No difference in S100 expression was seen in trametinib- and cannabinoid-treated cells compared to vehicle.

Tumors were immunohistochemically stained for the melanoma marker S100 and automated quantification of immunohistochemical staining of tumor sections using QuPath Software was

performed as described previously.[187,225] *Results from eight mice per treatment group. (Figure 30 corresponds to SFigure 4E of the original publication by Richtig et al*[183]*)*

Although we could demonstrate that the A2058 melanoma cells were resistant to MEK inhibitor treatment in vitro, we did not see this effect in vivo (**Figure 31A & B**). CT treatment with and without MEK inhibition significantly reduced tumor growth over the time period investigated. When mice were sacrificed and tumors resected, we observed that the MEK inhibitor alone significantly reduced tumor mass compared to the group treated with CT alone (**Figure 31C**). However, all treatments significantly reduced the tumor mass after 11 days of treatment compared to the vehicle group.

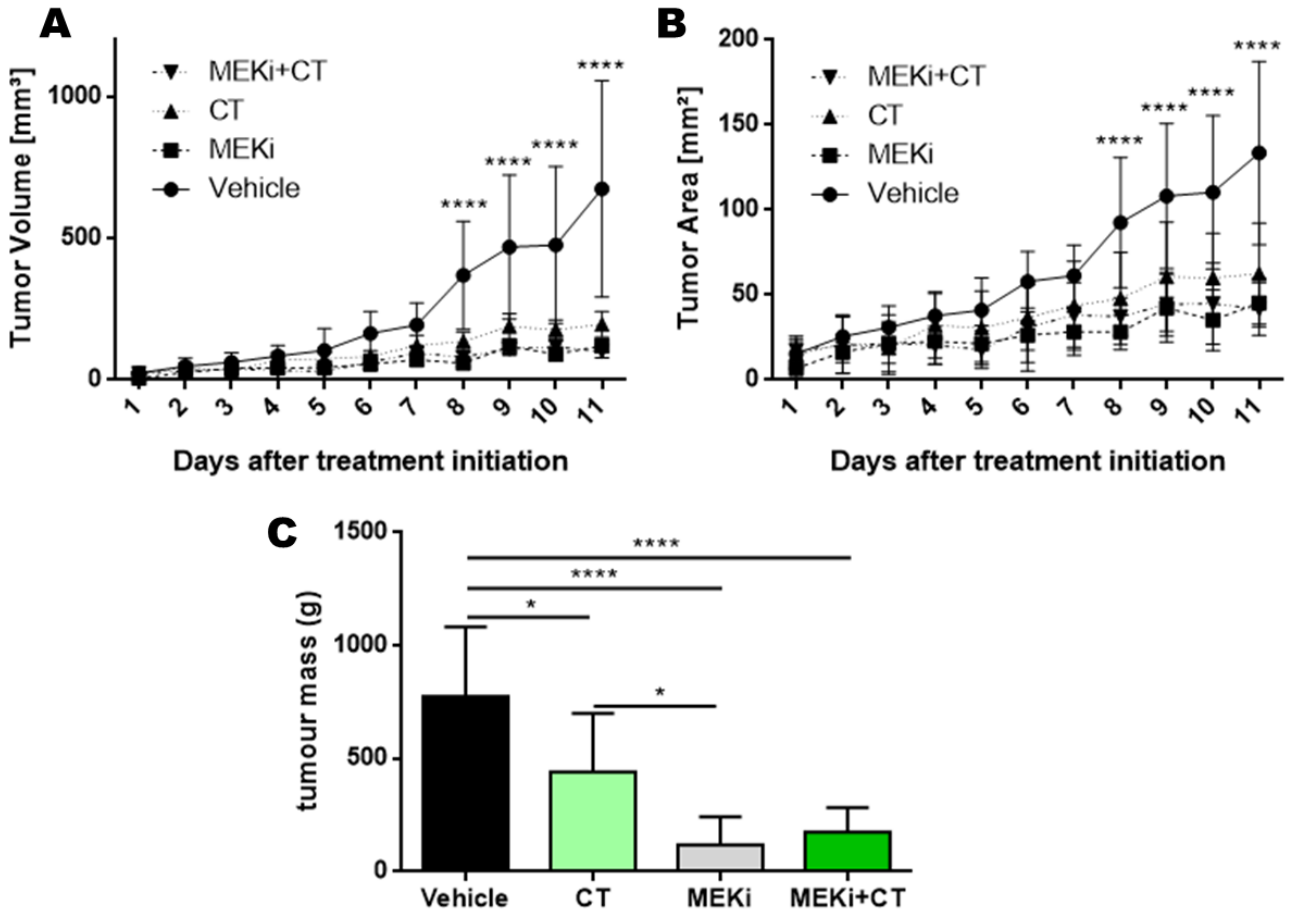

Figure 31. Cannabinoids and MEK-inhibitor treatment significantly reduced cancer growth.

*Tumor diameter was measured every day using a caliper and tumor volume and area was calculated. There was no significant difference in tumor volume (A) and tumor area (B) before day 8. From day 8 on there was a significant difference between vehicle and all other treatment groups. There was no statistically significant difference between different drug treatments. When mice were sacrificed and tumors resected, there was a statistically significant difference in tumor mass in comparison to the vehicle group and all treatments and between the CT and the MEKi group. Eight mice per treatment group. Statistically significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*
(Figure 31 corresponds to Figure 6B-C and SFigure 4D of the original publication by Richtig et al [183])

In addition, CT treatment had no significant influence on the Ki67[226] proliferation index when compared to vehicle group (**Figure 32**). In contrast, trametinib significantly reduced the number of Ki67 positive cells and CT was not able to further increase or reverse this effect. However, it seems that influencing Ki67 expression is independent of caspase 3 since we could not see any significant changes in caspase 3 IHC staining (**Figure 33**).

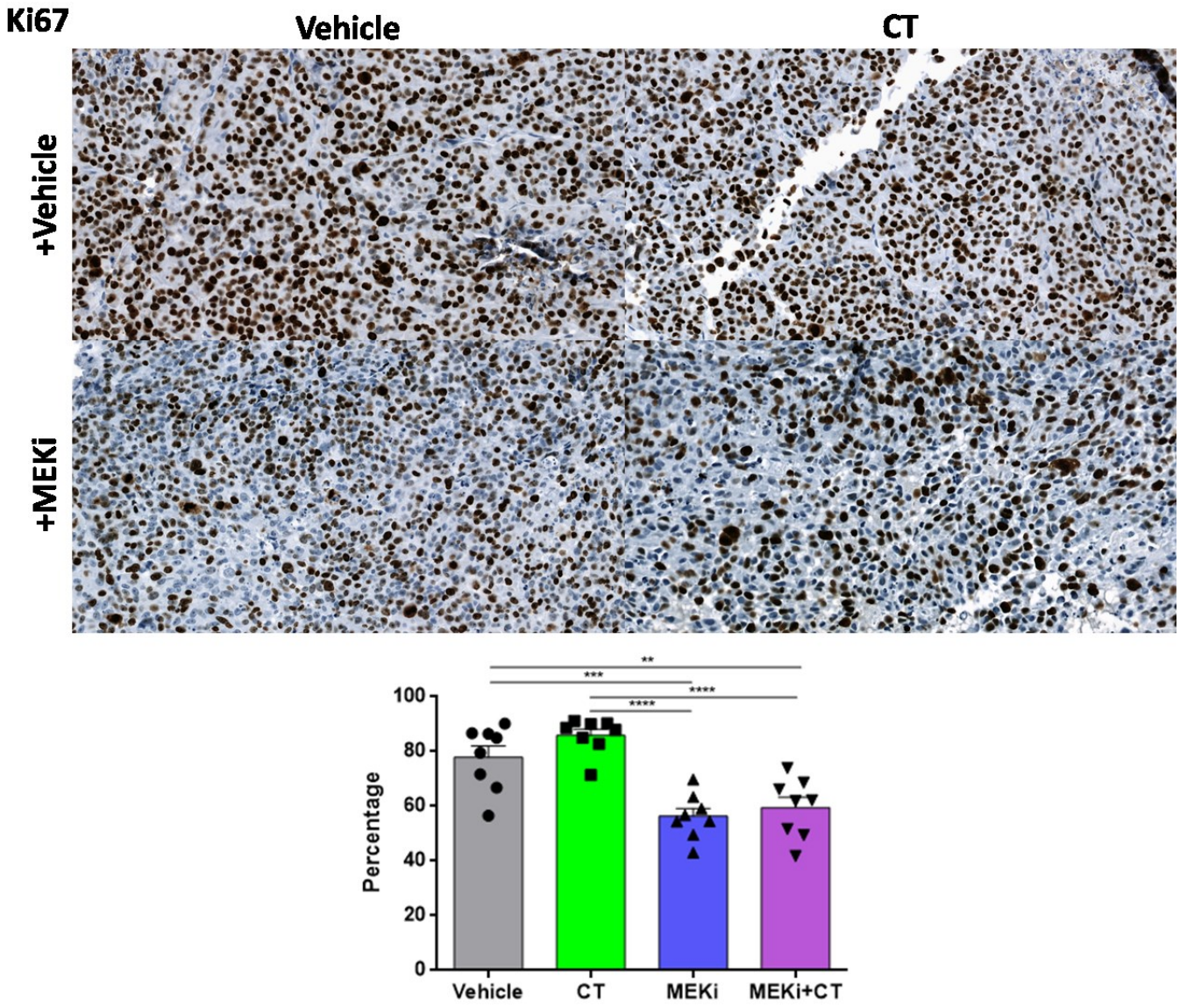

Figure 32. Trametinib significantly reduces the number of Ki67 positive cells in-vivo.

Tumours were immunohistochemically stained for the proliferation marker Ki67 and automated quantification was performed using QuPath Software as previously described.[187,225] *Results from eight mice per treatment group. Statistically significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*

(Figure 32 corresponds to Figure 6D of the original publication by Richtig et al [183])

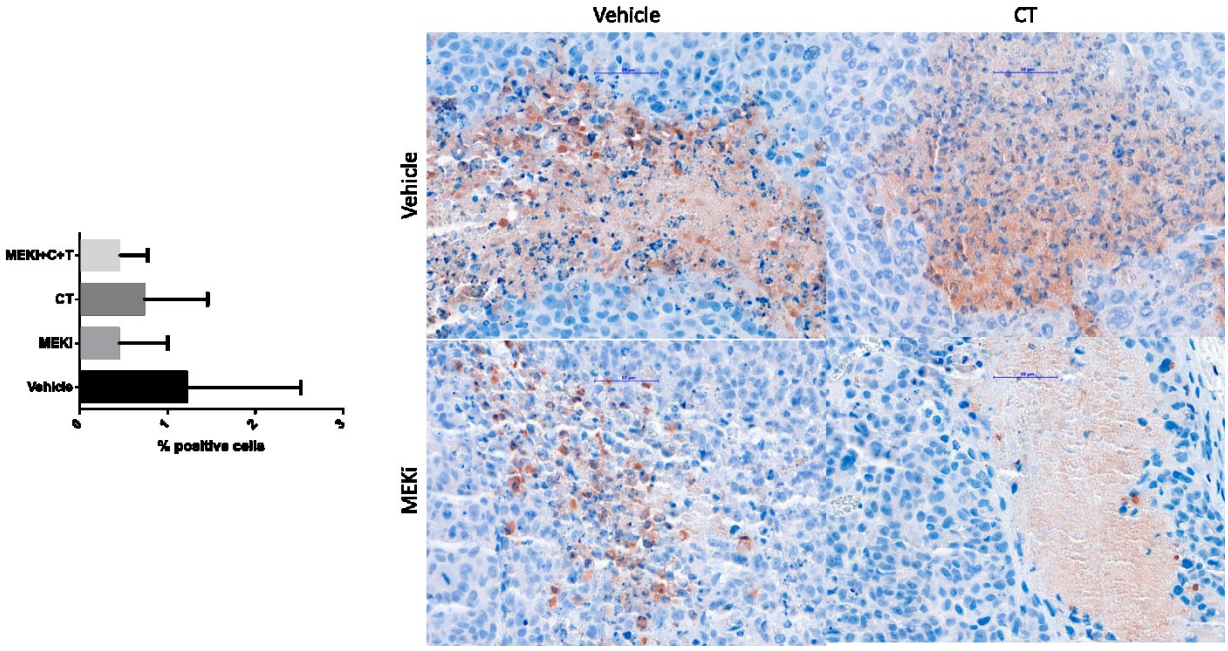

Figure 33. Trametinib and cannabinoid treatment had no influence on caspase-3 expression in vivo.

Tumors were immunohistochemically stained for the proliferation marker Ki67 and automated quantification of immunohistochemical staining of tumor sections using QuPath Software was performed as previously described.[187,225] *Results from eight mice per treatment group. Statistically significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*

Immunomodulatory effects of cannabinoids on melanoma cells

In addition to the direct tumoricidal effect of a given treatment, it is also of interest how it affects the tumor microenvironment. Cannabinoids have been described as anti-inflammatory agents having beneficial effects in many inflammatory diseases like asthma, colitis ulcerosa and others.[227,228]

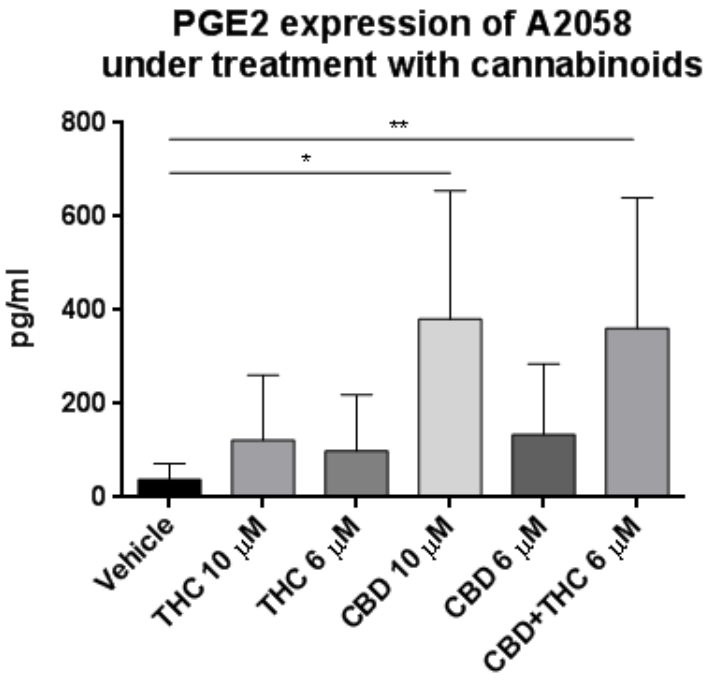

Figure 34. Cannabinoid treatment increases PGE_2 production in melanoma cells.

*A2058 cells were treated with different concentrations of cannabinoids (THC 6 and 10 uM, CBD 6 and 6 uM, CBD+THC 6 uM) for 24 hours. PGE2 was quantified by radioimmunoassay. CT and high-concentration CBD significantly increased PGE2 production after 24 hours. Data are presented as mean ± SD. Experiment was performed five times. Statistically significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*

Treatment with different concentrations of cannabinoids over 24 hours demonstrated an increase of PGE2 concentration in the supernatant of cells treated with CBD 10 µM and CT 6 µM (**Figure 34**).

Another important mechanism, although highly controversial regarding its predictive value of immunotherapy response, is the expression of PD-L1 on the surface of cancer cells.[229]

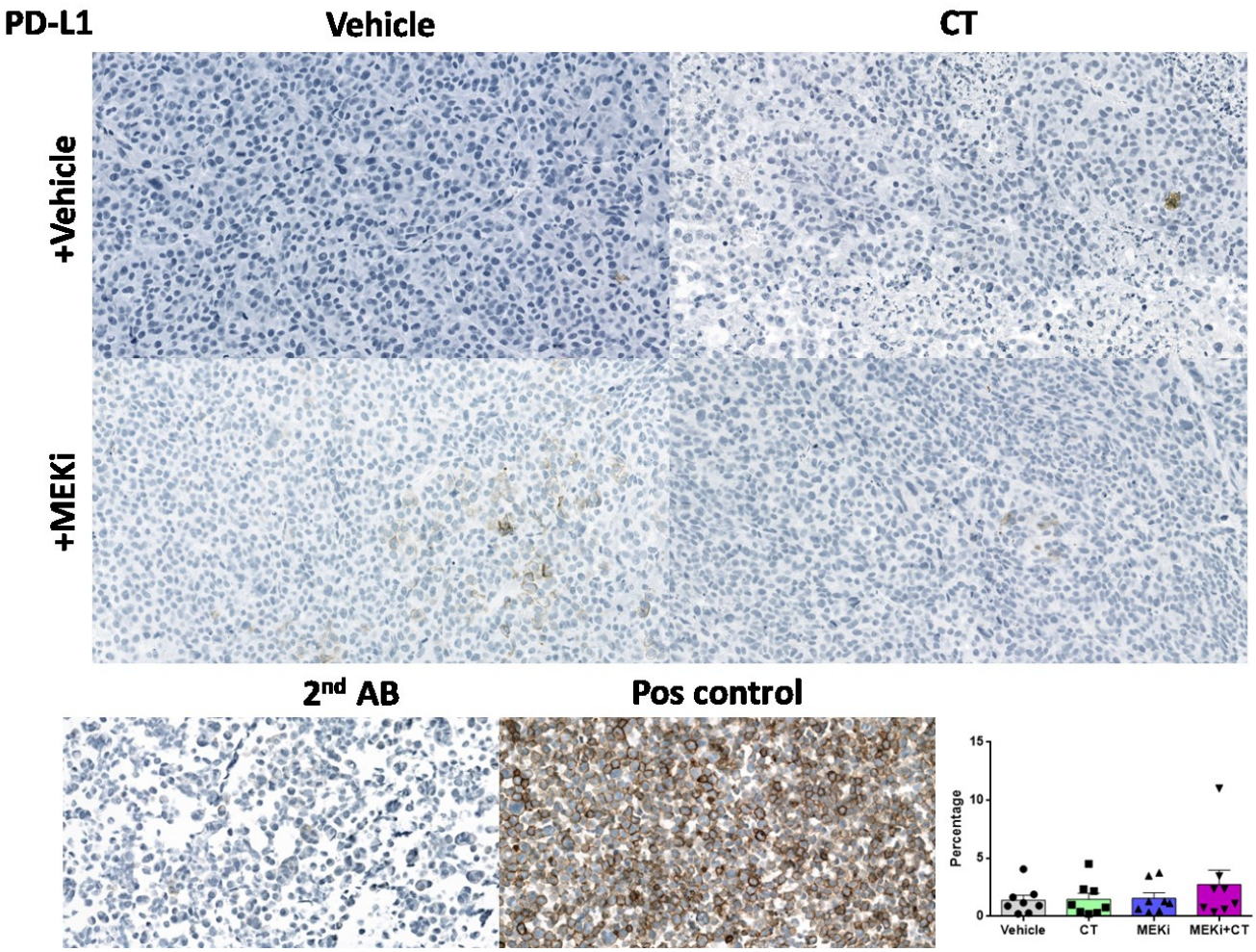

Figure 35. Trametinib and cannabinoid expression did not significantly increase PD-L1 expression in-vivo.

Tumors stained immunohistochemically for programmed death ligand 1 (PD-L1) and automated quantification of immunohistochemical staining using QuPath Software was performed as previously described.[187,225] *Results from eight mice per treatment group. Statistically significant levels: * = p<0.05; ** = p<0.01; *** = p<0.001 and **** = p<0.0001. ns = not significant.*

Interestingly, there was no significant difference in PD-L1 expression between any treatment group when A2058 cells were used in vivo (**Figure 35**).

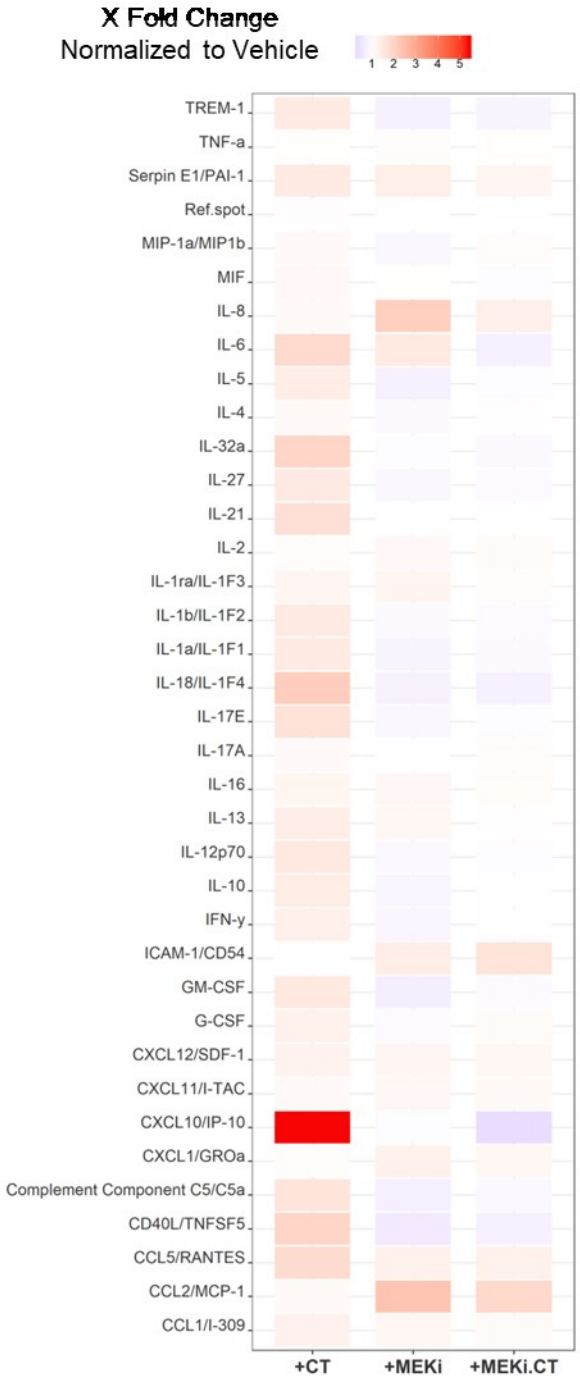

Figure 36. Cannabinoid and MEK inhibitor treatment alter cytokine levels in melanoma cells.

Human A2058 melanoma cells were treated with a combination of CBD and THC (6 μM, each) with or without MEK inhibitor (Trametinib)(30 nM) for 24 hours. Supernatant was collected and

Human Cytokine Array was used to assess different cytokine levels among treatment groups. Results were normalized to vehicle and plotted as a heatmap using ggplot2. Cannabinoids and MEK inhibitor treatment were able to alter cytokine levels.

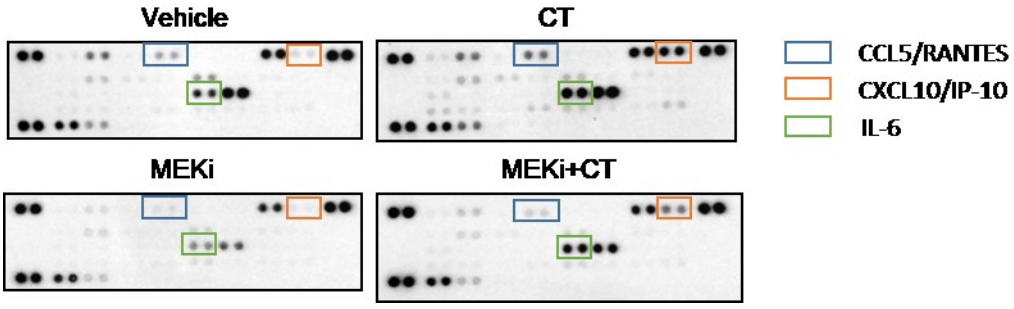

Figure 37. Cannabinoid and MEK inhibitor treatment alter cytokine levels in melanoma cells.

Raw images of the Human Cytokine Array divided by treatment groups used as template for heatmap plotting. Circled in blue is the spot for CCL5/RANTES, circled in orange CXCL10/IP-10 and circled in green IL-6.

Another important mechanism of immune evasion in cancer are cytokines and other factors enhancing immune evasion in the tumor environment.[230,231] It has been shown that melanoma cells can express IL-6 and PGE2 to decrease the likelihood of lymphocyte infiltration and therefore might be less responsive to immune therapy.[232] Therefore, we were interested if cannabis – a known anti-inflammatory substance – can stimulate melanoma cells to express anti-inflammatory cytokines. Treatment with CT (6 µM) for 24 hours resulted in an 5x fold increase in CXCL10/IP-10 expression, followed by IL-18/IL-1F4 (2.1x), IL-32a (1.9x) and CD40L/TNFSF5 (1.9x). The clinically used MEK-inhibitor trametinib increased CCL2/MCP-1 (2.3x), IL-8 (2.1x) and IL-6 (1.5x) and reduced CD40L/TNFSF5 (0.61x), GM-CSF (0.68x) and complement component C5/C5a (0.70x)(**Figure 36 & Figure 37**). When MEKi was added to CT, the increased expression of CXCL10/IP-10, IL-6, IL-18/IL-1F4 reversed to baseline expression. However, the MEKi alone could further reduce these three levels demonstrating that cannabinoids might counterplay the MEKi effects on melanoma cells (**Figure 38 & Figure 39**).

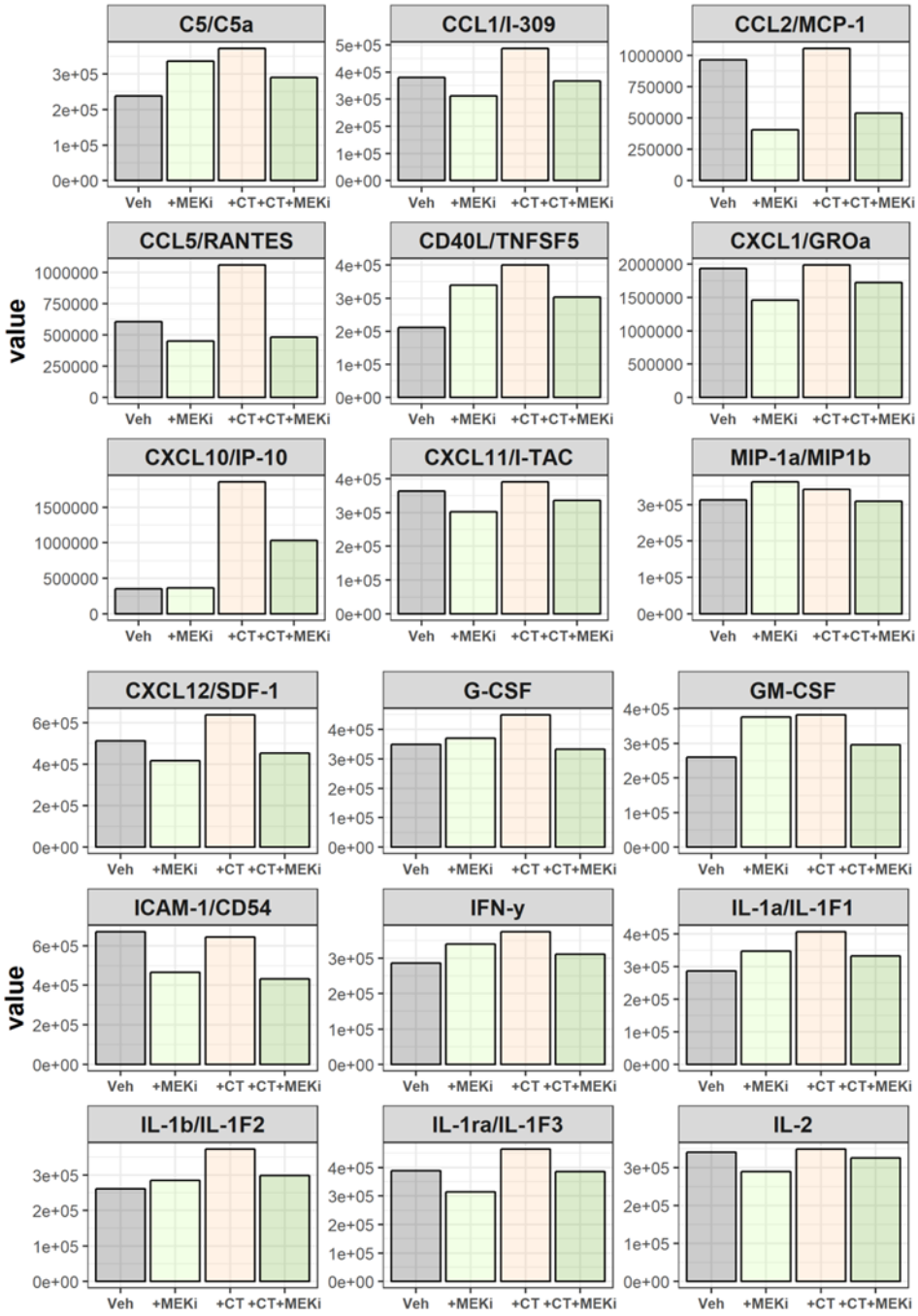

Figure 38. Cannabinoid and MEK inhibitor treatment alter cytokine levels in melanoma cells.

Raw density spot values of each cytokine antibody spot were plotted as a single bar chart using ggplot2. Veh = Vehicle, MEKi = trametinib treatment, CT = CBD and THC 6 μM treatment, CT+MEKi = CBD and THC 6 μM and trametinib (30nM) treatment. Experiment was once performed in duplicates.

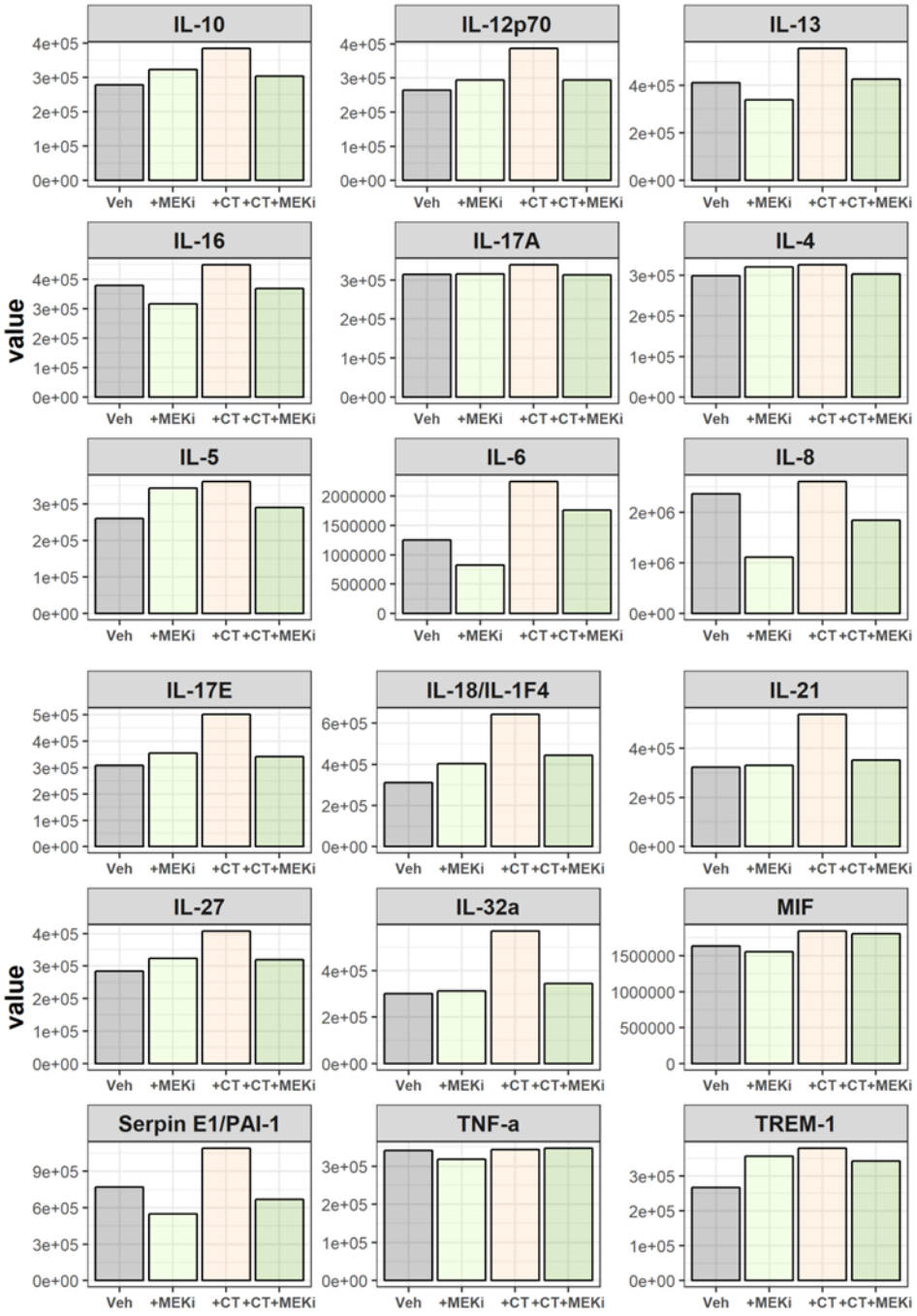

Figure 39. Cannabinoid and MEK inhibitor treatment alter cytokine levels in melanoma cells.

Raw density spot values of each cytokine antibody spot were plotted as a single bar chart using ggplot2. Veh = Vehicle, MEKi = trametinib treatment, CT = CBD and THC 6 μM treatment, CT+MEKi = CBD and THC 6 μM and trametinib (30nM) treatment. The Experiment was performed once in duplicates.

Discussion

Therapeutic options for melanoma were limited for many years and the only curable approach was early detection in combination with surgical removal.[233] Although there has been plenty of research performed on pharmacological therapeutic options, there was no real breakthrough for decades and, therefore, melanoma has been considered as one of the most difficult cancer types to treat. This changed dramatically when two key scientific discoveries were made: On one hand, that a high proportion – around 40 to 50% - of melanomas is harboring a highly conserved somatic hotspot mutation on the *BRAF* gene at position 600 with an exchange of valine to glutamic acid[234,235], which is potentially suitable for therapy. On the other hand, it was discovered that tumor cells can express molecules hiding them from immune surveillance.[104,236] Blocking these proteins on T-cells can reinduce tumor cell surveillance[237]. Both findings led to the development of highly efficient drugs called after their respective targets: targeted therapy and immune checkpoint inhibitors.

Cannabinoids have been studied for decades for different purposes. It has been proposed that cannabinoids might help against autoimmunological disorders including psoriasis, scleroderma[238] or allergic asthma[239]. The second large focus of cannabinoid research has traditionally been cancer. The majority of studies suggest that there is an anti-cancerous effect but there are also some studies suggesting an opposite, tumor-promoting effect. To date, there is no approved cannabinoid compound or cannabinoid formula for the causal therapy of cancer. Cancer itself is a highly complex disease composing of two main mechanisms – apart from many other important mechanisms: Firstly, the biology of cancer itself which includes its uncontrolled growth and immortalization, and, secondly, its effects on its environment leading to evasion from the immune system.[240]

Cannabinoid treatment of melanoma cells

In melanoma research the most commonly used cell line is A375. Therefore, we used the two most studied cannabinoid compounds THC and CBD and tested its effect on the A375 cell line. Importantly this was also performed to validate our results in comparison with other studies, which have been conducted in this field. As shown by other studies, there was a concentration-dependent decrease in cell viability for CBD as well as THC.[169] Another study could demonstrate

that 1 µM of THC had no effect on A375 cells, which is also in line with our results.[241] Importantly, melanoma is the tumor type with the highest tumor mutational burden (TMB), and TMB is increasing with stage and aggressiveness.[16,41]

A higher TMB increases the likelihood of primary or secondary resistance to therapies which directly target melanoma cells. With this in mind we tested the effects of cannabinoids in various melanoma cell lines with different genetical background. Not surprisingly, there was a huge diversity in response to cannabinoid treatment in different cell lines. There were cell lines that responded to cannabinoid treatment such as the A375 cell line but there were also cell lines resistant against cannabinoid treatment like the cell line UACC-62. This demonstrates an important point in cancer therapy: Drugs should be used always bearing in mind that one size usually does not fit all. Therefore, there might be some melanoma cells sensitive to cannabinoid therapy but definitely not all will respond.

In cancer therapy, there are three therapeutic options: surgery, radiation and pharmacological therapy. Melanoma is particular, because it has two growth phases[242]: an early horizontal growth phase, where the tumor spreads on the surface of the skin but does not grow invasively, and a second vertical growth phase, where the tumor primarily no longer grows on the surface (and accordingly it is no longer recognized as a benign process), but grows invasively and, thus, the metastatic potential increases.

These facts have led to the classification of melanoma primarily according to its depth of invasion. Superficial melanomas have a very good prognosis because they can be surgically removed, whereas melanomas with deep cutaneous invasion have a poorer prognosis because they may have already spread despite apparent complete surgical removal.

This also implies that horizontally growing melanomas do not require pharmacological therapy. For this reason, melanoma cell lines characterized by vertical growth were also included in our work. We could show that cannabinoids can have growth-inhibitory effects, but not in all of them. This is comparable with results from the literature where A375 cell line were more sensitive to THC and CBD in comparison to the SK-Mel-28 cell line.[169] Importantly, A375 cells are non-metastatic cells in comparison to SK-Mel-28 which has been isolated from a lymph node and has therefore a higher likelihood of being resistant to any therapy.[169]

Cannabinoids are said to have many beneficial properties.[243]
One of these properties is their anti-inflammatory effect and their effect on immune cells.[244] Thus, cannabinoids are being researched for use in bronchial asthma, where they have an immunomodulatory effect on eosinophils.[244] On the other hand, eosinophils have been shown to play an important role in tumor defense.[191] It could be demonstrated that the more eosinophils someone has in the blood stream, the likelier he was to respond to immunotherapy.[191] Thus, it was of interest to include eosinophils as a control in our study and we were able to show that the combination of THC and CBD resulted in a biphasic loss of eosinophil viability but had no impact on PBMCs.

Cannabinoid signaling pathways in melanoma cells

Currently, two cannabinoid receptors are known in the literature that mediate their effect via G-proteins and are well characterized[123–125]: CB1 and CB2. The CB1 receptor is mainly expressed in various regions of the brain, but also in cardio-, hepato-, and adipocytes, as well as in other cells.[245] In comparison, the CB2 receptor is mainly expressed on immune cells.[140] In our study, we demonstrated that the effects of THC and, to a lesser extent, CBD are mediated via the CB1 receptor. This is not surprising, since melanoma cells are of neuronal origin.[246] Further data has been provided recently by Costas-Insua et al[247], who could demonstrate that growth of melanoma brain metastases can be inhibited by CB1 receptor signaling.

On the other hand, there are also studies that attribute a pro-tumorogenic effect to cannabinoid receptor 1.[248] According to a study by Carpi et al, this pro-tumorogenic effect is accompanied by a consecutive activation of ERK and AKT.[248] This is interesting because a large proportion of melanomas have a MAPK pathway-activating mutation in the *BRAF* gene and/or the AKT pathway. This is a known escape pathway in melanomas with primary, as well as secondary resistance to BRAF inhibitors.[98] Thus, it can be speculated that patients with regular cannabis use are more likely to have melanomas that do not have a $BRAF^{V600E}$ mutation because they already have the necessary oncogenic MAPK pathway activation from regular cannabis use. However, this also means that these patients are no longer amenable to an important line of therapy. In our data, we can see that THC alone tended to increase growth in the majority of our cells. Interestingly, it was the cells that had a metastatic $BRAF^{V600E}$ mutant phenotype, whereas THC seems to have more of an antitumor effect on the A375 $BRAF^{V600E}$ mutant non-metastatic cell

line.

However, the effect mediated by CBD seems to be independent of CB1 receptor signaling. There have been multiple targets of cannabinoid signaling described in the literature including the peroxisome proliferator-activated receptors (PPAR) α and PPARγ, transient receptor potential vanilloid-1 (TRPV1) channels, G protein-coupled receptor (GPR) 55 and GPR35.[128] Interestingly, we could demonstrate that CBD as well as THC mediate their apoptotic effect through TRPV1. It was shown that overexpression of TRPV1 in colon adenocarcinoma cell line CaCo-2, the pancreatic cancer cell line PANC-1 and in A2058, as well as A375 melanoma cell line resulted in growth inhibition. In CaCo-2 cells, cannabidiol TRPV1 activation led to reduced phosphorylation of AKT by TRPV1, CB1 and PPARγ activation, which finally resulted in a lower proliferation rate.[249] In melanoma, TRPV1 receptor expression was shown to be higher in primary melanomas than in metastases.[250] In concordance, it was shown that melanomas with high TRPV1 receptor expression had a better prognosis than those with low levels. Activation of TRPV1 in A2058 and A375 melanoma cells led to an increase in cleaved caspase-3 levels as well as P53 expression and therefore induced apoptosis in this cell lines.[250] In our study, we could not only show that cannabidiol and THC activate the TRPV1- and CB1-receptor but also that the PPARα receptor plays a crucial role in apoptosis. This is in line with other reports, which could demonstrate that PPARα activation is associated with a pro-apoptotic function by promoting Bcl-2 degradation and activation of caspase-3.[251] Specifically in melanoma, Grabacka et al demonstrated that PPARα activation resulted in decreased metastatic potential, as well as decreased cell growth via the down-regulation of AKT. Interestingly, the effects could be reversed with the PPARα antagonist GW9662.[252]

From a clinical point of view, it has been shown that decreased PPARα expression in the tumor tissue of breast cancer patients results in significantly shortened overall survival.[253]

Apoptosis mediated by Cannabidiol and THC treatment in melanoma

Different pathways are involved in initiating apoptosis, but it is mainly mediated either by activation of the intrinsic pathway or by activation of the extrinsic pathway.[254] The extrinsic pathway is triggered by external stimuli to the cell. This is followed by the activation of death receptors at the cell membrane. These receptors mostly belong to the tumor necrosis factor (TNF) receptor superfamily. Other known death receptors are TRAIL and CD95. Interestingly, these

death receptors can also be activated by various – well known – tumor suppressor proteins such as P53 or myc.[255]

Following extrinsic activation, Fas-associated death domain (FADD) is recruited, which subsequently activates caspase-8, activating further down-stream caspases and ultimately leading to apoptosis.[256]

In our study, we used different inhibitors to investigate if the extrinsic pathway was activated by cannabinoids. Jun N-terminal kinases (JNK) play a critical role in death receptor-initiated extrinsic apoptosis.[257] In another study by a group around Fallahi-Sichani et al[258] was able to show that JNK has an important role in resistance development after BRAF inhibitor use. However, we were not able to show that JNK has a role in cannabinoid mediated effects.

The intrinsic pathway has always been an interesting therapeutic target because of its important protective role in carcinogenesis. Stress signals can activate the intrinsic pathway which in turn lead to the release of pro-apoptotic factors such as apoptosis-inducing factor (AIF), Smac/DIABLO or cytochrome c from the mitochondrial intermembrane space, thereby quickly initiating mitochondrial membrane depolarization leading to apoptosis.[259] A major part of melanoma research aimed to make melanomas more susceptible to apoptosis through the intrinsic pathway. A major focus has been on the Bcl-2 family type of proteins as they protect mitochondrial integrity and thus prevent apoptosis.[260] When we treated A2058 melanoma cells with different cannabinoids, we observed that mitochondrial depolarization was disrupted when treated with the combination of CBD and THC as early as 2 h post treatment and for up to 24 h. Importantly, we did not see any reduction in cell count until 24 h of cannabinoid treatment suggesting that after mitochondrial depolarization further processes are necessary to finally carry out apoptosis. Similar findings were made by Olivas-Aguirre et al. who could demonstrate that cannabidiol treatment lead to mitochondrial cytochrome c release in acute lymphoblastic leukemia cells.[261]

Due to their highly lipophilic nature, it has been proposed that cannabinoids can interact directly with the mitochondrial membrane, leading to cytochrome c release rather than via a specific receptor.[262] In Contrast, activation of CB1 or TRPV1 can lead to mitochondria-mediated cell death as suggested by other studies.[262–264]

Ramírez-Barrantes et al could show that TRPV1 activation led to TRPV1-dependent Ca^{2+} signaling modifications which in turn led to a plasma membrane potential depolarization resulting in cell death.[264] In another study, it was also shown that TRPV1 activation led to a Ca^{2+} increase in mitochondria and subsequently led to cytochrome c release of mitochondria with subsequential activation of caspase-3.[263]

Another mechanism proposed by some groups is that cannabinoids might mediate their apoptotic effects by cytochrome c release from the mitochondrial membrane. This is not a caspase-3 downstream mediated apoptotic effect but rather an activation of autophagy leading to cell death.[265,266]

Dando et al demonstrated that cannabinoids were able to disrupt the energy balance of pancreatic cancer cell lines leading to an induction of AMPK-mediated autophagy.[265] Interestingly, the metabolic disturbance takes place in the Krebs cycle by AKT activation with consecutive activation of myc.[265] Similar findings were made in glioma cells treated with THC, also mediating its apoptotic effect by autophagy and AKT suppression.[266] Interestingly, we were not able to confirm these previous findings that autophagy is involved in cannabinoid mediated cell death in metastatic melanoma cells.

Autophagy depends on the activation of PTEN and P53[267], but both proteins are commonly mutated in melanoma. Importantly, loss of function mutations in *PTEN* and *TP53* are associated with a more aggressive and invasive phenotype.[16] This is interesting because it has been shown that cannabinoid-mediated autophagy can work in melanoma.[169] However, the melanoma cell line A375 is a non-metastatic $TP53^{wt}$ cell line. In contrast, the A2058 is a metastatic $TP53^{mt}$ cell line and thus it is not surprising that cannabinoid-induced autophagy is functional in the A375 cell line, but not in the A2058 cell line suggesting that cannabinoid-induced autophagy might only work in cancer cells with non-metastatic phenotype since *TP53* mutation is commonly associated with a metastatic phenotype.[268]

On the other hand, there have been reports that cannabinoids mediate their apoptotic effects through caspase activation in many types of cancers.[269] Caspases are a conserved family of cysteine proteases that are well known to be involved in cell death as well as in inflammation.[270] The different caspases can be divided into caspases involved in inflammation (caspase-1, -4, -5, -

11), caspase involved in apoptosis (caspase-3, -6, -7, -8, -9, -10) and caspases with other functions (caspase-2, -12, -14). The caspases involved in apoptosis can be further divided into initiator and executer caspases. Initiator caspases activate the executer caspases, which are responsible for the characteristic changes of apoptosis. These include cell shrinkage, chromosomal DNA fragmentation, membrane bleeding and the formation of "apoptotic bodies".[271]

In our study, we could demonstrate that cannabinoids can activate the intrinsic caspase pathway through mitochondrial disruption. Importantly, this effect could be blocked by the pan-caspase inhibitor ZVAD. In fact, there are several reports in the literature that cannabinoids can mediate their apoptotic effect through caspase activation in many different types of cancers.[269]

Interestingly, it seems that caspase activation is a double-edged sword. It has been shown that failed apoptosis after caspase activation leads to a more aggressive phenotype.[272] We were able to show that cannabinoids leads to caspase 3/7 activity, but that this could also be blocked by a pan-caspase inhibitor and that blockade restored cell viability. Caspase 3/7 activation by cannabinoids in melanoma has been described previously.[169,273]

Previously, caspase activation in melanoma has been associated with autophagy activation.[169] Interestingly, we did not see increased autophagic activity, nor did we see that an autophagy-specific inhibitor like BafA1 increased cell viability after cannabinoid treatment. BafA1 is an ATPase inhibitor that blocks the autophagic flux acutely by inhibiting autolysosome acidification and autophagosome–lysosome fusion and in our study BafA1 was able to reverse the autophagic effect of chloroquine but not of cannabinoids.[203–205] In contrast to Armstrong et al.[169], we used a metastatic cell line instead of the non-metastatic A375 cell line and this could be the reason why autophagy was not involved in the apoptotic process.

The effect of commonly used targeted therapy and cannabinoids in melanoma

Patients with oncologic disease use cannabinoids for three purposes: 1) for physical and/or psychical relief, 2) as an alternative cancer therapy, or 3) to stimulate appetite while undergoing chemotherapy.[156] Interestingly, a large proportion of patients believes that cannabinoids are a good alternative to commonly used therapies including targeted therapy as well as checkpoint inhibitor therapy.[156] Acute side effects of cannabis intake can include sedation, euphoria, lethargy

and intensification of ordinary sensory experiences as well as perceptual distortion, or continuous laughter and talkativeness.[274] However, for cancer patients – depending of the prognosis of their cancer - the long-term effects of cannabis might be more important. It could be demonstrated that chronic cannabis consumption can lead to the development of psychosis.[275] For cancer patients, this is of importance since it has been suggested in the literature that lifetime use of cannabis increases the risk of depression including higher incidences of suicidal ideation.[276] Especially important for cancer patients, which are a psychological highly vulnerable group, is the fact that lifetime consumption seems to be a relevant risk factor for suicidal attempts.[277] It has to be kept in mind that, if cancer patients receive such supporting therapies, they may need continuous monitoring as well as psychological support besides their anti-cancerous and other clinical therapy.[157,277] For clinicians, this is of great importance since in a recent study it was demonstrated that the efficacy of a commonly used immune checkpoint inhibitor is reduced when combined with cannabinoids suggesting that cannabinoids might interfere with potential curative anti-cancerous therapies and should, therefore, be avoided.[177]

Importantly, this raises two questions when it comes to clinical cannabinoid usage: Firstly, can cannabinoid intake reduce cancer cell growth in vivo, and secondly, does cannabinoid intake interfere with commonly used modern targeted therapy?

In our in vivo model, we could demonstrate that the clinically used combination of CBD and THC significantly reduced tumor growth in the $BRAF^{V600E}$ mutated metastatic melanoma cell line A2058.

Blazquez et al. showed in their study that the synthetic cannabinoids WIN-55,212–2 and JWH-133 could reduce cell proliferation and the metastatic capacity of B16 murine melanoma cell line in a mouse model.[241] In line with this result, Simmerman et al. could demonstrate that CBD monotherapy can prolong survival of mice bearing B16F10 melanoma cells.[172] Interestingly, although there was survival prolongation compared to vehicle, CBD was less effective than cisplatin.[172]

This is both interesting and important because chemotherapy no longer plays a role in melanoma, with few exceptions.[278] From a clinical perspective, one could ask whether CBD has any clinically significant added value or survival benefit at all.

Glodde et al. could show that THC significantly inhibited tumor growth of transplanted HCmel12 melanomas but not of murine B16 melanoma cells in a CB receptor-dependent manner in vivo.[279] In addition, they demonstrated that THC could also have antagonistic effects on the characteristic pro-inflammatory microenvironment of HCmel12 cell growth.[279]

In line with these results, Armstrong et al. could further demonstrate that THC could reduce tumor growth of CHL-1 melanoma cells in a xenograft mouse model.[169] The effect was even more pronounced when THC was given in combination with CBD.[169]

With the background of all these results, we investigated in our in vivo model the effect of cannabinoids on tumor growth and also the efficacy of cannabinoids in combination with a clinically used MEK inhibitor. For this purpose, we used the combination of CBD and THC, which is clinically used and available as Sativex[280] and treated the $BRAF^{V600E}$ mutated A2058 metastatic melanoma cells with the MEK inhibitor trametinib in a xenograft in-vivo model.

Although every therapeutic group show significantly reduced tumor growth in vivo, we could not find any significant difference between the single therapeutic groups. This is interesting since there have been reports suggesting that cannabinoids might mediate their apoptotic effect by activation of the MAPK-Pathway including MEK and ERK which would lead to a synergistic apoptotic effect of cannabinoids and MEKi which we did not observe in our study.[281,282] On the other hand, however, there are also reports that show that cannabinoids can inhibit the MAPK pathway in colon cancer cell lines, suggesting that there are cell type-specific cannabinoid-induced effects or at least not every cell type responds in a similar predictive way to cannabinoids.[283]

Limitations of cannabinoid research

Several problems from in-vitro and in-vivo data arise when translating cannabis models to cancer treatment: i), reports of "successful" treatment in cancer patients with cannabis highly depend rather on the patients' history than on objective blood serum concentrations of cannabinoids.[167,168] ii), cannabis seems to have anti-cancerous effects as well as anti-inflammatory effects.[173,174] As we know today, the immune system plays a pivotal role in suppressing tumor growth as well as

combats metastatic spread.[175,176] In line with these findings, it opens up the question if the direct anti-cancerous effects of cannabis are abolished by the anti-inflammatory effects. More importantly, a recent retrospective analysis demonstrated that cannabinoid intake can negatively impact immune checkpoint inhibitor therapy.[177] Whether this is due to direct inhibitory and anti-inflammatory effects on the immune system or interference at a pharmacokinetic level has to be elucidated by further investigations. In our study, we could demonstrate that cannabinoids have an impact on several proteins involved in immune signaling. However, we were also able to show that MEK inhibitors have an influence on immune signaling and thus a prediction of an overall immune effect with simultaneous intake of cannabinoids and MEK inhibitors appears to be impossible.. iii), it is known that cannabinoids have a strong effect on the cytochrome p450 enzyme system.[178,179] This can result in unintentionally low or high plasma levels of the initial cancer therapy, resulting in increased side effects.

iv), in melanoma, non-metastatic disease is mainly treatment by surgical removal of the primary tumor.[180] Therefore, there is no clinical need for topical cannabinoid treatment of surgically removable primary melanoma. v), when it comes to the question if patients should take cannabinoids as therapeutic agent or therapeutic supplement, it has to be kept in mind that – if cannabis has any anti-apoptotic effect on cancer cells – that prior continuous usage of cannabis may make cancer cells resistant to any further cannabinoid intake. In addition, it has to be kept in mind that long-term cannabinoid use might act in a different way than cancer immunotherapy.[177] vi), although there might be no net effect on cancer cell death, cannabinoids might be suitable to reduce tumor heterogeneity. However, this can be a double edged sword since cannabinoids could kill clones that might be harboring primary resistance to immune checkpoint blockade or, on the other hand promote clones with primary resistance to immune checkpoint blockade.[181,182] vii), cannabis has been advertised as an agent that can kill all types of cancer neglecting the fact that different types of cancer have different genetic drivers and different immunogenicity. Therefore, it is highly unlikely that cannabinoids can fulfil this promise.

In conclusion, all these issues have to be kept in mind when it comes to cannabinoid research and the question how cannabinoids should or can be used in daily clinical practice.

Conclusions

In summary, our study demonstrated that cannabinoids had an pro-apoptotic effect on different melanoma cell lines in vitro and to some extend their growth in vivo. From a clinical point of view, the rational is given to use cannabinoids at least as a supportive therapy in patients with metastatic melanoma in combination with modern targeted therapy. Mechanistically, cannabinoids mediate their effects in a CB1-, TRPV1- and PPARα-receptor-dependent way, independently of autophagy but by activation of caspases and mitochondrial cytochrome c release.

Bibliography

1. Liu J, Fukunaga-Kalabis M, Li L, Herlyn M. Developmental pathways activated in melanocytes and melanoma. *Arch Biochem Biophys*. 2014;563:13-21. doi:10.1016/j.abb.2014.07.023
2. Dorsky RI, Moon RT, Raible DW. Control of neural crest cell fate by the Wnt signalling pathway. *Nature*. 1998;396(6709):370-373. doi:10.1038/24620
3. Pandey M, Mathew A, Abraham EK, Ahamed IM, Nair KM. Primary malignant melanoma of the mucous membranes. *Eur J Surg Oncol*. 1998;24(4):303-307.
4. Kawakami A, Fisher DE. Key Discoveries in Melanocyte Development. *Journal of Investigative Dermatology*. 2011;131:E2-E4. doi:10.1038/skinbio.2011.2
5. Richtig G, Richtig E, Massone C, Hofmann-Wellenhof R. Analysis of clinical, dermoscopic and histopathological features of primary melanomas of patients with metastatic disease - A retrospective study at the Department of Dermatology, Medical University of Graz, 2000-2010. *Journal of the European Academy of Dermatology and Venereology*. 2014;28(12):1776-1781. doi:10.1111/jdv.12413
6. Deeks ED. Nivolumab: A review of its use in patients with malignant melanoma. *Drugs*. 2014;74(11):1233-1239. doi:10.1007/s40265-014-0234-4
7. Gershenwald JE, Scolyer RA, Hess KR, et al. Melanoma staging: Evidence-based changes in the American Joint Committee on Cancer eighth edition cancer staging manual. *CA Cancer J Clin*. 2017;67(6):472-492. doi:10.3322/caac.21409
8. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature*. 2002;417(6892):949-954. doi:10.1038/nature00766

9. Colombino M, Capone M, Lissia A, et al. BRAF/NRAS mutation frequencies among primary tumors and metastases in patients with melanoma. *Journal of Clinical Oncology*. 2012;30(20):2522-2529. doi:10.1200/JCO.2011.41.2452

10. Long G V., Menzies AM, Nagrial AM, et al. Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. *Journal of Clinical Oncology*. 2011;29(10):1239-1246. doi:10.1200/JCO.2010.32.4327

11. Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev*. 2011;75(1):50-83. doi:10.1128/MMBR.00031-10

12. Wan PTC, Garnett MJ, Roe SM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell*. 2004;116(6):855-867. doi:10.1016/S0092-8674(04)00215-6

13. Ellerhorst JA, Greene VR, Ekmekcioglu S, et al. Clinical correlates of NRAS and BRAF mutations in primary human melanoma. *Clinical Cancer Research*. 2011;17(2):229-235. doi:10.1158/1078-0432.CCR-10-2276

14. Rafnar T, Sulem P, Stacey SN, et al. Sequence variants at the TERT-CLPTM1L locus associate with many cancer types. *Nat Genet*. 2009;41(2):221-227. doi:10.1038/ng.296

15. Van Raamsdonk CD, Griewank KG, Crosby MB, et al. Mutations in GNA11 in Uveal Melanoma. *New England Journal of Medicine*. 2010;363(23):2191-2199. doi:10.1056/NEJMoa1000584

16. Shain AH, Yeh I, Kovalyshyn I, et al. The Genetic Evolution of Melanoma from Precursor Lesions. *New England Journal of Medicine*. 2015;373(20):1926-1936. doi:10.1056/NEJMoa1502583

17. Masliah-Planchon J, Garinet S, Pasmant E. RAS-MAPK pathway epigenetic activation in cancer: miRNAs in action. *Oncotarget*. 2015;7(25):38892-38907. doi:10.18632/oncotarget.6476

18. McKay MM, Morrison DK. Integrating signals from RTKs to ERK/MAPK. *Oncogene*. 2007;26(22):3113-3121. doi:10.1038/sj.onc.1210394

19. Nazarian R, Shi H, Wang Q, et al. Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature*. 2010;468(7326):973-977. doi:10.1038/nature09626

20. Kamisawa T, Wood LD, Itoi T, Takaori K. Pancreatic cancer. *Lancet*. 2016;388(10039):73-85. doi:10.1016/S0140-6736(16)00141-0

21. Moura MM, Cavaco BM, Leite V. RAS proto-oncogene in medullary thyroid carcinoma. *Endocr Relat Cancer*. 2015;22(5):R235-52. doi:10.1530/ERC-15-0070

22. Fakih MG. Metastatic colorectal cancer: current state and future directions. *J Clin Oncol*. 2015;33(16):1809-1824. doi:10.1200/JCO.2014.59.7633

23. Malumbres M, Barbacid M. RAS oncogenes: the first 30 years. *Nat Rev Cancer*. 2003;3(6):459-465. doi:10.1038/nrc1097

24. Young LC, Hartig N, Muñoz-Alegre M, et al. An MRAS, SHOC2, and SCRIB complex coordinates ERK pathway activation with polarity and tumorigenic growth. *Mol Cell*. 2013;52(5):679-692. doi:10.1016/j.molcel.2013.10.004

25. Karnoub AE, Weinberg RA. Ras oncogenes: split personalities. *Nat Rev Mol Cell Biol*. 2008;9(7):517-531. doi:10.1038/nrm2438

26. Johnson DB, Puzanov I. Treatment of NRAS-Mutant Melanoma. *Curr Treat Options Oncol*. 2015;16(4):15. doi:10.1007/s11864-015-0330-z

27. Fedorenko I V, Gibney GT, Smalley KSM. NRAS mutant melanoma: biological behavior and future strategies for therapeutic management. *Oncogene*. 2013;32(25):3009-3018. doi:10.1038/onc.2012.453

28. Forbes SA, Bindal N, Bamford S, et al. COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. *Nucleic Acids Res*. 2011;39(Database):D945-D950. doi:10.1093/nar/gkq929

29. Colicelli J. Human RAS superfamily proteins and related GTPases. *Sci STKE*. 2004;2004(250):RE13. doi:10.1126/stke.2502004re13

30. Wennerberg K, Rossman KL, Der CJ. The Ras superfamily at a glance. *J Cell Sci*. 2005;118(Pt 5):843-846. doi:10.1242/jcs.01660

31. Haarberg HE, Paraiso KHT, Wood E, et al. Inhibition of Wee1, AKT, and CDK4 Underlies the Efficacy of the HSP90 Inhibitor XL888 in an In Vivo Model of NRAS-Mutant Melanoma. *Mol Cancer Ther*. 2013;12(6):901-912. doi:10.1158/1535-7163.MCT-12-1003

32. Rodriguez-Viciana P, Warne PH, Dhand R, et al. Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature*. 1994;370(6490):527-532. doi:10.1038/370527a0

33. Kelley GG, Reks SE, Ondrako JM, Smrcka A V. Phospholipase C(epsilon): a novel Ras effector. *EMBO J*. 2001;20(4):743-754. doi:10.1093/emboj/20.4.743

34. Malliri A, van der Kammen RA, Clark K, van der Valk M, Michiels F, Collard JG. Mice deficient in the Rac activator Tiam1 are resistant to Ras-induced skin tumours. *Nature*. 2002;417(6891):867-871. doi:10.1038/nature00848

35. Downward J. Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer*. 2003;3(1):11-22. doi:10.1038/nrc969

36. Dumaz N, Hayward R, Martin J, et al. In Melanoma, RAS Mutations Are Accompanied by Switching Signaling from BRAF to CRAF and Disrupted Cyclic AMP Signaling. *Cancer Res*. 2006;66(19):9483-9491. doi:10.1158/0008-5472.CAN-05-4227

37. Kyriakis JM, App H, Zhang XF, et al. Raf-1 activates MAP kinase-kinase. *Nature*. 1992;358(6385):417-421. doi:10.1038/358417a0

38. Lito P, Saborowski A, Yue J, et al. Disruption of CRAF-mediated MEK activation is required for effective MEK inhibition in KRAS mutant tumors. *Cancer Cell*. 2014;25(5):697-710. doi:10.1016/j.ccr.2014.03.011

39. Wang HG, Rapp UR, Reed JC. Bcl-2 Targets the Protein Kinase Raf-1 to Mitochondria. *Cell*. 1996;87(4):629-638. doi:10.1016/S0092-8674(00)81383-5

40. Jin S, Zhuo Y, Guo W, Field J. p21-activated Kinase 1 (Pak1)-dependent phosphorylation of Raf-1 regulates its mitochondrial localization, phosphorylation of BAD, and Bcl-2 association. *J Biol Chem*. 2005;280(26):24698-24705. doi:10.1074/jbc.M413374200

41. Alexandrov LB, Nik-Zainal S, Wedge DC, et al. Signatures of mutational processes in human cancer. *Nature*. 2013;500(7463):415-421. doi:10.1038/nature12477

42. Curtin JA, Fridlyand J, Kageshita T, et al. Distinct Sets of Genetic Alterations in Melanoma. *New England Journal of Medicine*. 2005;353(20):2135-2147. doi:10.1056/NEJMoa050092

43. Brash DE. UV Signature Mutations. *Photochem Photobiol*. 2015;91(1):15-26. doi:10.1111/php.12377

44. Alexandrov LB, Ju YS, Haase K, et al. Mutational signatures associated with tobacco smoking in human cancer. *Science (1979)*. 2016;354(6312):618-622. doi:10.1126/science.aag0299

45. Wu J, Rosenbaum E, Begum S, Westra WH. Distribution of BRAF T1799A(V600E) Mutations Across Various Types of Benign Nevi: Implications for Melanocytic Tumorigenesis. *Am J Dermatopathol*. 2007;29(6):534-537. doi:10.1097/DAD.0b013e3181584950

46. Haenssle HA, Kraus SL, Brehmer F, et al. Dynamic Changes in Nevi of a Patient With Melanoma Treated With Vemurafenib. *Arch Dermatol*. 2012;148(10):1183. doi:10.1001/archdermatol.2012.2649

47. Pollock PM, Harper UL, Hansen KS, et al. High frequency of BRAF mutations in nevi. *Nat Genet*. 2003;33(1):19-20. doi:10.1038/ng1054

48. Poynter JN, Elder JT, Fullen DR, et al. BRAF and NRAS mutations in melanoma and melanocytic nevi. *Melanoma Res*. 2006;16(4):267-273. doi:10.1097/01.cmr.0000222600.73179.f3

49. Bevona C. Cutaneous Melanomas Associated With Nevi. *Arch Dermatol*. 2003;139(12):1620. doi:10.1001/archderm.139.12.1620

50. Thomas NE, Edmiston SN, Tsai YS, et al. Utility of TERT Promoter Mutations for Cutaneous Primary Melanoma Diagnosis. *Am J Dermatopathol*. Published online September 2018:1. doi:10.1097/DAD.0000000000001259

51. Huang FW, Hodis E, Xu MJ, Kryukov G V, Chin L, Garraway LA. Highly recurrent TERT promoter mutations in human melanoma. *Science*. 2013;339(6122):957-959. doi:10.1126/science.1229259

52. Begg CB, Orlow I, Hummer AJ, et al. Lifetime Risk of Melanoma in CDKN2A Mutation Carriers in a Population-Based Sample. *JNCI: Journal of the National Cancer Institute*. 2005;97(20):1507-1515. doi:10.1093/jnci/dji312

53. Monzon J, Liu L, Brill H, et al. CDKN2A Mutations in Multiple Primary Melanomas. *New England Journal of Medicine*. 1998;338(13):879-887. doi:10.1056/NEJM199803263381305

54. Zerp SF, Elsas A van, Peltenburg LTC, Schrier PI. p53 mutations in human cutaneous melanoma correlate with sun exposure but are not always involved in melanomagenesis. *Br J Cancer*. 1999;79(5-6):921-926. doi:10.1038/sj.bjc.6690147

55. Kim DW, Haydu LE, Joon AY, et al. Clinicopathological features and clinical outcomes associated with TP 53 and BRAF N on- V 600 mutations in cutaneous melanoma patients. *Cancer*. Published online December 2016. doi:10.1002/cncr.30463

56. Xiao W, Du N, Huang T, et al. TP53 Mutation as Potential Negative Predictor for Response of Anti-CTLA-4 Therapy in Metastatic Melanoma. *EBioMedicine*. 2018;32:119-124. doi:10.1016/j.ebiom.2018.05.019

57. Akbani R, Akdemir KC, Aksoy BA, et al. Genomic Classification of Cutaneous Melanoma. *Cell*. 2015;161(7):1681-1696. doi:10.1016/j.cell.2015.05.044

58. Weber JS, D'Angelo SP, Minor D, et al. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): A randomised, controlled, open-label, phase 3 trial. *Lancet Oncol*. 2015;16(4):375-384. doi:10.1016/S1470-2045(15)70076-8

59. Robert C, Long G V., Brady B, et al. Nivolumab in Previously Untreated Melanoma without *BRAF* Mutation. *New England Journal of Medicine*. 2015;372(4):320-330. doi:10.1056/NEJMoa1412082

60. Robert C, Schachter J, Long G V., et al. Pembrolizumab versus Ipilimumab in Advanced Melanoma. *New England Journal of Medicine*. 2015;372(26):2521-2532. doi:10.1056/NEJMoa1503093

61. Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Eng J Med*. 2011;364(26):2507-2516. doi:10.1056/NEJMoa1103782.Improved

62. Kim KB, Kefford R, Pavlick AC, et al. Phase II study of the MEK1/MEK2 inhibitor Trametinib in patients with metastatic BRAF-mutant cutaneous melanoma previously treated with or without a BRAF inhibitor. *J Clin Oncol*. 2013;31(4):482-489. doi:10.1200/JCO.2012.43.5966

63. McGettigan S. Dabrafenib: A New Therapy for Use in BRAF-Mutated Metastatic Melanoma. *J Adv Pract Oncol*. 2014;5(3):211-215.

64. Livingstone E, Zimmer L, Piel S, Schadendorf D. PLX4032: does it keep its promise for metastatic melanoma treatment? *Expert Opin Investig Drugs*. 2010;19(11):1439-1449. doi:10.1517/13543784.2010.527945

65. Dummer R, Ascierto PA, Gogas HJ, et al. Encorafenib plus binimetinib versus vemurafenib or encorafenib in patients with BRAF -mutant melanoma (COLUMBUS): a multicentre, open-label, randomised phase 3 trial. *Lancet Oncol*. 2018;19(5):603-615. doi:10.1016/S1470-2045(18)30142-6

66. McArthur GA, Chapman PB, Robert C, et al. Safety and efficacy of vemurafenib in BRAFV600E and BRAFV600K mutation-positive melanoma (BRIM-3): Extended follow-up of a phase 3, randomised, open-label study. *Lancet Oncol*. 2014;15(3):323-332. doi:10.1016/S1470-2045(14)70012-9

67. Hauschild A, Grob JJ, Demidov L V., et al. Dabrafenib in BRAF-mutated metastatic melanoma: A multicentre, open-label, phase 3 randomised controlled trial. *The Lancet*. 2012;380(9839):358-365. doi:10.1016/S0140-6736(12)60868-X

68. Li Z, Jiang K, Zhu X, et al. Encorafenib (LGX818), a potent BRAF inhibitor, induces senescence accompanied by autophagy in BRAFV600E melanoma cells. *Cancer Lett*. 2016;370(2):332-344. doi:10.1016/j.canlet.2015.11.015

69. Zimmer L, Hillen U, Livingstone E, et al. Atypical melanocytic proliferations and new primary melanomas in patients with advanced melanoma undergoing selective BRAF Inhibition. *Journal of Clinical Oncology*. 2012;30(19):2375-2383. doi:10.1200/JCO.2011.41.1660

70. Hatzivassiliou G, Song K, Yen I, et al. RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature*. 2010;464(7287):431-435. doi:10.1038/nature08833

71. Ascierto PA, Schadendorf D, Berking C, et al. MEK162 for patients with advanced melanoma harbouring NRAS or Val600 BRAF mutations: A non-randomised, open-label phase 2 study. *Lancet Oncol*. 2013;14(3):249-256. doi:10.1016/S1470-2045(13)70024-X

72. Abdel-Wahab O, Klimek VM, Gaskell AA, et al. Efficacy of intermittent combined RAF and MEK inhibition in a patient with concurrent BRAF- and NRAS-mutant malignancies. *Cancer Discov*. 2014;4(5):538-545. doi:10.1158/2159-8290.CD-13-1038

73. Flaherty KT, Infante JR, Daud A, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med*. 2012;367(18):1694-1703. doi:10.1056/NEJMoa1210093

74. Ascierto PA, Marincola FM, Atkins MB. What's new in melanoma? Combination! *J Transl Med*. 2015;13:213. doi:10.1186/s12967-015-0582-1

75. Ribas A, Gonzalez R, Pavlick A, et al. Combination of vemurafenib and cobimetinib in patients with advanced BRAFV600-mutated melanoma: A phase 1b study. *Lancet Oncol*. 2014;15(9):954-965. doi:10.1016/S1470-2045(14)70301-8

76. Larkin J, Ascierto PA, Dréno B, et al. Combined vemurafenib and cobimetinib in BRAF-mutated melanoma. *N Engl J Med*. 2014;371(20):1867-1876. doi:10.1056/NEJMoa1408868

77. Robert C, Karaszewska B, Schachter J, et al. Improved Overall Survival in Melanoma with Combined Dabrafenib and Trametinib. *New England Journal of Medicine*. 2014;372(1):141116004513004. doi:10.1056/NEJMoa1412690

78. Curtin JA, Busam K, Pinkel D, Bastian BC. Somatic activation of KIT in distinct subtypes of melanoma. *Journal of Clinical Oncology*. 2006;24(26):4340-4346. doi:10.1200/JCO.2006.06.2984

79. Bastian BC, Esteve-Puig R. Targeting activated KIT signaling for melanoma therapy. *J Clin Oncol*. 2013;31(26):3288-3290. doi:10.1200/JCO.2013.50.3227

80. Posch C, Moslehi H, Sanlorenzo M, et al. Pharmacological inhibitors of c-KIT block mutant c-KIT mediated migration of melanocytes and melanoma cells in vitro and in vivo. *Oncotarget*. 2016;7(29):45916-45925. doi:10.18632/oncotarget.10001

81. Hodi FS, Corless CL, Giobbie-Hurder A, et al. Imatinib for Melanomas Harboring Mutationally Activated or Amplified KIT Arising on Mucosal, Acral, and Chronically Sun-Damaged Skin. *Journal of Clinical Oncology*. 2013;31(26):3182-3190. doi:10.1200/JCO.2012.47.7836

82. Deinlein T, Wolf IH, Rainer B, et al. Treatment of Primary and Metastatic Multifocal Mucosal Melanoma of the Oral Cavity with Imatinib. *Case Rep Oncol*. 2017;10(2):558-563. doi:10.1159/000477563

83. Comparison of Two Doses of Imatinib for the Treatment of Unresectable or Metastatic Gastrointestinal Stromal Tumors: A Meta-Analysis of 1,640 Patients. *Journal of Clinical Oncology*. 2010;28(7):1247-1253. doi:10.1200/JCO.2009.24.2099

84. Minor DR, Kashani-Sabet M, Garrido M, O'Day SJ, Hamid O, Bastian BC. Sunitinib therapy for melanoma patients with KIT mutations. *Clinical Cancer Research*. 2012;18(5):1457-1463. doi:10.1158/1078-0432.CCR-11-1987

85. Carvajal RD, Lawrence DP, Weber JS, et al. Phase II study of nilotinib in melanoma harboring KIT alterations following progression to prior KIT inhibition. *Clinical Cancer Research*. 2015;21(10):2289-2296. doi:10.1158/1078-0432.CCR-14-1630

86. Lippert T, Ruoff HJ, Volm M. Intrinsic and Acquired Drug Resistance in Malignant Tumors. *Arzneimittelforschung*. 2011;58(06):261-264. doi:10.1055/s-0031-1296504

87. Longley D, Johnston P. Molecular mechanisms of drug resistance. *J Pathol*. 2005;205(2):275-292. doi:10.1002/path.1706

88. Boussemart L, Girault I, Malka-Mahieu H, et al. Secondary Tumors Arising in Patients Undergoing BRAF Inhibitor Therapy Exhibit Increased BRAF–CRAF Heterodimerization. *Cancer Res*. 2016;76(6):1476-1484. doi:10.1158/0008-5472.CAN-15-2900-T

89. Robert C, Grob JJ, Stroyakovskiy D, et al. Five-Year Outcomes with Dabrafenib plus Trametinib in Metastatic Melanoma. *New England Journal of Medicine*. 2019;381(7):626-636. doi:10.1056/NEJMoa1904059

90. Manzano JL, Layos L, Bugés C, et al. Resistant mechanisms to BRAF inhibitors in melanoma. *Ann Transl Med*. 2016;4(12):237-237. doi:10.21037/atm.2016.06.07

91. Villanueva J, Infante JR, Krepler C, et al. Concurrent MEK2 Mutation and BRAF Amplification Confer Resistance to BRAF and MEK Inhibitors in Melanoma. *Cell Rep*. 2013;4(6):1090-1099. doi:10.1016/j.celrep.2013.08.023

92. Richtig G, Hoeller C, Kashofer K, et al. Beyond the BRAFV600E hotspot - Biology and clinical implications of rare BRAF gene mutations in melanoma patients. *British Journal of Dermatology*. Published online 2017. doi:10.1111/bjd.15436

93. Posch C, Moslehi H, Feeney L, et al. Combined targeting of MEK and PI3K/mTOR effector pathways is necessary to effectively inhibit NRAS mutant melanoma in vitro and in vivo. *Proc Natl Acad Sci U S A*. 2013;110(10):4015-4020. doi:10.1073/pnas.1216013110

94. Wagle N, Van Allen EM, Treacy DJ, et al. MAP Kinase Pathway Alterations in BRAF-Mutant Melanoma Patients with Acquired Resistance to Combined RAF/MEK Inhibition. *Cancer Discov*. 2014;4(1):61-68. doi:10.1158/2159-8290.CD-13-0631

95. Atefi M, von Euw E, Attar N, et al. Reversing Melanoma Cross-Resistance to BRAF and MEK Inhibitors by Co-Targeting the AKT/mTOR Pathway. Brandner JM, ed. *PLoS One*. 2011;6(12):e28973. doi:10.1371/journal.pone.0028973

96. Ji Z, Njauw CN, Taylor M, Neel V, Flaherty KT, Tsao H. p53 Rescue through HDM2 Antagonism Suppresses Melanoma Growth and Potentiates MEK Inhibition. *Journal of Investigative Dermatology*. 2012;132(2):356-364. doi:10.1038/jid.2011.313

97. Kwong LN, Costello JC, Liu H, et al. Oncogenic NRAS signaling differentially regulates survival and proliferation in melanoma. *Nat Med*. 2012;18(10):1503-1510. doi:10.1038/nm.2941

98. Richtig G, Hoeller C, Kashofer K, et al. Beyond the BRAF(V)(600E) hotspot - Biology and clinical implications of rare BRAF gene mutations in melanoma patients. *Br J Dermatol*. Published online March 9, 2017. doi:10.1111/bjd.15436

99. Atkins MB, Kunkel L, Sznol M, Rosenberg S a. High-dose recombinant interleukin-2 therapy in patients with metastatic melanoma: long-term survival update. *Cancer J Sci Am*. 2000;6 Suppl 1:S11-S14.

100. Robert C, Thomas L, Bondarenko I, et al. Ipilimumab plus Dacarbazine for Previously Untreated Metastatic Melanoma. *New England Journal of Medicine*. 2011;364(26):2517-2526. doi:10.1056/NEJMoa1104621

101. O'Day SJ, Hamid O, Urba WJ. Targeting cytotoxic T-lymphocyte antigen-4 (CTLA-4). *Cancer*. 2007;110(12):2614-2627. doi:10.1002/cncr.23086

102. Schadendorf D, Hodi FS, Robert C, et al. Pooled Analysis of Long-Term Survival Data From Phase II and Phase III Trials of Ipilimumab in Unresectable or Metastatic Melanoma. *Journal of Clinical Oncology*. 2015;33(17):1889-1894. doi:10.1200/JCO.2014.56.2736

103. Ribas A, Kefford R, Marshall MA, et al. Phase III randomized clinical trial comparing tremelimumab with standard-of-care chemotherapy in patients with advanced melanoma. *Journal of Clinical Oncology*. 2013;31(5):616-622. doi:10.1200/JCO.2012.44.6112

104. Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med*. 2002;8(8):793-800. doi:10.1038/nm730

105. Blank C, Kuball J, Voelkl S, et al. Blockade of PD-L1 (B7-H1) augments human tumor-specific T cell responses in vitro. *Int J Cancer*. 2006;119(2):317-327. doi:10.1002/ijc.21775

106. Robert C, Soria JC, Eggermont AMM. Drug of the year: Programmed Death-1 receptor/Programmed Death-1 Ligand-1 receptor monoclonal antibodies. *Eur J Cancer*. 2013;49(14):2968-2971. doi:10.1016/j.ejca.2013.07.001

107. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, Activity, and Immune Correlates of Anti–PD-1 Antibody in Cancer. *New England Journal of Medicine*. 2012;366(26):2443-2454. doi:10.1056/NEJMoa1200690

108. Galluzzi L, Eggermont A, Kroemer G. Doubling the blockade for melanoma immunotherapy. *Oncoimmunology*. 2016;5(1):e1106127. doi:10.1080/2162402X.2015.1106127

109. Wolchok JD, Kluger H, Callahan MK, et al. Nivolumab plus Ipilimumab in Advanced Melanoma. *New England Journal of Medicine*. 2013;369(2):122-133. doi:10.1056/NEJMoa1302369

110. Hamid O, Robert C, Daud A, et al. Supplementary - Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N Engl J Med*. 2013;369(2):134-144. doi:10.1056/NEJMoa1305133

111. Tawbi HA, Schadendorf D, Lipson EJ, et al. Relatlimab and Nivolumab versus Nivolumab in Untreated Advanced Melanoma. *New England Journal of Medicine*. 2022;386(1):24-34. doi:10.1056/NEJMoa2109970

112. Xu Z, Fisher DE. mRNA melanoma vaccine revolution spurred by the COVID-19 pandemic. *Front Immunol*. 2023;14. doi:10.3389/fimmu.2023.1155728

113. Romero D. Immunotherapy: PD-1 says goodbye, TIM-3 says hello. *Nat Rev Clin Oncol*. 2016;13(4):202-203. doi:10.1038/nrclinonc.2016.40

114. Mittal D, Sinha D, Barkauskas D, et al. Adenosine 2B receptor expression on cancer cells promotes metastasis. *Cancer Res*. 2016;76(15):4372-4382. doi:10.1158/0008-5472.CAN-16-0544

115. Radhakrishnan R, Wilkinson ST, D'Souza DC. Gone to Pot - A Review of the Association between Cannabis and Psychosis. *Front Psychiatry*. 2014;5(May):54. doi:10.3389/fpsyt.2014.00054

116. Bezemer KDB, Smink BE, van Maanen R, Verschraagen M, de Gier JJ. Prevalence of medicinal drugs in suspected impaired drivers and a comparison with the use in the general Dutch population. *Forensic Sci Int*. 2014;241:203-211. doi:10.1016/j.forsciint.2014.06.004

117. Kleber HD, Dupont RL. Physicians and medical marijuana. *American Journal of Psychiatry*. 2012;169(6):564-568. doi:10.1176/appi.ajp.2012.12030373

118. Hazekamp A, Heerdink ER. The prevalence and incidence of medicinal cannabis on prescription in the Netherlands. *Eur J Clin Pharmacol*. 2013;69(8):1575-1580. doi:10.1007/s00228-013-1503-y

119. Tramèr MR, Carroll D, Campbell FA, Reynolds DJ, Moore RA, McQuay HJ. Cannabinoids for control of chemotherapy induced nausea and vomiting: quantitative systematic review. *BMJ*. 2001;323(7303):16-21. doi:10.1136/bmj.323.7303.16

120. Radbruch L, Elsner F. Emerging analgesics in cancer pain management. *Expert Opin Emerg Drugs*. 2005;10(1):151-171. doi:EMD100112 [pii]

121. Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R. Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. *Trends Pharmacol Sci*. 2009;30(10):515-527. doi:10.1016/j.tips.2009.07.006

122. Sharma P, Murthy P, Bharath MMS. Chemistry, metabolism, and toxicology of cannabis: clinical implications. *Iran J Psychiatry*. 2012;7(4):149-156.

123. Matsuda L a, Lolait SJ, Brownstein MJ, Young a C, Bonner TI. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*. 1990;346(6284):561-564. doi:10.1038/346561a0

124. Aso E, Ferrer I. Cannabinoids for treatment of alzheimer's disease: Moving toward the clinic. *Front Pharmacol*. 2014;5 MAR. doi:10.3389/fphar.2014.00037

125. Grotenhermen F. Pharmacokinetics and pharmacodynamics of cannabinoids. *Clin Pharmacokinet*. 2003;42(4):327-360. doi:10.2165/00003088-200342040-00003

126. Glass M, Felder CC. Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor. *JNeurosci*. 1997;17(14):5327-5333.

127. Bosier B, Muccioli GG, Hermans E, Lambert DM. Functionally selective cannabinoid receptor signalling: Therapeutic implications and opportunities. *Biochem Pharmacol*. 2010;80(1):1-12. doi:10.1016/j.bcp.2010.02.013

128. Pertwee RG, Howlett a C, Abood ME, et al. International Union of Basic and Clinical Pharmacology . LXXIX . Cannabinoid Receptors and Their Ligands : Beyond CB 1 and CB 2. *Pharmacol Rev*. 2010;62(4):588-631. doi:10.1124/pr.110.003004.588

129. Barann M, Molderings G, Brüss M, Bönisch H, Urban BW, Göthert M. Direct inhibition by cannabinoids of human 5-HT3A receptors: probable involvement of an allosteric modulatory site. *Br J Pharmacol*. 2002;137(5):589-596. doi:10.1038/sj.bjp.0704829

130. Oz M. The Endogenous Cannabinoid Anandamide Inhibits 7 Nicotinic Acetylcholine Receptor-Mediated Responses in Xenopus Oocytes. *Journal of Pharmacology and Experimental Therapeutics*. 2003;306(3):1003-1010. doi:10.1124/jpet.103.049981

131. Hillard CJ. Stress regulates endocannabinoid-CB1 receptor signaling. *Semin Immunol*. 2014;26(5):380-388. doi:10.1016/j.smim.2014.04.001

132. Moldrich G, Wenger T. Localization of the CB1 cannabinoid receptor in the rat brain. An immunohistochemical study☆. *Peptides (NY)*. 2000;21(11):1735-1742. doi:10.1016/S0196-9781(00)00324-7

133. Huestis MA, Gorelick DA, Heishman SJ, et al. Blockade of Effects of Smoked Marijuana by the CB1-Selective Cannabinoid Receptor Antagonist SR141716. *Arch Gen Psychiatry*. 2001;58(4):322. doi:10.1001/archpsyc.58.4.322

134. Gérard CM, Mollereau C, Vassart G, Parmentier M. Molecular cloning of a human cannabinoid receptor which is also expressed in testis. *Biochemical Journal*. 1991;279(1):129-134. doi:10.1042/bj2790129

135. Jeong W il, Osei-Hyiaman D, Park O, et al. Paracrine Activation of Hepatic CB1 Receptors by Stellate Cell-Derived Endocannabinoids Mediates Alcoholic Fatty Liver. *Cell Metab*. 2008;7(3):227-235. doi:10.1016/j.cmet.2007.12.007

136. Waldman M, Hochhauser E, Fishbein M, Aravot D, Shainberg A, Sarne Y. An ultra-low dose of tetrahydrocannabinol provides cardioprotection. *Biochem Pharmacol*. 2013;85(11):1626-1633. doi:10.1016/j.bcp.2013.03.014

137. Gary-Bobo M. The Cannabinoid CB1 Receptor Antagonist Rimonabant (SR141716) Inhibits Cell Proliferation and Increases Markers of Adipocyte Maturation in Cultured Mouse 3T3 F442A Preadipocytes. *Mol Pharmacol*. 2005;69(2):471-478. doi:10.1124/mol.105.015040

138. Ziegler CG, Mohn C, Lamounier-Zepter V, et al. Expression and Function of Endocannabinoid Receptors in the Human Adrenal Cortex. *Hormone and Metabolic Research*. 2010;42(02):88-92. doi:10.1055/s-0029-1241860

139. BOUABOULA M, RINALDI M, CARAYON P, et al. Cannabinoid-receptor expression in human leukocytes. *Eur J Biochem*. 1993;214(1):173-180. doi:10.1111/j.1432-1033.1993.tb17910.x

140. Malfitano AM, Basu S, Maresz K, Bifulco M, Dittel BN. What we know and do not know about the cannabinoid receptor 2 (CB2). *Semin Immunol*. 2014;26(5):369-379. doi:10.1016/j.smim.2014.04.002

141. Galiegue S, Mary S, Marchand J, et al. Expression of Central and Peripheral Cannabinoid Receptors in Human Immune Tissues and Leukocyte Subpopulations. *Eur J Biochem*. 1995;232(1):54-61. doi:10.1111/j.1432-1033.1995.tb20780.x

142. Hart S, Fischer OM, Ullrich A. Cannabinoids induce cancer cell proliferation via tumor necrosis factor alpha-converting enzyme (TACE/ADAM17)-mediated transactivation of the epidermal growth factor receptor. *Cancer Res*. 2004;64(6):1943-1950. doi:10.1158/0008-5472.CAN-03-3720

143. McKallip RJ, Nagarkatti M, Nagarkatti PS. Δ-9-Tetrahydrocannabinol Enhances Breast Cancer Growth and Metastasis by Suppression of the Antitumor Immune Response. *The Journal of Immunology*. 2005;174(6):3281-3289. doi:10.4049/jimmunol.174.6.3281

144. Carracedo A, Lorente M, Egia A, et al. The stress-regulated protein p8 mediates cannabinoid-induced apoptosis of tumor cells. *Cancer Cell*. 2006;9(4):301-312. doi:10.1016/j.ccr.2006.03.005

145. Salazar M, Carracedo A, Salanueva ÍJ, et al. Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells. *Journal of Clinical Investigation*. 2009;119(5):1359-1372. doi:10.1172/JCI37948

146. Casanova ML, Blázquez C, Martínez-Palacio J, et al. Inhibition of skin tumor growth and angiogenesis in vivo by activation of cannabinoid receptors. *J Clin Invest*. 2003;111(1):43-50. doi:10.1172/JCI16116

147. Blázquez C, Salazar M, Carracedo A, et al. Cannabinoids inhibit glioma cell invasion by down-regulating matrix metalloproteinase-2 expression. *Cancer Res*. 2008;68(6):1945-1952. doi:10.1158/0008-5472.CAN-07-5176

148. Preet A, Ganju R, Groopman J. D 9 -Tetrahydrocannabinol inhibits epithelial growth factor-induced lung cancer cell migration in vitro as well as its growth and metastasis in vivo. *Oncogene*. 2008;27:339-346. doi:10.1038/sj.onc.1210641

149. Qamri Z, Preet A, Nasser MW, et al. Synthetic cannabinoid receptor agonists inhibit tumor growth and metastasis of breast cancer. *Mol Cancer Ther*. 2009;8(11):3117-3129. doi:10.1158/1535-7163.MCT-09-0448

150. Ramer R, Hinz B. Inhibition of cancer cell invasion by cannabinoids via increased expression of tissue inhibitor of matrix metalloproteinases-1. *J Natl Cancer Inst*. 2008;100(1):59-69. doi:10.1093/jnci/djm268

151. Newton CA, Chou PJ, Perkins I, Klein TW. CB1 and CB2 Cannabinoid Receptors Mediate Different Aspects of Delta-9-Tetrahydrocannabinol (THC)-Induced T Helper Cell Shift Following Immune Activation by Legionella Pneumophila Infection. *Journal of Neuroimmune Pharmacology*. 2009;4(1):92-102. doi:10.1007/s11481-008-9126-2

152. Hegde VL, Nagarkatti M, Nagarkatti PS. Cannabinoid receptor activation leads to massive mobilization of myeloid-derived suppressor cells with potent immunosuppressive properties. *Eur J Immunol*. 2010;40(12):3358-3371. doi:10.1002/eji.201040667

153. Blázquez C, Carracedo A, Barrado L, et al. Cannabinoid receptors as novel targets for the treatment of melanoma. *The FASEB Journal*. 2006;20(14):2633-2635. doi:10.1096/fj.06-6638fje

154. Velasco G, Sánchez C, Guzmán M. Anticancer mechanisms of cannabinoids. *Current Oncology*. 2016;23:S23-S32. doi:10.3747/co.23.3080

155. Shrivastava A, Kuzontkoski PM, Groopman JE, Prasad A. Cannabidiol Induces Programmed Cell Death in Breast Cancer Cells by Coordinating the Cross-talk between Apoptosis and Autophagy. *Mol Cancer Ther*. 2011;10(7):1161-1172. doi:10.1158/1535-7163.MCT-10-1100

156. Cortellini A, Porzio G, Cofini V, et al. What cancer patients actually know regarding medical cannabis? A cross-sectional survey with a critical analysis of the current attitudes. *Journal of Oncology Pharmacy Practice*. 2019;25(6):1439-1444. doi:10.1177/1078155219843161

157. Pergam SA, Woodfield MC, Lee CM, et al. Cannabis use among patients at a comprehensive cancer center in a state with legalized medicinal and recreational use. *Cancer*. 2017;123(22):4488-4497. doi:10.1002/cncr.30879

158. Shi S, Brant AR, Sabolch A, Pollom E. False News of a Cannabis Cancer Cure. *Cureus*. Published online January 19, 2019. doi:10.7759/cureus.3918

159. Singh Y, Bali C. Cannabis Extract Treatment for Terminal Acute Lymphoblastic Leukemia with a Philadelphia Chromosome Mutation. *Case Rep Oncol*. 2013;6(3):585-592. doi:10.1159/000356446

160. Abrams D, Guzman M. Cannabis in cancer care. *Clin Pharmacol Ther*. 2015;97(6):575-586. doi:10.1002/cpt.108

161. Baram L, Peled E, Berman P, et al. The heterogeneity and complexity of Cannabis extracts as antitumor agents. *Oncotarget*. 2019;10(41):4091-4106. doi:10.18632/oncotarget.26983

162. Lukhele ST, Motadi LR. Cannabidiol rather than Cannabis sativa extracts inhibit cell growth and induce apoptosis in cervical cancer cells. *BMC Complement Altern Med*. 2016;16(1):335. doi:10.1186/s12906-016-1280-0

163. Guzmán M, Duarte MJ, Blázquez C, et al. A pilot clinical study of Δ9-tetrahydrocannabinol in patients with recurrent glioblastoma multiforme. *Br J Cancer*. 2006;95(2):197-203. doi:10.1038/sj.bjc.6603236

164. Yung WKA, Albright RE, Olson J, et al. A phase II study of temozolomide vs. procarbazine in patients with glioblastoma multiforme at first relapse. *Br J Cancer*. 2000;83(5):588-593. doi:10.1054/bjoc.2000.1316

165. Twelves C, Short S, Wright S. A two-part safety and exploratory efficacy randomized double-blind, placebo-controlled study of a 1:1 ratio of the cannabinoids cannabidiol and delta-9-tetrahydrocannabinol (CBD:THC) plus dose-intense temozolomide in patients with recurrent glioblastoma m. *Journal of Clinical Oncology*. 2017;35(15_suppl):2046-2046. doi:10.1200/JCO.2017.35.15_suppl.2046

166. Dall'Stella PB, Docema MFL, Maldaun MVC, Feher O, Lancellotti CLP. Case Report: Clinical Outcome and Image Response of Two Patients With Secondary High-Grade Glioma Treated With Chemoradiation, PCV, and Cannabidiol. *Front Oncol*. 2019;8. doi:10.3389/fonc.2018.00643

167. Foroughi M, Hendson G, Sargent MA, Steinbok P. Spontaneous regression of septum pellucidum/forniceal pilocytic astrocytomas—possible role of Cannabis inhalation. *Child's Nervous System*. 2011;27(4):671-679. doi:10.1007/s00381-011-1410-4

168. Sulé-Suso J, Watson NA, van Pittius DG, Jegannathen A. Striking lung cancer response to self-administration of cannabidiol: A case report and literature review. *SAGE Open Med Case Rep*. 2019;7:2050313X1983216. doi:10.1177/2050313X19832160

169. Armstrong JL, Hill DS, McKee CS, et al. Exploiting cannabinoid-induced cytotoxic autophagy to drive melanoma cell death. *J Invest Dermatol*. 2015;135(6):1629-1637. doi:10.1038/jid.2015.45

170. Verykiou S, Alexander M, Edwards N, et al. Harnessing autophagy to overcome mitogen-activated protein kinase kinase inhibitor-induced resistance in metastatic melanoma. *British Journal of Dermatology*. 2019;180(2):346-356. doi:10.1111/bjd.17333

171. Glodde N, Jakobs M, Bald T, Tüting T, Gaffal E. Differential role of cannabinoids in the pathogenesis of skin cancer. *Life Sci*. 2015;138:35-40. doi:10.1016/j.lfs.2015.04.003

172. Simmerman E, Qin X, Yu JC, Baban B. Cannabinoids as a Potential New and Novel Treatment for Melanoma: A Pilot Study in a Murine Model. *Journal of Surgical Research*. 2019;235:210-215. doi:10.1016/j.jss.2018.08.055

173. Nagarkatti P, Pandey R, Rieder SA, Hegde VL, Nagarkatti M. Cannabinoids as novel anti-inflammatory drugs. *Future Med Chem*. 2009;1(7):1333-1349. doi:10.4155/fmc.09.93

174. Pellati F, Borgonetti V, Brighenti V, Biagi M, Benvenuti S, Corsi L. Cannabis sativa L. and Nonpsychoactive Cannabinoids: Their Chemistry and Role against Oxidative Stress, Inflammation, and Cancer. *Biomed Res Int*. 2018;2018:1-15. doi:10.1155/2018/1691428

175. Janssen LME, Ramsay EE, Logsdon CD, Overwijk WW. The immune system in cancer metastasis: friend or foe? *J Immunother Cancer*. 2017;5(1):79. doi:10.1186/s40425-017-0283-9

176. Blomberg OS, Spagnuolo L, de Visser KE. Immune regulation of metastasis: mechanistic insights and therapeutic opportunities. *Dis Model Mech*. 2018;11(10):dmm036236. doi:10.1242/dmm.036236

177. Taha T, Meiri D, Talhamy S, Wollner M, Peer A, Bar-Sela G. Cannabis Impacts Tumor Response Rate to Nivolumab in Patients with Advanced Malignancies. *Oncologist*. Published online January 22, 2019:theoncologist.2018-0383. doi:10.1634/theoncologist.2018-0383

178. Jiang R, Yamaori S, Okamoto Y, Yamamoto I, Watanabe K. Cannabidiol is a potent inhibitor of the catalytic activity of cytochrome P450 2C19. *Drug Metab Pharmacokinet*. 2013;28(4):332-338.

179. Yamaori S, Okamoto Y, Yamamoto I, Watanabe K. Cannabidiol, a Major Phytocannabinoid, As a Potent Atypical Inhibitor for CYP2D6. *Drug Metabolism and Disposition*. 2011;39(11):2049-2056. doi:10.1124/dmd.111.041384

180. Schadendorf D, Fisher DE, Garbe C, et al. Melanoma. *Nat Rev Dis Primers*. Published online April 23, 2015:15003. doi:10.1038/nrdp.2015.3

181. McGranahan N, Furness AJS, Rosenthal R, et al. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. *Science (1979)*. 2016;351(6280):1463-1469. doi:10.1126/science.aaf1490

182. Riaz N, Havel JJ, Makarov V, et al. Tumor and Microenvironment Evolution during Immunotherapy with Nivolumab. *Cell*. 2017;171(4):934-949.e16. doi:10.1016/j.cell.2017.09.028

183. Richtig G, Kienzl M, Rittchen S, et al. Cannabinoids Reduce Melanoma Cell Viability and Do Not Interfere with Commonly Used Targeted Therapy in Metastatic Melanoma In Vivo and In Vitro. *Biology (Basel)*. 2023;12(5):706. doi:10.3390/biology12050706

184. Theiler A, Bärnthaler T, Platzer W, et al. Butyrate ameliorates allergic airway inflammation by limiting eosinophil trafficking and survival. *Journal of Allergy and Clinical Immunology*. Published online May 2019. doi:10.1016/j.jaci.2019.05.002

185. Schuligoi R. Effect of colchicine on nerve growth factor – induced leukocyte accumulation and thermal hyperalgesia in the rat. *Naunyn Schmiedebergs Arch Pharmacol*. 1998;358(2):264-269. doi:10.1007/PL00005251

186. Jensen MM, Jørgensen JT, Binderup T, Kjær A. Tumor volume in subcutaneous mouse xenografts measured by microCT is more accurate and reproducible than determined by 18F-FDG-microPET or external caliper. *BMC Med Imaging*. 2008;8(1):16. doi:10.1186/1471-2342-8-16

187. Bankhead P, Loughrey MB, Fernández JA, et al. QuPath: Open source software for digital pathology image analysis. *Sci Rep*. 2017;7(1):16878. doi:10.1038/s41598-017-17204-5

188. Costa L, Amaral C, Teixeira N, Correia-da-Silva G, Fonseca BM. Cannabinoid-induced autophagy: Protective or death role? *Prostaglandins Other Lipid Mediat*. 2016;122:54-63. doi:10.1016/j.prostaglandins.2015.12.006

189. Sini MC, Doneddu V, Paliogiannis P, et al. Genetic alterations in main candidate genes during melanoma progression. *Oncotarget*. 2018;9(9). doi:10.18632/oncotarget.23989

190. Ahmed F, Haass NK. Microenvironment-Driven Dynamic Heterogeneity and Phenotypic Plasticity as a Mechanism of Melanoma Therapy Resistance. *Front Oncol*. 2018;8. doi:10.3389/fonc.2018.00173

191. Delyon J, Mateus C, Lefeuvre D, et al. Experience in daily practice with ipilimumab for the treatment of patients with metastatic melanoma: an early increase in lymphocyte and eosinophil counts is associated with improved survival. *Ann Oncol*. 2013;24(6):1697-1703. doi:10.1093/annonc/mdt027

192. Kakadia S, Yarlagadda N, Awad R, et al. Mechanisms of resistance to BRAF and MEK inhibitors and clinical update of US Food and Drug Administration-approved targeted therapy in advanced melanoma. *Onco Targets Ther*. 2018;Volume 11:7095-7107. doi:10.2147/OTT.S182721

193. Cook KL, Warri A, Soto-Pantoja DR, et al. Chloroquine Inhibits Autophagy to Potentiate Antiestrogen Responsiveness in ER+ Breast Cancer. *Clinical Cancer Research*. 2014;20(12):3222-3232. doi:10.1158/1078-0432.CCR-13-3227

194. Fonseca BM, Correia-da-Silva G, Teixeira NA. Cannabinoid-induced cell death in endometrial cancer cells: involvement of TRPV1 receptors in apoptosis. *J Physiol Biochem*. 2018;74(2):261-272. doi:10.1007/s13105-018-0611-7

195. O'Sullivan SE. An update on PPAR activation by cannabinoids. *Br J Pharmacol*. 2016;173(12):1899-1910. doi:10.1111/bph.13497

196. Salvesen GS, Duckett CS. IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol*. 2002;3(6):401-410. doi:10.1038/nrm830

197. Apoptosis: A Target for Anticancer Therapy. *Int J Mol Sci*. 2018;19(2):448. doi:10.3390/ijms19020448

198. Hassan M, Watari H, AbuAlmaaty A, Ohba Y, Sakuragi N. Apoptosis and Molecular Targeting Therapy in Cancer. *Biomed Res Int*. 2014;2014:1-23. doi:10.1155/2014/150845

199. Wang C, Youle RJ. The Role of Mitochondria in Apoptosis. *Annu Rev Genet*. 2009;43(1):95-118. doi:10.1146/annurev-genet-102108-134850

200. Sotthibundhu A, McDonagh K, von Kriegsheim A, et al. Rapamycin regulates autophagy and cell adhesion in induced pluripotent stem cells. *Stem Cell Res Ther*. 2016;7(1):166. doi:10.1186/s13287-016-0425-x

201. Mauthe M, Orhon I, Rocchi C, et al. Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy*. 2018;14(8):1435-1455. doi:10.1080/15548627.2018.1474314

202. Kabeya Y. LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. *J Cell Sci*. 2004;117(13):2805-2812. doi:10.1242/jcs.01131

203. Papandreou ME, Tavernarakis N. Monitoring Autophagic Responses in Caenorhabditis elegans. In: ; 2017:429-444. doi:10.1016/bs.mie.2016.10.039

204. Wilkinson DS, Jariwala JS, Anderson E, et al. Phosphorylation of LC3 by the Hippo Kinases STK3/STK4 Is Essential for Autophagy. *Mol Cell*. 2015;57(1):55-68. doi:10.1016/j.molcel.2014.11.019

205. Gutierrez E, Richardson DR, Jansson PJ. The Anticancer Agent Di-2-pyridylketone 4,4-Dimethyl-3-thiosemicarbazone (Dp44mT) Overcomes Prosurvival Autophagy by Two Mechanisms. *Journal of Biological Chemistry*. 2014;289(48):33568-33589. doi:10.1074/jbc.M114.599480

206. Wu YC, Wu WKK, Li Y, et al. Inhibition of macroautophagy by bafilomycin A1 lowers proliferation and induces apoptosis in colon cancer cells. *Biochem Biophys Res Commun*. 2009;382(2):451-456. doi:10.1016/j.bbrc.2009.03.051

207. Marino ML, Pellegrini P, Di Lernia G, et al. Autophagy Is a Protective Mechanism for Human Melanoma Cells under Acidic Stress. *Journal of Biological Chemistry*. 2012;287(36):30664-30676. doi:10.1074/jbc.M112.339127

208. Luan Q, Jin L, Jiang CC, et al. RIPK1 regulates survival of human melanoma cells upon endoplasmic reticulum stress through autophagy. *Autophagy*. 2015;11(7):975-994. doi:10.1080/15548627.2015.1049800

209. Tsapras P, Nezis IP. Caspase involvement in autophagy. *Cell Death Differ*. 2017;24(8):1369-1379. doi:10.1038/cdd.2017.43

210. McIlwain DR, Berger T, Mak TW. Caspase Functions in Cell Death and Disease. *Cold Spring Harb Perspect Biol*. 2013;5(4):a008656-a008656. doi:10.1101/cshperspect.a008656

211. Yan Y, Jiang K, Liu P, et al. Bafilomycin A1 induces caspase-independent cell death in hepatocellular carcinoma cells via targeting of autophagy and MAPK pathways. *Sci Rep*. 2016;6(1):37052. doi:10.1038/srep37052

212. Choi H, Chaiyamongkol W, Doolittle AC, et al. COX-2 expression mediated by calcium-TonEBP signaling axis under hyperosmotic conditions serves osmoprotective function in nucleus pulposus cells. *Journal of Biological Chemistry*. 2018;293(23):8969-8981. doi:10.1074/jbc.RA117.001167

213. LaCasse EC, Mahoney DJ, Cheung HH, Plenchette S, Baird S, Korneluk RG. IAP-targeted therapies for cancer. *Oncogene*. 2008;27(48):6252-6275. doi:10.1038/onc.2008.302

214. Oberoi-Khanuja TK, Murali A, Rajalingam K. IAPs on the move: role of inhibitors of apoptosis proteins in cell migration. *Cell Death Dis*. 2013;4(9):e784-e784. doi:10.1038/cddis.2013.311

215. Martínez-García D, Manero-Rupérez N, Quesada R, Korrodi-Gregório L, Soto-Cerrato V. Therapeutic strategies involving survivin inhibition in cancer. *Med Res Rev*. 2019;39(3):887-909. doi:10.1002/med.21547

216. JNK-signaling: A multiplexing hub in programmed cell death. *Genes Cancer*. Published online November 2, 2017. doi:10.18632/genesandcancer.155

217. Dienstmann R, Rodon J, Serra V, Tabernero J. Picking the Point of Inhibition: A Comparative Review of PI3K/AKT/mTOR Pathway Inhibitors. *Mol Cancer Ther*. 2014;13(5):1021-1031. doi:10.1158/1535-7163.MCT-13-0639

218. Kwong LN, Davies MA. Navigating the Therapeutic Complexity of PI3K Pathway Inhibition in Melanoma. *Clinical Cancer Research*. 2013;19(19):5310-5319. doi:10.1158/1078-0432.CCR-13-0142

219. Irvine M, Stewart A, Pedersen B, Boyd S, Kefford R, Rizos H. Oncogenic PI3K/AKT promotes the step-wise evolution of combination BRAF/MEK inhibitor resistance in melanoma. *Oncogenesis*. 2018;7(9):72. doi:10.1038/s41389-018-0081-3

220. Obenauf AC, Zou Y, Ji AL, et al. Therapy-induced tumour secretomes promote resistance and tumour progression. *Nature*. 2015;520(7547):368-372. doi:10.1038/nature14336

221. Kosnopfel C, Sinnberg T, Sauer B, et al. Human melanoma cells resistant to MAPK inhibitors can be effectively targeted by inhibition of the p90 ribosomal S6 kinase. *Oncotarget*. 2017;8(22). doi:10.18632/oncotarget.16204

222. Su F, Bradley WD, Wang Q, et al. Resistance to Selective BRAF Inhibition Can Be Mediated by Modest Upstream Pathway Activation. *Cancer Res*. 2012;72(4):969-978. doi:10.1158/0008-5472.CAN-11-1875

223. Berger M, Richtig G, Kashofer K, Aigelsreiter A, Richtig E. The window of opportunities for targeted therapy in BRAFwt/NRASwt/KITwt melanoma. Biology and clinical implications of fusion proteins and other mutations. *G Ital Dermatol Venereol*. Published online March 29, 2018. doi:10.23736/S0392-0488.18.05970-9

224. Harpio R, Einarsson R. S100 proteins as cancer biomarkers with focus on S100B in malignant melanoma. *Clin Biochem*. 2004;37(7):512-518. doi:10.1016/j.clinbiochem.2004.05.012

225. Richtig G, Aigelsreiter A, Schwarzenbacher D, et al. SOX9 is a proliferation and stem cell factor in hepatocellular carcinoma and possess widespread prognostic significance in different cancer types. Coleman WB, ed. *PLoS One*. 2017;12(11):e0187814. doi:10.1371/journal.pone.0187814

226. Melling N, Kowitz CM, Simon R, et al. High Ki67 expression is an independent good prognostic marker in colorectal cancer. *J Clin Pathol*. 2016;69(3):209-214. doi:10.1136/jclinpath-2015-202985

227. Frei RB, Luschnig P, Parzmair GP, et al. Cannabinoid receptor 2 augments eosinophil responsiveness and aggravates allergen-induced pulmonary inflammation in mice. *Allergy*. 2016;71(7):944-956. doi:10.1111/all.12858

228. Hasenoehrl C, Storr M, Schicho R. Cannabinoids for treating inflammatory bowel diseases: where are we and where do we go? *Expert Rev Gastroenterol Hepatol*. 2017;11(4):329-337. doi:10.1080/17474124.2017.1292851

229. Patel SP, Kurzrock R. PD-L1 Expression as a Predictive Biomarker in Cancer Immunotherapy. *Mol Cancer Ther*. 2015;14(4):847-856. doi:10.1158/1535-7163.MCT-14-0983

230. Elias EG, Hasskamp JH, Sharma BK. Cytokines and Growth Factors Expressed by Human Cutaneous Melanoma. *Cancers (Basel)*. 2010;2(2):794-808. doi:10.3390/cancers2020794

231. Nicholas C, Lesinski GB. Immunomodulatory cytokines as therapeutic agents for melanoma. *Immunotherapy*. 2011;3(5):673-690. doi:10.2217/imt.11.45

232. Zelenay S, van der Veen AG, Böttcher JP, et al. Cyclooxygenase-Dependent Tumor Growth through Evasion of Immunity. *Cell*. 2015;162(6):1257-1270. doi:10.1016/j.cell.2015.08.015

233. Keung EZ, Gershenwald JE. The eighth edition American Joint Committee on Cancer (AJCC) melanoma staging system: implications for melanoma treatment and care. *Expert Rev Anticancer Ther*. 2018;18(8):775-784. doi:10.1080/14737140.2018.1489246

234. Richtig G, Hoeller C, Kashofer K, et al. Beyond the BRAF V 600E hotspot - Biology and clinical implications of rare BRAF gene mutations in melanoma patients. *British Journal of Dermatology*. Published online March 2017. doi:10.1111/bjd.15436

235. Wan PTC, Garnett MJ, Roe SM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell*. 2004;116(6):855-867.

236. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proceedings of the National Academy of Sciences*. 2002;99(19):12293-12297. doi:10.1073/pnas.192461099

237. Leach DR, Krummel MF, Allison JP. Enhancement of Antitumor Immunity by CTLA-4 Blockade. *Science (1979)*. 1996;271(5256):1734-1736. doi:10.1126/science.271.5256.1734

238. Burstein SH. Ajulemic acid: potential treatment for chronic inflammation. *Pharmacol Res Perspect*. 2018;6(2):e00394. doi:10.1002/prp2.394

239. Vuolo F, Abreu SC, Michels M, et al. Cannabidiol reduces airway inflammation and fibrosis in experimental allergic asthma. *Eur J Pharmacol*. 2019;843:251-259. doi:10.1016/j.ejphar.2018.11.029

240. Hanahan D. Hallmarks of Cancer: New Dimensions. *Cancer Discov*. 2022;12(1):31-46. doi:10.1158/2159-8290.CD-21-1059

241. Blázquez C, Carracedo A, Barrado L, et al. Cannabinoid receptors as novel targets for the treatment of melanoma. *The FASEB Journal*. 2006;20(14):2633-2635. doi:10.1096/fj.06-6638fje

242. Richtig G, Richtig E, Massone C, Hofmann-Wellenhof R. Analysis of clinical, dermoscopic and histopathological features of primary melanomas of patients with metastatic disease--a retrospective study at the Department of Dermatology, Medical University of Graz, 2000-2010. *J Eur Acad Dermatol Venereol*. 2014;28(12):1776-1781. doi:10.1111/jdv.12413

243. Legare CA, Raup-Konsavage WM, Vrana KE. Therapeutic Potential of Cannabis, Cannabidiol, and Cannabinoid-Based Pharmaceuticals. *Pharmacology*. 2022;107(3-4):131-149. doi:10.1159/000521683

244. Vuolo F, Abreu SC, Michels M, et al. Cannabidiol reduces airway inflammation and fibrosis in experimental allergic asthma. *Eur J Pharmacol*. 2019;843:251-259. doi:10.1016/j.ejphar.2018.11.029

245. Howlett AC, Abood ME. CB 1 and CB 2 Receptor Pharmacology. In: ; 2017:169-206. doi:10.1016/bs.apha.2017.03.007

246. Shakhova O. Neural crest stem cells in melanoma development. *Curr Opin Oncol*. 2014;26(2):215-221. doi:10.1097/CCO.0000000000000046

247. Costas-Insua C, Seijo-Vila M, Blázquez C, et al. Neuronal Cannabinoid CB1 Receptors Suppress the Growth of Melanoma Brain Metastases by Inhibiting Glutamatergic Signalling. *Cancers (Basel)*. 2023;15(9):2439. doi:10.3390/cancers15092439

248. Carpi S, Fogli S, Polini B, et al. Tumor-promoting effects of cannabinoid receptor type 1 in human melanoma cells. *Toxicology in Vitro*. 2017;40:272-279. doi:10.1016/j.tiv.2017.01.018

249. Aviello G, Romano B, Borrelli F, et al. Chemopreventive effect of the non-psychotropic phytocannabinoid cannabidiol on experimental colon cancer. *J Mol Med*. 2012;90(8):925-934. doi:10.1007/s00109-011-0856-x

250. Yang Y, Guo W, Ma J, et al. Downregulated TRPV1 Expression Contributes to Melanoma Growth via the Calcineurin-ATF3-p53 Pathway. *Journal of Investigative Dermatology*. 2018;138(10):2205-2215. doi:10.1016/j.jid.2018.03.1510

251. Gao J, Liu Q, Xu Y, et al. PPARα induces cell apoptosis by destructing Bcl2. *Oncotarget*. 2015;6(42):44635-44642. doi:10.18632/oncotarget.5988

252. Grabacka M, Plonka PM, Urbanska K, Reiss K. Peroxisome Proliferator–Activated Receptor α Activation Decreases Metastatic Potential of Melanoma Cells In vitro via Down-Regulation of Akt. *Clinical Cancer Research*. 2006;12(10):3028-3036. doi:10.1158/1078-0432.CCR-05-2556

253. Baker BG, Ball GR, Rakha EA, et al. Lack of expression of the proteins GMPR2 and PPARα are associated with the basal phenotype and patient outcome in breast cancer. *Breast Cancer Res Treat*. 2013;137(1):127-137. doi:10.1007/s10549-012-2302-3

254. Singh R, Letai A, Sarosiek K. Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins. *Nat Rev Mol Cell Biol*. 2019;20(3):175-193. doi:10.1038/s41580-018-0089-8

255. Cavalcante GC, Schaan AP, Cabral GF, et al. A Cell's Fate: An Overview of the Molecular Biology and Genetics of Apoptosis. *Int J Mol Sci*. 2019;20(17):4133. doi:10.3390/ijms20174133

256. Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene*. 2006;25(34):4798-4811. doi:10.1038/sj.onc.1209608

257. Dhanasekaran DN, Reddy EP. JNK signaling in apoptosis. *Oncogene*. 2008;27(48):6245-6251. doi:10.1038/onc.2008.301

258. Fallahi-Sichani M, Moerke NJ, Niepel M, Zhang T, Gray NS, Sorger PK. Systematic analysis of <scp> BRAF ^{V} </scp> ^{600E} melanomas reveals a role for <scp>JNK</scp> /c-Jun pathway in adaptive resistance to drug-induced apoptosis. *Mol Syst Biol*. 2015;11(3). doi:10.15252/msb.20145877

259. Perelman A, Wachtel C, Cohen M, Haupt S, Shapiro H, Tzur A. JC-1: alternative excitation wavelengths facilitate mitochondrial membrane potential cytometry. *Cell Death Dis*. 2012;3(11):e430-e430. doi:10.1038/cddis.2012.171

260. Mohana-Kumaran N, Hill DS, Allen JD, Haass NK. Targeting the intrinsic apoptosis pathway as a strategy for melanoma therapy. *Pigment Cell Melanoma Res*. 2014;27(4):525-539. doi:10.1111/pcmr.12242

261. Olivas-Aguirre M, Torres-López L, Valle-Reyes JS, Hernández-Cruz A, Pottosin I, Dobrovinskaya O. Cannabidiol directly targets mitochondria and disturbs calcium homeostasis in acute lymphoblastic leukemia. *Cell Death Dis*. 2019;10(10):779. doi:10.1038/s41419-019-2024-0

262. Hebert-Chatelain E, Marsicano G, Desprez T. Cannabinoids and Mitochondria. In: *Endocannabinoids and Lipid Mediators in Brain Functions*. Springer International Publishing; 2017:211-235. doi:10.1007/978-3-319-57371-7_8

263. Kim SR, Kim SU, Oh U, Jin BK. Transient Receptor Potential Vanilloid Subtype 1 Mediates Microglial Cell Death In Vivo and In Vitro via Ca 2+ -Mediated Mitochondrial Damage and Cytochrome c Release. *The Journal of Immunology*. 2006;177(7):4322-4329. doi:10.4049/jimmunol.177.7.4322

264. Ramírez-Barrantes R, Córdova C, Gatica S, et al. Transient Receptor Potential Vanilloid 1 Expression Mediates Capsaicin-Induced Cell Death. *Front Physiol*. 2018;9. doi:10.3389/fphys.2018.00682

265. Dando I, Donadelli M, Costanzo C, et al. Cannabinoids inhibit energetic metabolism and induce AMPK-dependent autophagy in pancreatic cancer cells. *Cell Death Dis*. 2013;4(6):e664-e664. doi:10.1038/cddis.2013.151

266. Salazar M, Carracedo A, Salanueva ÍJ, et al. Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells. *Journal of Clinical Investigation*. 2009;119(5):1359-1372. doi:10.1172/JCI37948

267. Mrakovcic M, Fröhlich LF. p53-Mediated Molecular Control of Autophagy in Tumor Cells. *Biomolecules*. 2018;8(2):14. doi:10.3390/biom8020014

268. Kandoth C, McLellan MD, Vandin F, et al. Mutational landscape and significance across 12 major cancer types. *Nature*. 2013;502(7471):333-339. doi:10.1038/nature12634

269. Sreevalsan S, Joseph S, Jutooru I, Chadalapaka G, Safe SH. Induction of apoptosis by cannabinoids in prostate and colon cancer cells is phosphatase dependent. *Anticancer Res*. 2011;31(11):3799-3807. http://www.ncbi.nlm.nih.gov/pubmed/22110202

270. Van Opdenbosch N, Lamkanfi M. Caspases in Cell Death, Inflammation, and Disease. *Immunity*. 2019;50(6):1352-1364. doi:10.1016/j.immuni.2019.05.020

271. Ramirez MLG, Salvesen GS. A primer on caspase mechanisms. *Semin Cell Dev Biol*. 2018;82:79-85. doi:10.1016/j.semcdb.2018.01.002

272. Berthenet K, Castillo Ferrer C, Fanfone D, et al. Failed Apoptosis Enhances Melanoma Cancer Cell Aggressiveness. *Cell Rep*. 2020;31(10):107731. doi:10.1016/j.celrep.2020.107731

273. Adinolfi B, Romanini A, Vanni A, et al. Anticancer activity of anandamide in human cutaneous melanoma cells. *Eur J Pharmacol*. 2013;718(1-3):154-159. doi:10.1016/j.ejphar.2013.08.039

274. Johns A. Psychiatric effects of cannabis. *British Journal of Psychiatry*. 2001;178(2):116-122. doi:10.1192/bjp.178.2.116

275. Large M, Sharma S, Compton MT, Slade T, Nielssen O. Cannabis Use and Earlier Onset of Psychosis. *Arch Gen Psychiatry*. 2011;68(6):555. doi:10.1001/archgenpsychiatry.2011.5

276. Price C, Hemmingsson T, Lewis G, Zammit S, Allebeck P. Cannabis and suicide: longitudinal study. *British Journal of Psychiatry*. 2009;195(6):492-497. doi:10.1192/bjp.bp.109.065227

277. Serafini G, Pompili M, Innamorati M, Rihmer Z, Sher L, Girardi P. Can Cannabis Increase the Suicide Risk in Psychosis? A Critical Review. *Curr Pharm Des*. 2012;18(32):5165-5187. doi:10.2174/138161212802884663

278. Pham JP, Joshua AM, da Silva IP, Dummer R, Goldinger SM. Chemotherapy in Cutaneous Melanoma: Is There Still a Role? *Curr Oncol Rep*. 2023;25(6):609-621. doi:10.1007/s11912-023-01385-6

279. Glodde N, Jakobs M, Bald T, Tüting T, Gaffal E. Differential role of cannabinoids in the pathogenesis of skin cancer. *Life Sci*. 2015;138:35-40. doi:10.1016/j.lfs.2015.04.003

280. Vermersch P. Sativex ® (tetrahydrocannabinol + cannabidiol), an endocannabinoid system modulator: basic features and main clinical data. *Expert Rev Neurother*. 2011;11(sup4):15-19. doi:10.1586/ern.11.27

281. Valjent E, Pagès C, Rogard M, Besson MJ, Maldonado R, Caboche J. Δ9-tetrahydrocannabinol-induced MAPK/ERK and Elk-1 activation in vivo depends on dopaminergic transmission. *European Journal of Neuroscience*. 2001;14(2):342-352. doi:10.1046/j.0953-816x.2001.01652.x

282. Vrechi TAM, Leão AHFF, Morais IBM, et al. Cannabidiol induces autophagy via ERK1/2 activation in neural cells. *Sci Rep*. 2021;11(1):5434. doi:10.1038/s41598-021-84879-2

283. Greenhough A, Patsos HA, Williams AC, Paraskeva C. The cannabinoid δ9-tetrahydrocannabinol inhibits RAS-MAPK and PI3K-AKT survival signalling and induces BAD-mediated apoptosis in colorectal cancer cells. *Int J Cancer*. 2007;121(10):2172-2180. doi:10.1002/ijc.22917

Appendix

Publications

Georg Richtig was involved in the following publications as a PhD student and employee of this institute, which are not directly related to this thesis:

Wagner NB, Lenders MM, Kühl K, Reinhardt L, André F, Dudda M, Ring N, Ebel C, Stäger R, Zellweger C, Lang R, Paar M, Gussek P, Richtig G, Stürmer SH, Kimeswenger S, Oellinger A, Forschner A, Leiter U, Weide B, Gassenmaier M, Schraag A, Klumpp B, Hoetzenecker W, Berking C, Richtig E, Ziemer M, Mangana J, Terheyden P, Loquai C, Nguyen VA, Gebhardt C, Meier F, Diem S, Cozzio A, Flatz L, Röcken M, Garbe C, Eigentler TK.
Pretreatment metastatic growth rate determines clinical outcome of advanced melanoma patients treated with anti-PD-1 antibodies: a multicenter cohort study.
J Immunother Cancer. 2021 May;9(5):e002350. doi: 10.1136/jitc-2021-002350.

Grübler MR, Zittermann A, Verheyen ND, Trummer C, Theiler-Schwetz V, Keppel MH, Malle O, Richtig G, Gängler S, Bischoff-Ferrari H, Scharnagl H, Meinitzer A, März W, Tomaschitz A, Pilz S.
Randomized trial of vitamin D versus placebo supplementation on markers of systemic inflammation in hypertensive patients.
Nutr Metab Cardiovasc Dis. 2021 Oct 28;31(11):3202-3209. doi: 10.1016/j.numecd.2021.07.028. Epub 2021 Aug 18.

Richtig G, Cerroni L, Schmidt H, Beham-Schmid C, Deinlein T, Vallant C, Richtig E.
Talimogene laherparepvec can initiate plasma cell invasion into infiltrated melanoma lesions - a case series.
J Eur Acad Dermatol Venereol. 2021 Mar;35(3):e209-e211. doi: 10.1111/jdv.16922. Epub 2020 Sep 22.

Richtig G, Richtig E, Neiss AN, Quehenberger F, Gmainer DG, Kamolz LP, Lumenta DB.
Does the time interval between sentinel lymph node biopsy and completion lymph node

dissection affect outcome in malignant melanoma? A retrospective cohort study.
Int J Surg. 2020 Mar;75:160-164. doi: 10.1016/j.ijsu.2020.01.146. Epub 2020 Feb 7.

Theiler A, Bärnthaler T, Platzer W, Richtig G, Peinhaupt M, Rittchen S, Kargl J, Ulven T, Marsh LM, Marsche G, Schuligoi R, Sturm EM, Heinemann A.
Butyrate ameliorates allergic airway inflammation by limiting eosinophil trafficking and survival.
J Allergy Clin Immunol. 2019 Sep;144(3):764-776. doi: 10.1016/j.jaci.2019.05.002.

Richtig G, Ramelyte E, Koch L, Greinix H, Ferrone S, Dummer R, Richtig E.
Unmasking of a primary desmoplastic melanoma tumour in the course of treatment of a metastatic disease with anti-PD-1 monoclonal antibody.
J Eur Acad Dermatol Venereol. 2019 Oct;33(10):e381-e383. doi: 10.1111/jdv.15675.

Richtig G, Aigelsreiter AM, Asslaber M, Weiland T, Pichler M, Eberhard K, Sygulla S, Schauer S, Hoefler G, Aigelsreiter A.
Hedgehog pathway proteins SMO and GLI expression as prognostic markers in head and neck squamous cell carcinoma.
Histopathology. 2019 Jul;75(1):118-127. doi: 10.1111/his.13860.

Richtig G, Hoeller C, Wolf M, Wolf I, Rainer BM, Schulter G, Richtig M, Grübler MR, Gappmayer A, Haidn T, Kofler J, Huegel R, Lange-Asschenfeldt B, Pichler M, Pilz S, Heinemann A, Richtig E.
Body mass index may predict the response to ipilimumab in metastatic melanoma: An observational multi-centre study.
PLoS One. 2018 Oct 1;13(10):e0204729. doi: 10.1371/journal.pone.0204729. eCollection 2018.

Richtig G, Richtig M, Hoetzenecker W, Saxinger W, Lange-Asschenfeldt B, Steiner A, Strohal R, Posch C, Bauer JW, Müllegger RR, Deinlein T, Sepp N, Volc-Platzer B, Nguyen VA, Schmuth M, Hoeller C, Pregartner G, Richtig E.
Knowledge and Influence of Predatory Journals in Dermatology: A Pan-Austrian Survey.
Acta Derm Venereol. 2019 Jan 1;99(1):58-62. doi: 10.2340/00015555-3037.

Richtig G, Berger M, Lange-Asschenfeldt B, Aberer W, Richtig E.
Problems and challenges of predatory journals.
J Eur Acad Dermatol Venereol. 2018 Sep;32(9):1441-1449. doi: 10.1111/jdv.15039.

Berger M, Richtig G, Kashofer K, Aigelsreiter A, Richtig E.
The window of opportunities for targeted therapy in BRAFwt/NRASwt/KITwt melanoma: biology and clinical implications of fusion proteins and other mutations.
G Ital Dermatol Venereol. 2018 Jun;153(3):349-360. doi: 10.23736/S0392-0488.18.05970-9.

Gutschner T, Richtig G, Haemmerle M, Pichler M.
From biomarkers to therapeutic targets-the promises and perils of long non-coding RNAs in cancer.
Cancer Metastasis Rev. 2018 Mar;37(1):83-105. doi: 10.1007/s10555-017-9718-5.

Richtig G, Aigelsreiter A, Schwarzenbacher D, Ress AL, Adiprasito JB, Stiegelbauer V, Hoefler G, Schauer S, Kiesslich T, Kornprat P, Winder T, Eisner F, Gerger A, Stoeger H, Stauber R, Lackner C, Pichler M.
SOX9 is a proliferation and stem cell factor in hepatocellular carcinoma and possess widespread prognostic significance in different cancer types.
PLoS One. 2017 Nov 9;12(11):e0187814. doi: 10.1371/journal.pone.0187814. eCollection 2017.

Goblirsch M, Richtig G, Slaby O, Berindan-Neagoe I, Gerger A, Pichler M.
MicroRNAs as a tool to aid stratification of colorectal cancer patients and to guide therapy.
Pharmacogenomics. 2017 Jul;18(10):1027-1038. doi: 10.2217/pgs-2017-0004.

Richtig G, Pichler M.
Prediction of Response in Melanoma Therapy by Systemic Inflammatory Response - One Size Fits Not All.
EBioMedicine. 2017 Apr;18:13-14. doi: 10.1016/j.ebiom.2017.03.032.

Richtig G, Ehall B, Richtig E, Aigelsreiter A, Gutschner T, Pichler M.
Function and Clinical Implications of Long Non-Coding RNAs in Melanoma.
Int J Mol Sci. 2017 Mar 28;18(4). pii: E715. doi: 10.3390/ijms18040715.

Richtig G, Hoeller C, Kashofer K, Aigelsreiter A, Heinemann A, Kwong LN, Pichler M, Richtig E.
Beyond the BRAFV600E hotspot: biology and clinical implications of rare BRAF gene mutations in melanoma patients.
Br J Dermatol. 2017 Oct;177(4):936-944. doi: 10.1111/bjd.15436.

Richtig G, Richtig E, Kashofer K, Koch L, Winter G, Hoefler G, Pichler M, Ehall B, Grübler MR, Heinemann A, Aigelsreiter A.
Testing and clinical implications for non-V600 BRAF mutations in metastatic NRASmt melanoma.
Br J Dermatol. 2017 Sep;177(3):860-861. doi: 10.1111/bjd.15222.

Grübler MR, Gaksch M, Kienreich K, Verheyen ND, Schmid J, Müllner C, Richtig G, Scharnagl H, Trummer C, Schwetz V, Meinitzer A, Pieske B, März W, Tomaschitz A, Pilz S.

Effects of Vitamin D3 on asymmetric- and symmetric dimethylarginine in arterial hypertension.
J Steroid Biochem Mol Biol. 2018 Jan;175:157-163. doi: 10.1016/j.jsbmb.2016.12.014.

Richtig G, Aigelsreiter A, Kashofer K, Talakic E, Kupsa R, Schaider H, Richtig E.
Two Case Reports of Rare BRAF Mutations in Exon 11 and Exon 15 with Discussion of Potential Treatment Options.
Case Rep Oncol. 2016 Sep 22;9(3):543-546. eCollection 2016 Sep-Dec.

Richtig G, Byrom L, Kupsa R, Schaider H, Hofmann-Wellenhof R, Wolf IH, Soyer HP, Richtig E.
Pregnancy as a driver for melanoma.
Br J Dermatol. 2017 Sep;177(3):854-857. doi: 10.1111/bjd.15124.

Grübler MR, Gaksch M, Kienreich K, Verheyen N, Schmid J, Ó Hartaigh B, Richtig G, Scharnagl H, Meinitzer A, Fahrleitner-Pammer A, März W, Tomaschitz A, Pilz S.
Effects of vitamin D supplementation on glycated haemoglobin and fasting glucose levels in hypertensive patients: a randomized controlled trial.
Diabetes Obes Metab. 2016 Oct;18(10):1006-12. doi: 10.1111/dom.12709.

Grübler MR, Gaksch M, Kienreich K, Verheyen N, Schmid J, Ó Hartaigh BW, Richtig G, Scharnagl H, Meinitzer A, Pieske B, Fahrleitner-Pammer A, März W, Tomaschitz A, Pilz S.
Effects of Vitamin D Supplementation on Plasma Aldosterone and Renin-A Randomized Placebo-Controlled Trial.
J Clin Hypertens (Greenwich). 2016 Jul;18(7):608-13. doi: 10.1111/jch.12825.

Reagents and Cell lines

Drugs	Company (Location)	Cat. No.
AM251	Tocris (Abingdon, UK)	1117
AM630	Tocris (Abingdon, UK)	1120
AMG9810	Tocris (Abingdon, UK)	2316
UC112	Tocris (Abingdon, UK)	5251
BI78D3	Tocris (Abingdon, UK)	3314
AZ10417808	Tocris (Abingdon, UK)	2172
Z-VAD-FMK	Selleckchem (Houston, TX)	S7023
Rapamycin	Tocris (Abingdon, UK)	1292
GW6471	Tocris (Abingdon, UK)	4618
GW9662	Tocris (Abingdon, UK)	1508
Cannabidiol	Tocris (Abingdon, UK)	1570
Tetrahydrocannabinol	Gatt-koller (Absam, Austria)	609030009
Chloroquine	Tocris (Abingdon, UK)	4109
Bafilomycin A1	Tocris (Abingdon, UK)	1334
Vemurafenib	Selleckchem (Houston, TX)	S1267
Trametinib	Selleckchem (Houston, TX)	S2673
UO126	Cell Signaling Technology Europe (Frankfurt am Main, Germany)	9903S
LY294002	Cell Signaling Technology Europe (Frankfurt am Main, Germany)	9901
Antibody	**Company (Location)**	**Cat. No.**
PD-L1 Rabbit mAb (E1L3N)	Cell Signaling Technology Europe (Frankfurt am Main, Germany)	13684
LC3A/B Rabbit mAb (D3U4C)	Cell Signaling Technology Europe (Frankfurt am Main, Germany)	12741
Ki-67 Mouse mAb (8D5)	Cell Signaling Technology Europe (Frankfurt am Main, Germany)	9449

Cytochrome c Mouse mAb (6H2.B4)	Cell Signaling Technology Europe (Frankfurt am Main, Germany)	12963
Goat anti-Mouse IgG, Alexa Fluor 488	Thermo Scientific (Vienna, Austria)	A-11001
Primer	**Company (Location)**	**Cat. No.**
CNR1	Bio-Rad Laboratories (Vienna, Austria)	#qhsaced0043777
TRPV1	Bio-Rad Laboratories (Vienna, Austria)	#qhsacid0022051
PPARA	Bio-Rad Laboratories (Vienna, Austria)	#qhsacid0011001
GAPDH	Bio-Rad Laboratories (Vienna, Austria)	#qhsaced0038674
TRPV2	Bio-Rad Laboratories (Vienna, Austria)	#qhsacid0010283
CNR2	Bio-Rad Laboratories (Vienna, Austria)	#qhsaced0038847
GPR55	Bio-Rad Laboratories (Vienna, Austria)	#qhsaced0001676
PPARG	Bio-Rad Laboratories (Vienna, Austria)	#qhsaced0044425
Miscellaneous		
Item	**Company (Location)**	**Cat. No.**
Nunc™ Lab-Tek™ II Chamber Slide™ System	Thermo Scientific (Vienna, Austria)	154534
Caspase-Glo® 3/7 Assay Systems	Promega GmbH (Mannheim, Germany)	G8091
CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS)	Promega GmbH (Mannheim, Germany)	G5421

Penicillin-Streptomycin, 10,000 U/ml Penicillin, 10 mg/ml Streptomycin	PAN-Biotech GmbH (Aidenbach, Germany)	P06-07050
Trypsin 0.05 %/EDTA 0.02 % in PBS, w/o: Ca and Mg	PAN-Biotech GmbH (Aidenbach, Germany)	P10-023100
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Scientific (Vienna, Austria)	41965039
DPBS, no calcium, no magnesium	Thermo Scientific (Vienna, Austria)	14190094
RPMI 1640 Medium	Thermo Scientific (Vienna, Austria)	21875034
Propidium iodide solution	Merck KGaA (Darmstadt, Germany)	P4864
Crystal Violet	Merck KGaA (Darmstadt, Germany)	C0775
Triton™ X-100	Merck KGaA (Darmstadt, Germany)	T8787
FITC Annexin V Apoptosis Detection Kit I	BD Bioscience (Schwechat, Austria)	556547
TRI Reagent®	Merck KGaA (Darmstadt, Germany)	93289
Fetal Bovine Serum (FBS)	Thermo Scientific (Vienna, Austria)	102701106
Minimum Essential Media (MEM), no glutamine, no phenol red	Thermo Scientific (Vienna, Austria)	51200046
JC-1 Dye	Thermo Scientific (Vienna, Austria)	T3168
RNeasy Mini Kit	Qiagen (Hilden, Germany)	74104
High-Capacity cDNA Reverse	Thermo Scientific (Vienna,	4368814

Transcription Kit	Austria)	
SsoAdvanced™ Universal SYBR® Green Supermix	Bio-Rad Laboratories (Vienna, Austria)	1725271
Histopaque®-1077	Merck KGaA (Darmstadt, Germany)	10771
4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Merck KGaA (Darmstadt, Germany)	D8417
Rhodamine Phalloidin	Thermo Scientific (Vienna, Austria)	R415
Eosinophil Isolation Kit, human	Miltenyi Biotec, Bergisch Gladbach, Germany	130-092-010
Kolliphor	Merck KGaA (Darmstadt, Germany)	C5135-500G
Proteome Profiler Human Cytokine Array Kit	R&D Systems (Minneapolis, MN, USA)	ARY005B
Proteome Profiler Human Phospho-Kinase Array Kit	R&D Systems (Minneapolis, MN, USA)	ARY003B
Cell lines		
Name	**Company/Institution**	**Cat. No./Donor**
A375	LGC Standards GmbH (Wesel, Germany)	CRL-1619
A2058	LGC Standards GmbH (Wesel, Germany)	CRL-11147
SK-Mel-28	LGC Standards GmbH (Wesel, Germany)	HTB-72
A375R	Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Vienna, Austria	Dr. Anna Obenauf
UACC-62	Research Institute of Molecular Pathology (IMP), Vienna	Dr. Anna Obenauf

	Biocenter (VBC), Vienna, Austria	
Colo-800	Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Vienna, Austria	Dr. Anna Obenauf
SK-Mel-30	Division of Oncology, Department of Internal Medicine, Medical University of Graz, Graz, Austria.	Prof. Martin Pichler
SBcl2	Biomedical Research, Medical University of Graz, Graz, Austria	Prof. Beate Rinner
MRC-5	LGC Standards GmbH (Wesel, Germany)	CCL-171

Devices

Devices	
Name	**Company**
BD FACSCalibur	BD Bioscience (Schwechat, Austria)
BD FACSCANTO II	BD Bioscience (Schwechat, Austria)
CLARIOstar Plus	BMG LABTECH (Ortenberg, Germany)
BioTek™ ELx800™	Thermo Scientific (Vienna, Austria)
T100™ Thermal Cycler	Bio-Rad Laboratories (Vienna, Austria)
CFX Connect™ Real-Time PCR Detection System	Bio-Rad Laboratories (Vienna, Austria)
VICTOR X5 Multilabel Plate Reader	Perkin Elmer (Waltham, MA)